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Molecular evaluation of the anticancer potential of 1,3,4-oxadiazole-2-thiones derivative and its synergetic action with Nigella sativa seed extract (Thymoquinone) in experimentally induced Murine Lung Cancer Cell Line Model.

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Abstract

Lung cancer persistently poses as a primary factor in cancer-linked deaths globally, necessitating novel treatment strategies. This investigation appraises the anti-tumor potential of Thymoquinone (TQ) and 1,3,4-oxadiazole derivatives independently and jointly against lung malignancy. immunohistochemical (IHC) analysis exhibited meaningful alteration of Bcl-2, CD4, and CD8 manifestation, signifying heightened apoptosis and immune response. Molecular studies demonstrated the compounds' ability to adjust key genes involved in cancer development, specifically Caspase-1 and mTOR pathways. The combined therapy exhibited synergistic impacts, suggesting a promising approach for lung cancer management. These conclusions highlight the potential of TQ and 1,3,4-oxadiazole as effective therapeutic agents, warranting further clinical exploration and research.

Keywords: Lung cancer, Thymoquinone, 1,3,4 oxadiazole, Bcl2, CD4, CD8, mTOR, Caspase1.

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1. Introduction

The most frequently diagnosed malignancy on a global scale has been lung cancer for the past several decades. With a global cancer burden of 12%, approximately 2.1 million new lung cancer diagnoses were recorded in 2018. Variations in the pattern were observed in various regions. This disease's high fatality rate and low survival are closely correlated with its global incidence. Lung cancer continues to be the most common cause of cancer-associated mortality in men and the second most common cause of cancer-associated death in women on a global scale. About 1.8 million deaths were recorded in 2018, with 1.2 million occurring in males and 576,100 in women. This figure represents one in five cancer-related deaths worldwide [1, 2]. The two primary histologic classes into which lung cancer tumors are dichotomized are SCLC and NSCLC. Adenocarcinoma accounts for approximately 40% of lung malignancies, squamous cell carcinoma for 25% to 30%, and large cell carcinomas for 10% to 15%, with NSCLC responsible for over 80% to 85% of lung malignancies [3, 4]. The most prevalent risk factor for lung cancer in the present day is without a doubt tobacco consumption. At the outset of the 20th century, lung cancer was a rare disease that was causally linked to tobacco use [5]. For the purpose

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of preventing, treating, and managing immunodeficiency and allergic diseases, there is a requirement for innovative methods that can influence specific pathological pathways and enhance immune responses... Throughout the Southwest Asian region, black seed or black cumin, (family Ranunculaceae), has been extensively used as a herbal medication and dietary constituent to treat a variety of inflammatory and allergic diseases [6]. It has been indicated in numerous reports that it has a wide variety of potential therapeutic benefits, such as antibacterial, antidiabetic, hypotensive, neuroprotective, hepatoprotective, antihistaminic, gastroprotective, anti-inflammatory, anticancer, and antioxidant qualities. The majority of the therapeutic benefits of N. sativa seed oil have been attributed to the presence of thymoquinone (TQ), a primary active constituent.

The oxadiazoles are heterocyclic compounds with five members, each of which contains one oxygen and two nitrogen elements. Four distinct isomers of oxadiazoles may exist, The compound's nomenclature is determined by the position of nitrogen atoms: 1,2,3-oxadiazole, 1,2,4-oxadiazole, 1,2,5-oxadiazole, and 1,3,4-oxadiazole. Their substantial influence is emphasized by their widespread application in a diverse array of scientific fields, such as the pharmaceutical industry and drug discovery [7]. Also, it is important to mention that compounds that contain 1,3,4-oxadiazole units demonstrate a diverse array of biological activities, including anti-tubercular antiparasitic, antibacterial, anti-inflammatory properties, antidepressant, and antifungal [7-9]. The anticancer activity may be enhanced by the combination of natural products and synthetic pharmaceuticals.

2. Material and Method

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

The B16/F10 melanoma cell line originating from a gp100-positive spontaneous murine melanoma was acquired from the cell culture department at NAWAH Scientific Center, Cairo, Egypt. The cells were maintained at 37°C in Dulbecco's Modified Eagle Medium, which was supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, in a humidified atmosphere containing 5% CO2. Subculturing was initiated when the cells reached 70-80% confluence. Cells were detached using a 0.25% trypsin-EDTA solution and subsequently neutralized with a total medium at this juncture. Subsequently, the trypan blue exclusion method was implemented to estimate cell viability. This method involves the addition of a 0.4% trypan blue solution to the detached cell suspension, and the subsequent counting of viable cells under a light microscope with a hemocytometer. Only cultures exhibiting over 95% viability were deemed suitable for use in experiments.

2.2. Chemicals and Reagents

The chemistry department of the faculty of science at Damanhour University, Egypt, supplied a 1,3,4-oxadiazole-2-thione derivative. Sigma-Aldrich Chemicals Company in St. Louis, USA, MO, was used to purchase thymoquinone (purity ≥98%), catalog number 274666.

2.3. Experimental Design and Animal Model

The investigation was conducted using 37 healthy-grown female C57BL/6 rodents, which were aged 6 to 7 weeks and had an average body weight of 25 grams. The rodents were procured from the animal residence of the National Research Center in Dokki, Cairo, Egypt. The animals had access to water at all times and were fed mouse chow. In Egypt, the Institutional Animal Ethics Committee of Damanhour University's Faculty of Science approved all research involving animals, as indicated by the reference numbers **DMU-SCI-CSRE-24-9-04**. All procedures adhered to the regulations set by the Committee for the Purpose of Control and Supervision of Experiments on Animals. The rodents were initially divided into two primary groups at the beginning of the experiment: The initial group (Control Group) consisted of seven rodents that served as the standard control.

The second group, 30 mice were intravenously administered with B16/F10 melanoma cells (1 × 106 cells/mL in 0.1 ml phosphate-buffered saline [PBS]) through the tail vein to form the Tumor-Bearing Group [10]. The tumor-bearing mice were monitored for 25 days to enable the evolution of lung tumor foci. After this period, two mice were randomly dissected to confirm the visibility of lung tumors. After confirmation, the remaining 28 mice in Group 2 were further separated into four subgroups (Groups 2–5), each containing 7 mice, to obtain particular treatments as outlined in the experimental protocol. Group 2: Served as lung cancer group without any treatment, Group 3: Lung cancer grouping treated with Nigella sativa seed extract (Thymoquinone) 40 mg/kg.bw [11] Group 4: Lung cancer group treated with 1,3,4-oxadiazole-2-thiones derivative (50 mg/kg) [12] Group 5: Lung cancer grouping treated with the combination of 1,3,4-oxadiazole-2-thiones derivative 50 mg/kg and Nigella sativa seed extract (Thymoquinone) 40 mg/ kg, This experimental design enabled the assessment of the therapeutic effects of Thymoquinone, the 1,3,4-oxadiazole-2-thione derivative, and their combination on lung tumor progression in the B16/F10 melanoma model. Four weeks after the final dose, all rodents were anesthetized with a 100 mg/mL solution of ketamine in sterilized saline and subsequently administered a dose of 120 mg/kg intraperitoneally (IP). Food was withheld from them overnight, and they were sacrificed through cervical decapitation [13].

2.4. Blood Sample Collection

Cardiac puncture using sterile capillary tubes was used to obtain blood samples. Part of the sample was mixed with dipotassium salt of EDTA as an anticoagulant for different hematological analyses.

2.5. Tissue Sample Collection and Preparation

Lung tissues were promptly extracted and cut into small pieces. Parts of the lung tissues were rinsed in ice-cold normal saline (0.9%) and stored for subsequent molecular and histological analyses.

2.6. Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was used to evaluate Bcl2, CD4, and CD8 protein expression in the lung tumor microenvironment. Ready to these targets use antibodies: Bcl2 (both up and down-regulation), CD4 and CD8 to confirm-directed association. Tissue blocks of formalin-fixed, paraffin-embedded lung tissue were sectioned at a thickness of 4 µm. The formation of a brown precipitate resulted from antibody binding, and the antigen was localized using 3,3'-diaminobenzidine (DAB) as a substrate. The precipitate was counterstained with Mayer's Hematoxylin.

2.7. Molecular Biology Investigations

The data was quantified using the comparative Ct (2-ΔΔCt) method, and the corresponding expression level of target genes was adjusted to GAPDH. The gene expression levels of m-TOR, Caspase 1, and GAPDH (which functions as an internal control) were analyzed using quantitative real-time PCR (qRT-PCR).

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

RNeasy Mini Kit (Qiagen, Hilden, Germany; Catalog no. 74104) was employed to extract total RNA from rodent tissue in the Physiology Laboratory of the Faculty of Science at Damanhour University, Egypt. To assess the integrity and concentration of RNA, the NanoPhotometer® NP80 (IMCERT Lab, Damanhour University) was employed. An A260/A280 ratio of 1.8 to 2.0 was employed to evaluate the purity of the RNA, with values in this range suggesting high-quality RNA.

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Invitrogen, USA; Catalog no. 4368814) was implemented to produce complementary DNA (cDNA) from the isolated RNA. The reverse transcription was conducted at the IMCERT Lab, Central Laboratories Building, Damanhour University. The Applied Biosystems StepOne™ Real-Time PCR system was employed to amplify the cDNA that was obtained using specific TaqMan primers. StepOne™ software

(IMCERT Lab, Damanhour University) was employed to analyze the data.

3. RESULTS

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

Table 1 showed that A pronounced drop in hemoglobin level was identified in the tumor group versus the control group. In contrast, the TQ and Oxadiazole treatment groups, either individually or in combination, demonstrated a significant increase in hemoglobin levels in comparison to the tumor group ($p \leq 0.05$). In contrast to the control group, the tumor group exhibited a significant reduction in red blood cell count. However, the RBC count in the treatment groups was considerably higher than that of the tumor group ($p \leq 0.05$). In contrast, the control group exhibited a substantially lower level of white blood cells (WBCs) than the tumor group. The interventions effectively reduced inflammation, as demonstrated by the substantial decrease in WBC counts between the treatment groups and the tumor group ($p \leq 0.05$).

In comparison to the control group, the tumor group exhibited a substantial increase in platelet count ($p \leq 0.05$). Conversely, the treatment groups exhibited significantly lower platelet counts than the tumor group.

Table 2 This demonstrates that the lymphocyte count in the tumor group was considerably lower than that of the control group ($p \leq 0.05$). However, the lymphocyte counts in the TQ and Oxadiazole treatment separately or in combination were greater than those in the tumor group. Finally, the total neutrophil count of the tumor group is markedly higher than that of the control group ($p \leq 0.05$). Additionally, the neutrophil count was significantly lower in the treatment groups than in the tumor group. In contrast, the control group exhibited a substantially lower monocyte count than the tumor group. The monocyte count in the treatment groups was markedly lower than that of the tumor group ($p \leq 0.05$). Furthermore, the basophil count was significantly higher in the tumor group than in the control group ($p \leq 0.05$). Conversely, Basophils were statistically decreased in the treatment groups compared to the tumor group. Furthermore, the eosinophil count of the tumor group was significantly elevated in comparison to that of the control group. In contrast, the tumor group exhibited a substantial decrease in eosinophil count in the treatment groups ($p \leq 0.05$).

Table 1. The effect of thymoquinone and 1,3,4-oxadiazole-2-thiones derivative treatment on complete blood count in all studied groups.

Groups	HGB (g\dl)	RBC (M/µL)	WBC (K/µL)	PLT (K/µL)
Control	13.83 ± 0.13	9.24 ± 0.13	8.33 ± 0.11	1170.9 ± 2.67
Tumor	8.33^{a} ± 0.18	6.19^{a} ± 0.11	12.41^{a} ± 0.16	1395.9^{a} ± 3.08
TQ	13.23^{ab} ± 0.15	9.07^{b} ± 0.11	8.29^{b} ± 0.17	1155.6^{ab} ± 3.87
Oxadiazole	13.13^{ab} ± 0.11	8.89^{abc} ± 0.11	8.20^{b} ± 0.12	1135.4^{abc} ± 3.82
Oxadiazole + TQ	13.84^{bcd} ± 0.10	9.20^{bd} ± 0.12	8.24^{b} ± 0.13	1159.7^{abd} ± 1.50
F	2073.441^{*}	928.423^{*}	1273.063^{*}	8476.509^{*}
p	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$

a: Significant with Control, b: Significant with Tumor, c: Significant with TQ, d: Significant with Oxadiazole.

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

Table 2. The effect of thymoquinone and 1,3,4-oxadiazole-2-thiones derivative treatment on absolute differential leukocyte count in all studied groups.

Groups	LYMPH	NEUT	MONO	BASO	EOSINO
Control	5.87 ± 0.09	1.59 ± 0.04	0.38 ± 0.02	0.114 ± 0.014	0.36 ± 0.02
Tumor	3.77^{a} ± 0.03	7.25^{a} ± 0.09	0.64^{a} ± 0.02	0.177^{a} ± 0.015	0.53^{a} ± 0.03
TQ	5.70^{ab} ± 0.12	1.68^{ab} ± 0.04	0.51^{ab} ± 0.01	0.109^{b} ± 0.011	0.27^{ab} ± 0.01
Oxadiazole	5.11^{abc} ± 0.08	2.15^{abc} ± 0.03	0.52^{ab} ± 0.01	0.093^{ab} ± 0.008	0.30^{abc} ± 0.01
Oxadiazole +TQ	4.39^{abcd} ± 0.06	2.82^{abcd} ± 0.06	0.58^{abcd} ± 0.02	0.096^{ab} ± 0.005	0.33^{abc} ± 0.01
F	824.393^{*}	12381.00^{*}	266.859^{*}	66.825^{*}	262.909^{*}
p	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$

a: Significant with Control, b: Significant with Tumor, c: Significant with TQ, d: Significant with Oxadiazole

3.3. Immunohistochemical study

3.3.1 The effect of lung cancer and treatments on the expression of Bcl2, CD4, and CD8 (IHC Score)

Figure 1 shows that the IHC Score of the immunostaining reactivity percentage of Bcl2 in the tumor group tissue (Fig. 5) was significantly increased compared to the control group (Fig. 4). This percentage value was significantly decreased in the tumor-treatment groups TQ (Fig.7) and, Oxadiazole (Fig. 6) individually or in combination (Fig. 8) when compared with the tumor group (Fig 5).

The immunohistochemical staining score for CD4 confirmed that the tumor-infiltrating T lymphocytes were predominantly located in the lung cancer group. (Fig. 2) . The number of infiltrating CD4 T cells in the tumor group (Fig. 10) was significantly higher than that of the control group (Fig. 9). Conversely, the number of infiltrating CD4 T cells in the treated groups, TQ (Fig. 12), oxadiazole (Fig. 11), or a combination of the two (Fig. 13), was significantly lower.

The tumor-infiltrating T lymphocytes were predominantly observed within the lung cancer group and the other treated groups (Fig. 3), confirmed by the immunohistochemical staining score for CD 8, where the number of infiltrating CD8 T cells in the tumor group (Fig 15) was overwhelmingly in comparison with that within control group (Fig.14). On the other hand, the number of infiltrating CD8 T cells in the treatment groups, oxadiazole (Fig.16), TQ (Fig.17) or in a combination of them (Fig.18) was reduced.

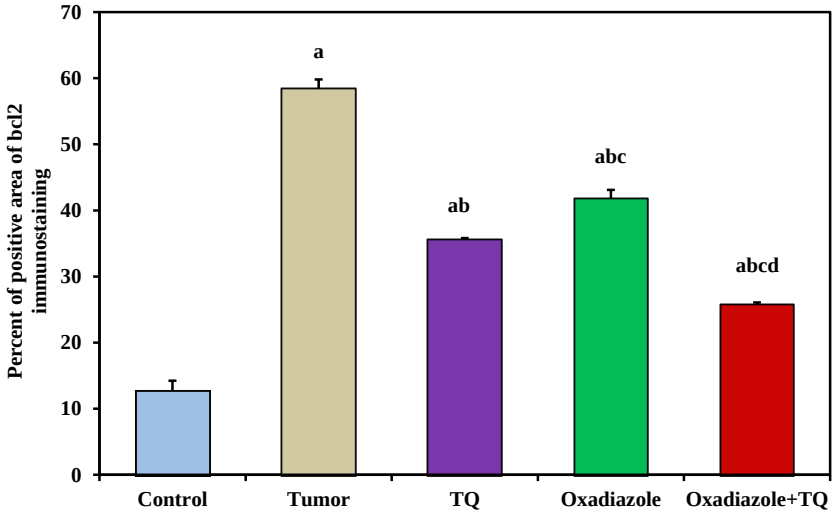

Figure 1. a: Significantly with Control, b: Significantly with Tumor, c: Significantly with TQ, and d: Significantly with Oxadiazole A comparison of the numerous research groups based on the percentage of the positive area of bcl2 immunostaining

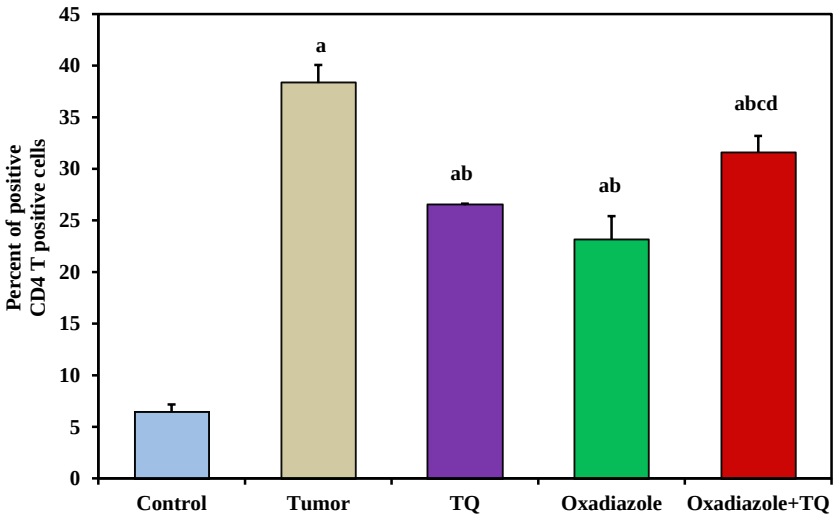

Figure 2. A comparison of the multiple analyzed groups based on the percentage of positive CD4 T cells. a: Significant with Control, b: Significant with Tumor, c: Significant with TQ, and d: Significant with Oxadiazole.

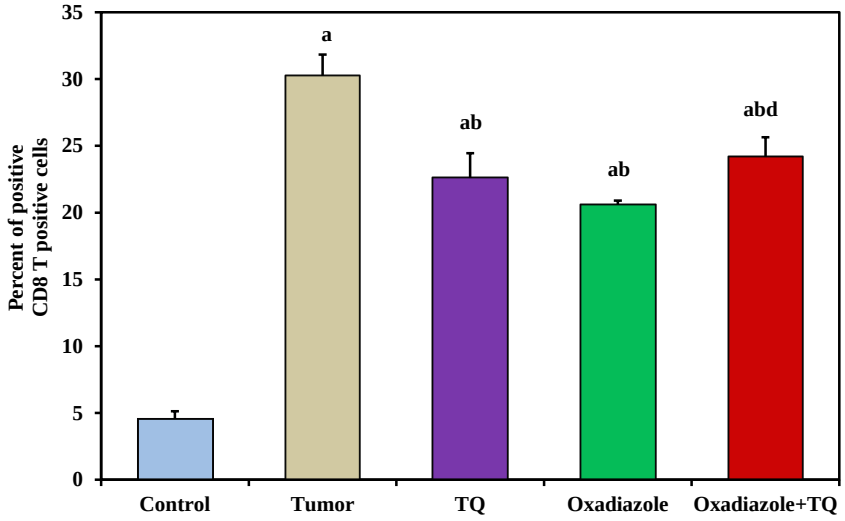

Figure 3. Comparison of the diverse groups that were analyzed according to the percentage of CD8 T cells that were positive. Significant with Control: a, Significant with Tumor: b, Significant with TQ: c, and d; Significant with Oxadiazole

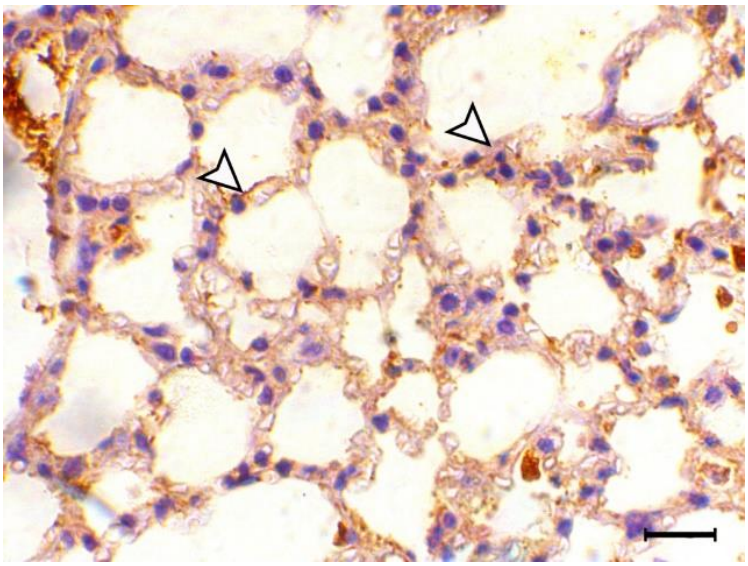

Figure 4. Lung of the control animal showing cytoplasmic expression of BCL2 antibody within the pneumocytes (arrowheads), BCL2 IHC, bar = 25 µm.

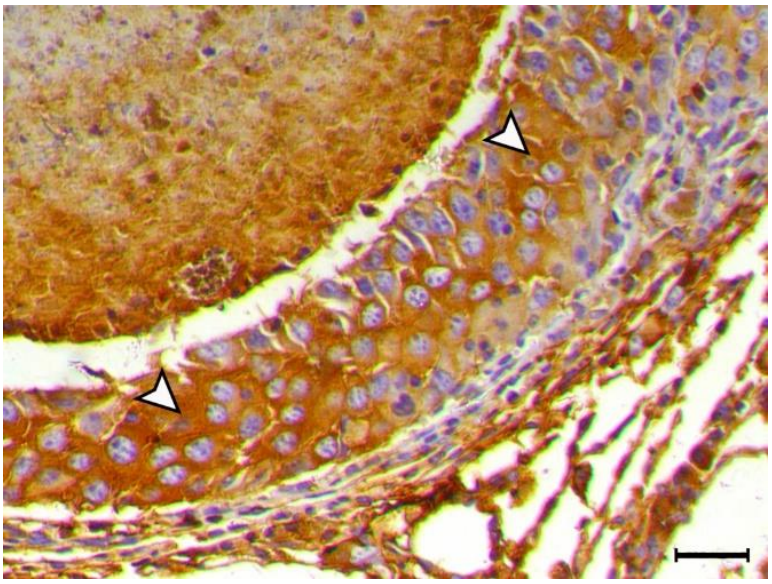

Figure 5. The lung of the diseased animal tumor group shows marked cytoplasmic expression of BCL2 antibody within the neoplastic cells (arrowheads), BCL2IHC, bar = 25 µm.

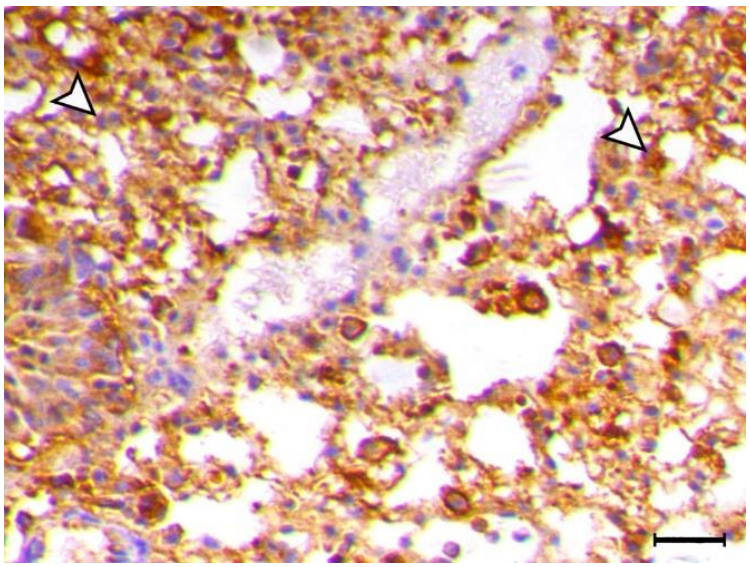

Figure 6. The lung of the oxadiazole-treated animal group shows decreased cytoplasmic expression of BCL2 antibody within the pneumocytes (arrowheads), BCL2 IHC, bar = 25 µm.

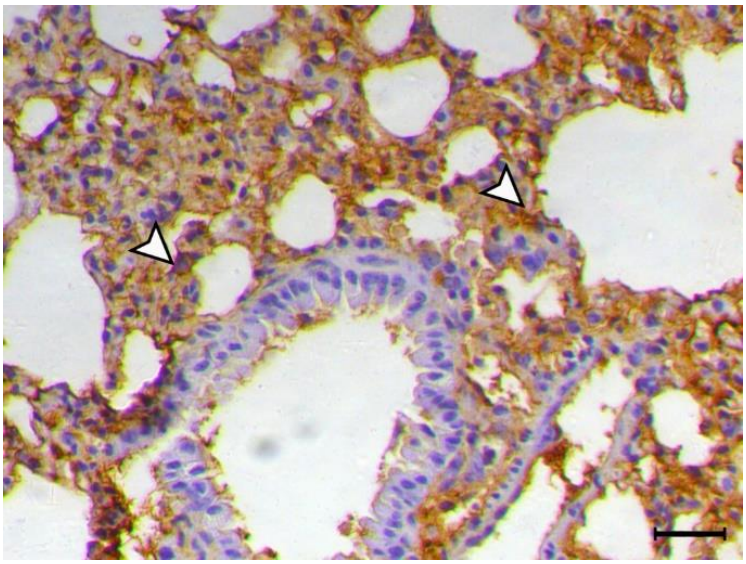

Figure 7. The lung of the TQ-treated animal group shows decreased cytoplasmic of BCL2 antibody within the pneumocytes (arrowheads), BCL2 IHC, bar = 25 µm.

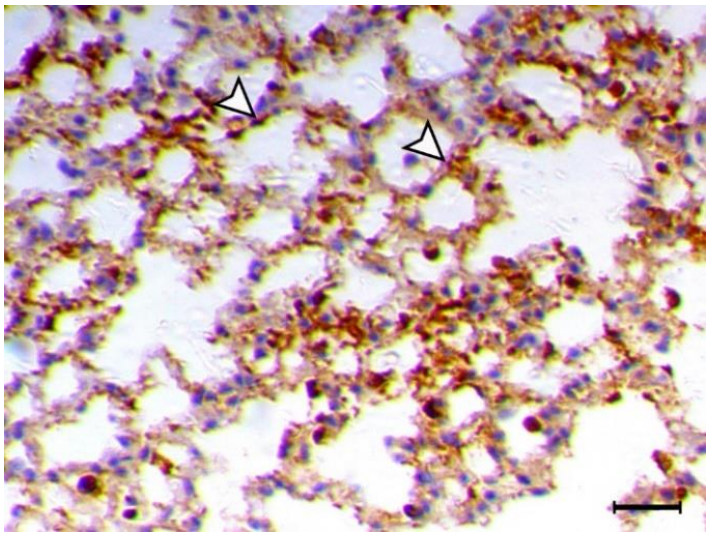

Figure 8. The lung of a diseased animal treated with a combination of TQ and oxadiazole group showing a noticeable decrease of cytoplasmic expression of BCL2 antibody within the pneumocytes (arrowheads), BCL2 IHC, bar = 25 µm.

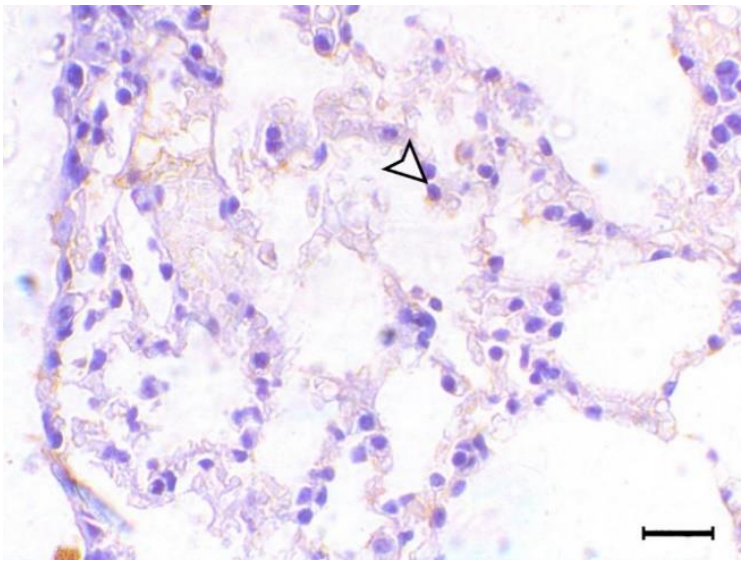

Figure 9. The lung of the control animal group shows a mild expression of CD4 antibody within the interstitial cells (arrowhead), CD4 IHC, bar = 25 µm.

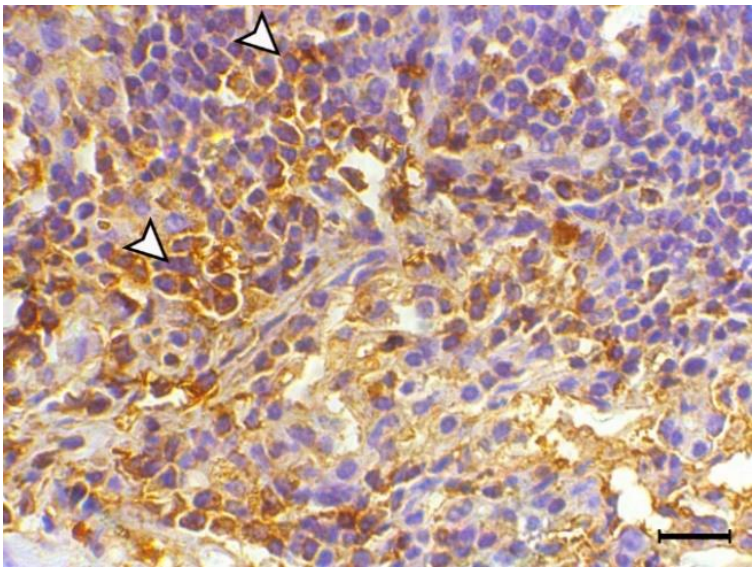

Figure 10. The lung of the diseased animal tumor group shows a marked expression of CD4 antibody within the pulmonary tissues (arrowheads), CD4IHC, bar = 25 µm.

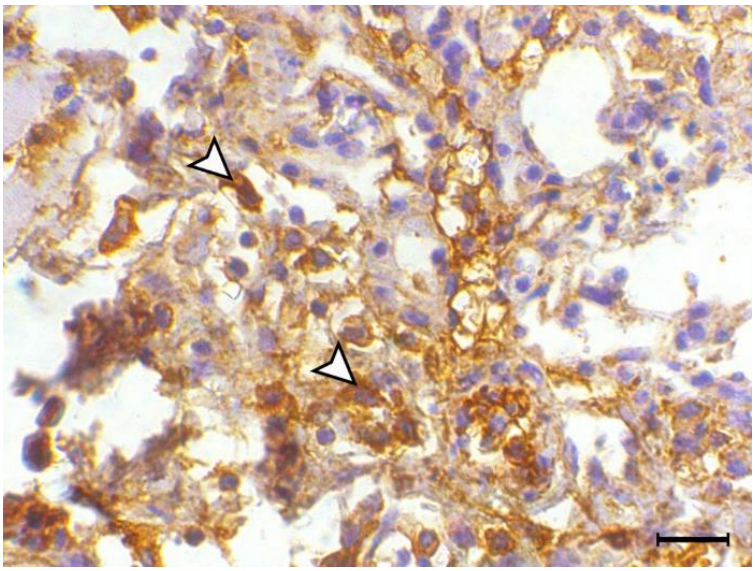

Figure 11. The lung of a diseased animal oxadiazole-treated group shows a decreased expression of CD4 antibody within the pulmonary tissues (arrowheads), CD4 IHC, bar = 25 µm.

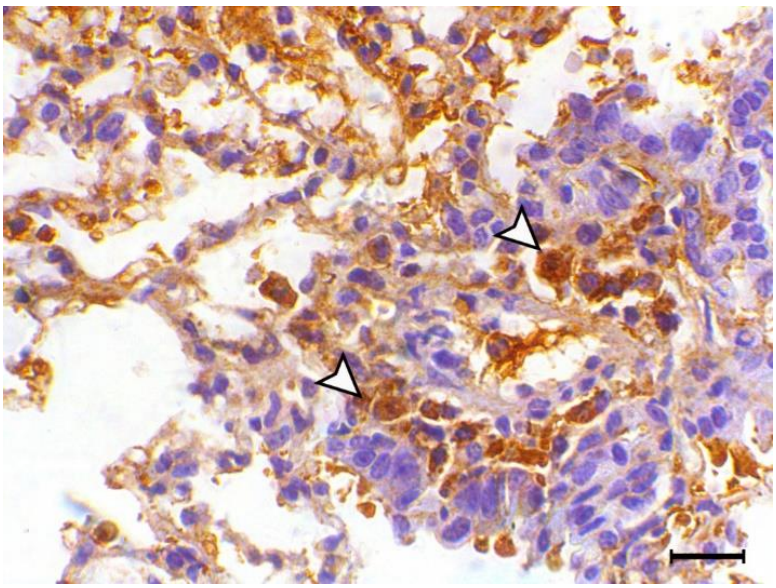

Figure 12. The lung of a diseased animal treated with TQ shows a decreased CD4 antibody immunostaining within the pulmonary tissues (arrowheads), CD4 IHC, bar = 25 µm.

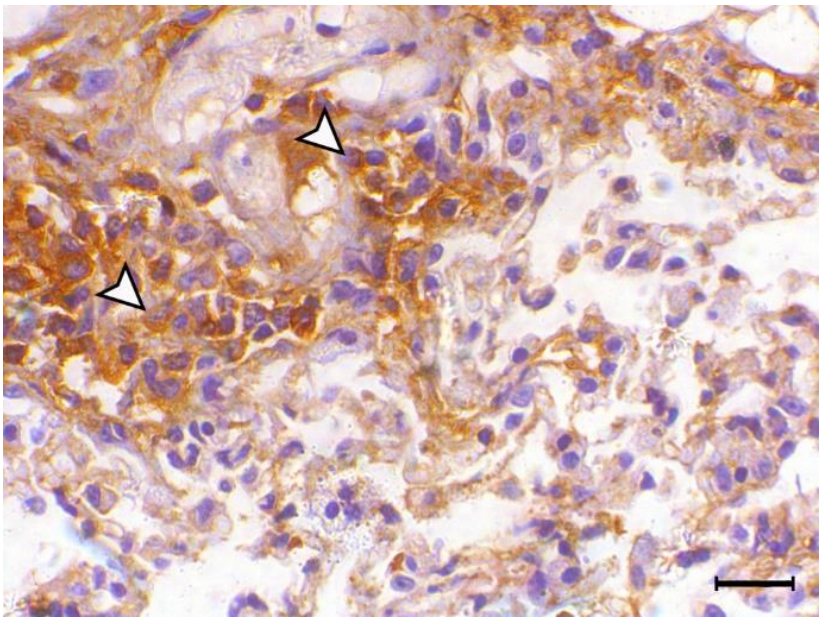

Figure 13. The lung of a diseased animal treated with a combination of TQ and oxadiazole shows a decreased expression of CD4 antibody within the pulmonary tissues (arrowheads), CD4 IHC, bar = 25 µm

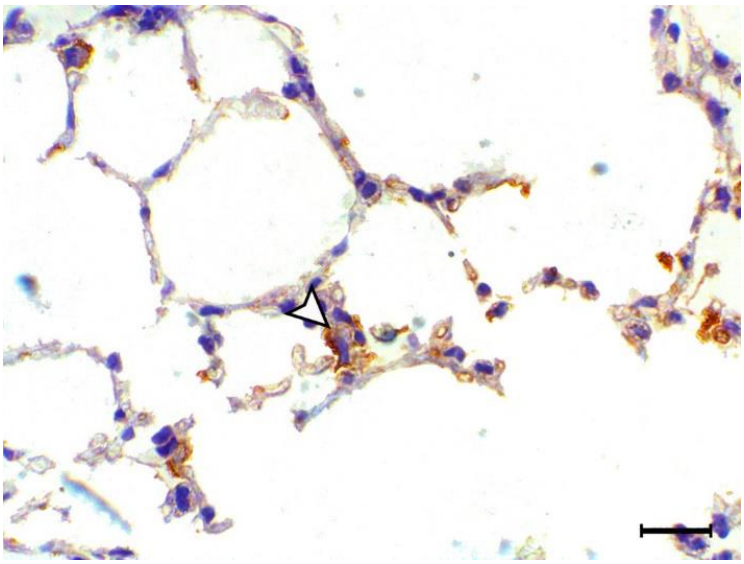

Figure 14. The lung of a control animal shows a mild expression of CD8 antibody within the interstitial cells (arrowhead), CD8 IHC, bar = 25 µm.

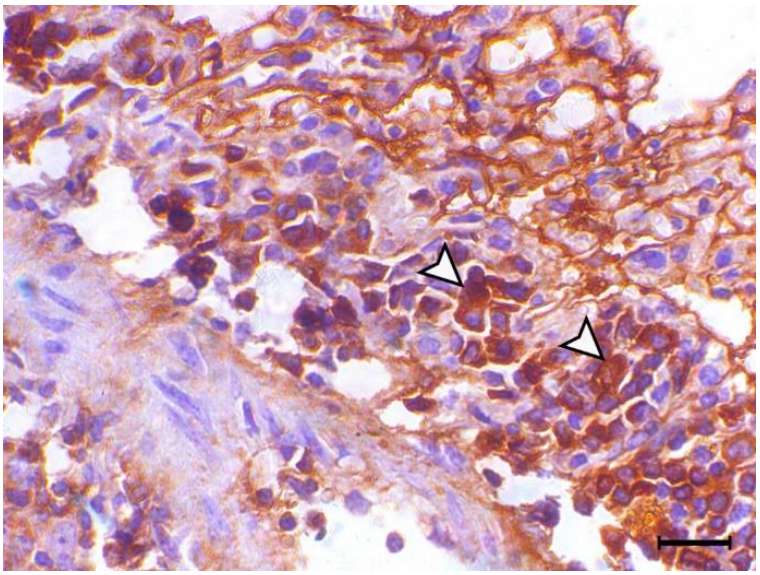

Figure 15. The lung of a diseased animal (tumor) group shows a marked expression of CD8 antibody within the pulmonary tissues (arrowheads), CD8IHC, bar = 25 µm.

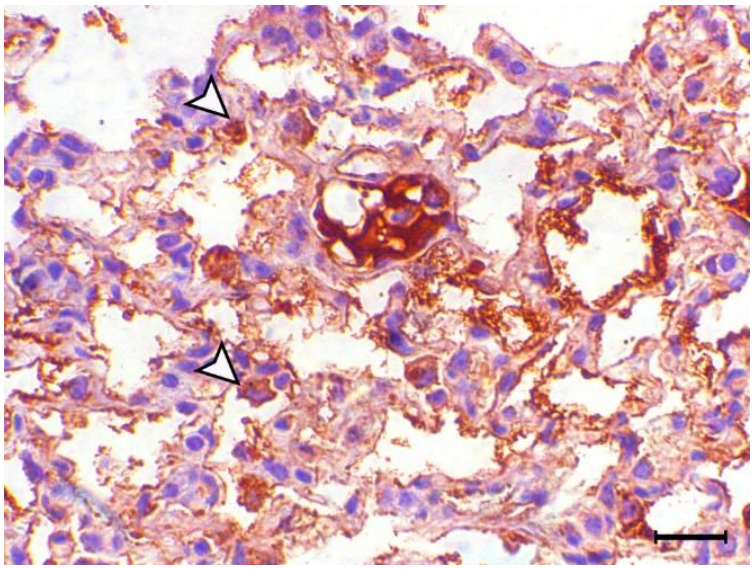

Figure 16. The lung of a diseased animal treated with oxadiazole shows a decreased expression of CD8 antibody within the pulmonary tissues (arrowheads), CD8 IHC, bar = 25 µm.

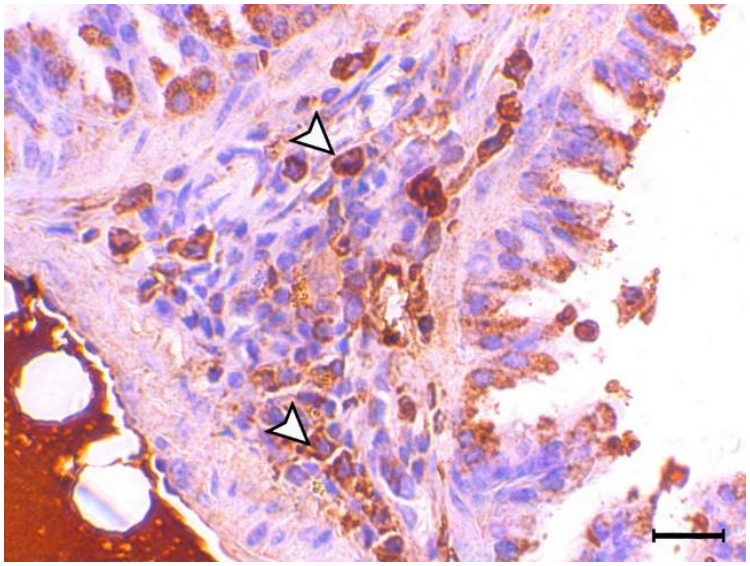

Figure 17. The lung of a diseased animal treated with TQ shows a decreased CD8 antibody immunostaining within the pulmonary tissues (arrowheads), CD8 IHC, bar = 25 µm.

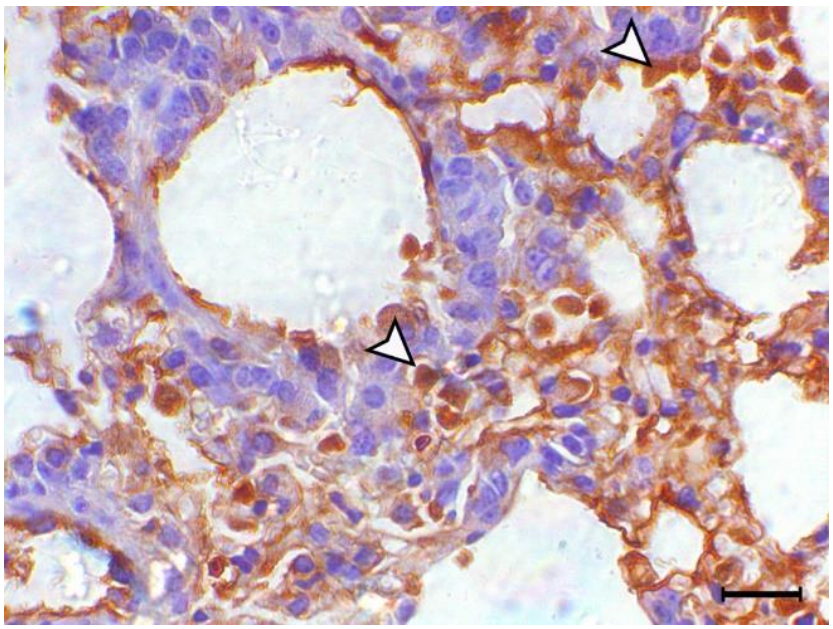

Figure 18. The lung of a diseased animal treated with a combination of TQ and oxadiazole shows a decreased expression of CD8 antibody within the pulmonary tissues (arrowheads), CD8 IHC, bar = 25 µm.

3.4. mTOR mRNA Expression in Lung Cancer and Healthy Lung Tissue Assessed by RT-PCR

In this investigation, the mTOR gene expression was identified in the lung tissues of all examined groups through real-time quantitative PCR. The tumor tissues and non-tumor tissues exhibited a substantial disparity in mTOR expression (Fig. 19). The two groups exhibited a substantial fold difference (4.84 vs. 1.032). Additionally, the treated groups, which were either oxadiazole alone or in combination, exhibited a significant fold difference in mTOR expression in comparison to the tumor group (3.22, 3.64, and 2.88)

3.5. Caspase 1 mRNA Expression in Lung Cancer and Healthy Lung Tissue Assessed by RT-PCR

Real-time quantitative PCR was employed to detect the expression of Caspase 1 mRNA in both tumor and normal lung tissues. The tumor tissues and non-tumor tissues exhibited a substantial disparity in Caspase 1 expression (Fig. 20). A substantial fold difference was observed between the two groups (3.38 vs. 0.988). Additionally, the treatment groups, which included TQ, oxadiazole alone, or a combination of the two, exhibited a significant difference. A substantial factor difference in Caspase 1 expression was observed in comparison to the tumor group (2.44, 2.62, and 2.26)

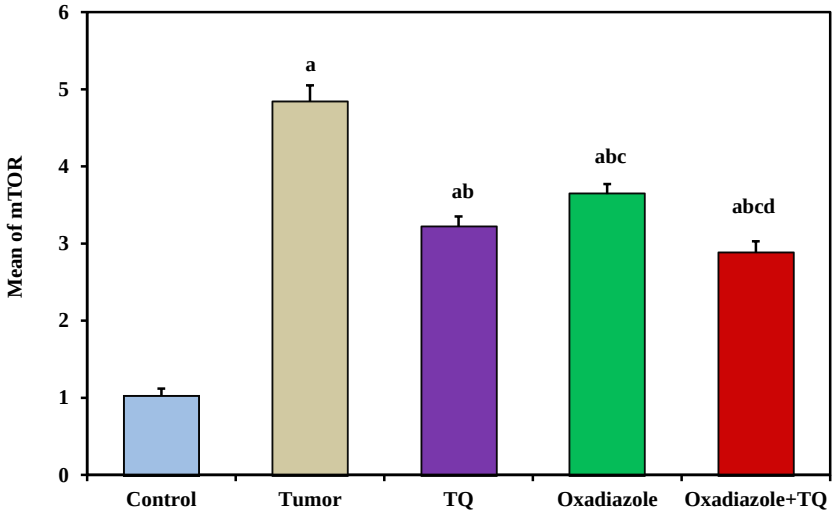

Figure 19. a: Significant with Control, b: Significant with Tumor, c: Significant with TQ, d: Significant with Oxadiazole Comparison of the various researched groups based on the fold change of mTOR

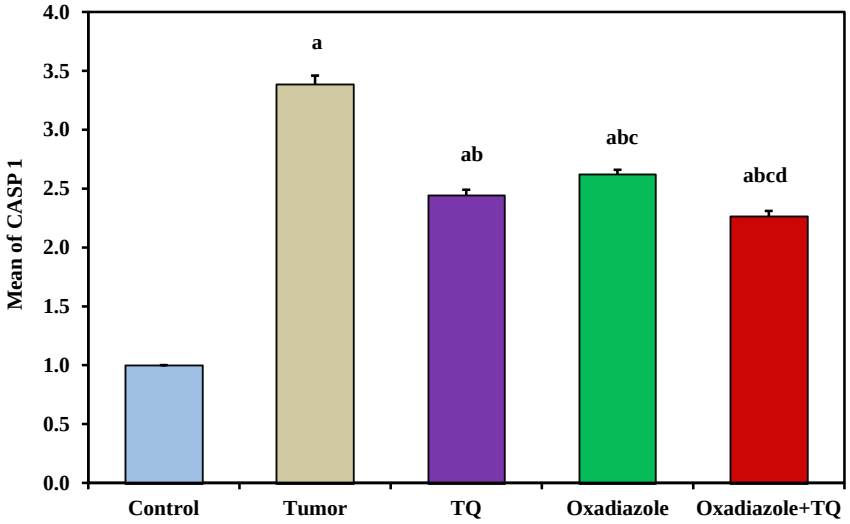

Figure 20. a: Significant with Control, b: Significant with Tumor, c: Significant with TQ, d: Significant with Oxadiazole Comparison of the various investigated groups based on the fold change of CASP 1

4. DISCUSSION

Lung cancer significantly elevates the levels of numerous pro-inflammatory cytokines, like interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-alpha (TNF-α). In the progression of cancer-related anemia (CRA) in bronchoalveolar lavage fluid, blood, and lung tissue, IL-6 is of particular importance. [14,15]. By inhibiting erythropoiesis, IL-6 reduces the proliferation of red blood cell (RBC) progenitors, reduces their response to erythropoietin (EPO), and affects iron homeostasis by inducing the synthesis of hepcidin, a hormone that prevents the availability of iron in the circulation for hemoglobin formation[16-18]. This is agreed and clarifies our Results which demonstrate a significant decrease in hemoglobin level and RBCs count in the B16F10 melanoma metastasis lung cancer group (gp2). Moreover, TNF-α and IL-1β decrease EPO production, which also decreases RBC production and induces oxidative stress, thereby stimulating eryptosis (premature death of RBCs), and worsening cancer-related anemia [19-21]. This process is further complicated by the hypoxia-inducible factor-1α (HIF-1α) induction in the tumor microenvironment, which promotes the expression of both EPO and EPO receptor (EPOR) [22]. Yet, this immune response can result in ineffective erythropoiesis, which results in anemia Systemic inflammation also induces hepcidin production in hepatocytes, which reduces the absorption of iron from the intestine and the discharge of iron from macrophages, limiting iron for erythropoiesis [23, 24].

Thymoquinone (TQ), a natural compound known for its potent anti-inflammatory and antioxidant properties, has shown potential in solving these problems. The significantly increased amount of hemoglobin and RBCs count in the TQ-treated group (gp3) confirmed the therapeutic role of thymoquinone as it significantly reduces the pro-inflammatory cytokines including interleukin-6 (IL-6), tumor necrosis-alpha (TNF-α), and interleukin-1 beta (IL-1β) levels, shown by both protein and mRNA expression respectively [25] TQ down-regulates IL-6 which ultimately reduces hepcidin level which enhances the available iron for RBC production. Moreover, TQ down-regulates the HIF-1α and NF-κB pathways, reducing inflammation and oxidative stress, both of which play pivotal roles in CRA [26] [27]. Likewise, the gp4 showed the protective effects of Oxadiazole derivative on hemoglobin level and RBCs count, this may be due to the anti-inflammatory effects of oxadiazole derivatives through inhibiting the activity of reactive oxygen species (ROS) and nitric oxide (NO) [28] [29] , IL-6 and TNF-α, in activated macrophages Additionally, oxadiazole may reduce IL-6, subsequently limiting hepcidin and improving iron metabolism, with the consequent beneficial effects on erythropoiesis. Co-administration of TQ and oxadiazole derivative may synergistically potentiate these effects and, therefore, may provide a potential therapeutic strategy for CRA in lung cancer patients as validated by the present study through a highly significant increase in the hemoglobin level and RBCs count in the (gp5) co-treated group.

The hematopoietic system is frequently dysregulated by lung cancer, as evidenced by an elevated white blood cell (WBC) and platelet (PLT) count. This association is confirmed by our Results (GP2), which indicate a substantial increase in the count of platelets and white blood cells. Tumor and immune cells secrete granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which promote the production of white blood cells (WBCs) and platelets, in addition, to inducing hyper hematopoiesis and extramedullaring hematopoiesis [30, 31]. Additionally, pro-inflammatory cytokines (e.g., IL-1β and IL-6) also stimulate thrombocytosis via TPO-dependent mechanisms [32-35].

The substantial ameliorative effects seen with TQ (GP3), the oxadiazole derivative (GP4), and the combination treatment (GP5) could be attributed to

their action of inhibiting these cytokines, thus normalizing WBC and platelet counts, facilitating balanced hematopoiesis [36] [28]. Furthermore, based on the properties of TQ and oxadiazole derivatives, including anti-inflammatory, antioxidant, anthelmintic, antibacterial, anticancer, and antiangiogenic activity, they are likely to provide phenotypic rescue for hemoglobin levels and hamper aberrant hematopoiesis in lung cancer patients. These compounds may offer a multifaceted strategy for addressing hematological malignancy-related complications through modulation of cytokine release and inflammatory signaling.

The present results also showed that the melanoma metastasized lung cancer group displayed the greater reactivity of Bcl-2 IHC, which is following the anti-apoptotic protein characteristics. Bcl-2 helps tumor survival by reducing apoptosis. it prevents the release of cytochrome c, and so cascades blocking caspases activation and Binds to the have (BH3) domains of pro-apoptotic proteins such as Bax neutralizing their effect [37, 38]. On the other hand, the groups received Thymoquinone (TQ), 1,3,4 oxadiazole derivatives, and the combination of both treatments revealed a significant decrease in Bcl-2 overexpression. TQ also inhibits the overexpression of Bcl-2, similar to the 1,3,4-oxadiazole derivatives. TQ binds to the residues that comprise the active site of Bcl-2, such as Ser205, which inhibits activity and reduces expression leading to apoptosis. In the same vein, 1,3,4-oxadiazole derivatives lower the expression of Bcl-2, while simultaneously increasing the levels of Bax activating caspase-3 and the pro-apoptotic protein.PARP is cleaved and apoptosis ensues as the result of this cascade. This mechanism suggests that in lung cancer cells, both TQ and 1,3,4-oxadiazole compounds induce apoptosis via a caspase-dependent pathway. [39-44].

In the context of anti-tumor immunity, the role of CD8+ and CD4+ T cells The immune system's primary mediators against tumor cells are CD4+ and CD8+ T cells, which serve as central effectors in the development of anti-tumor immunity. The primary effector cells for the anti-tumor response are CD8+ T cells or CTLs. The current results showed high IHC staining reactivity of CD4+ and CD8+ in tumor-induced mice group with CD4+ and CD8+ T cells, both of which are preferentially overexpressed in lung cancer tissues and are important mediators of antitumor immunity. By recognizing tumor-specific antigens on MHC class I molecules, these cells directly destroy tumor cells. This mechanism consists of the secretion of cytotoxic molecules, including perforin and granzymes that trigger apoptosis and kill neoplastic cells [45, 46]. By stimulating the activity of CD8+ T cells and other immune cells, CD4+ T cells or helper T cells promote anti-tumor immunity. They also emit cytokines, including IFN-γ and IL-2, which promote the activity and expansion of CD8+ T cells. In addition, they have the capacity to differentiate into Th1 CD4 + T cells, which offer supplementary anti-tumor responses [47]. CD8+ cytotoxic T cells, which are the main effectors targeting tumors, interact with HLA on the surface of cancer cells through HLA molecules to trigger HLA-restricted cytotoxic responses that lead to the inhibition of growth and metastasis of tumors [48]. CD4+ T cells can undergo additional differentiation into regulatory T cells or helper T cells, contingent upon their context within the tumor microenvironment. (Treg) that is known to suppress immune responses [49] [50].

T-cell recruitment and survival is driven by interleukin-6 (IL-6). It promotes chemokine expression to recruit T cells, inhibits apoptosis of T cells via the STAT3 pathway, and supports Th1/Th2 differentiation as well as B cell helper function. In animal studies [51-54], mice treated with TQ and mice treated with the 1,3,4-oxadiazole derivative showed activity related to the modulation of immune responses by suppressing IL-6 signaling and inhibiting pro-inflammatory substances including IL-6 and TNF-α. TQ could also inhibit NF-κB activation leading to a decrease in the

inflammatory-induced immunosuppression, which could further lead to increased anti-tumor immunity. Overall, these findings indicate that TQ and 1,3,4-oxadiazole derivatives can modulate T cell activity and reduce inflammation in the tumor microenvironment [26] [55] [28].

CD4+ and CD8+ T cells exhibit both stimulatory and inhibitory effects on anti-tumor immunity, and apoptosis is suppressed in lung cancer by the overexpression of Bcl-2. These mechanistic cellular pathways are targeted by both TQ and 1,3,4-oxadiazole derivatives which were observed to downregulate Bcl-2, induce apoptosis, and modulate immune responses via IL-6 and NF-κB inhibition. These effects suggest a new potential therapeutic agent for restoring immune balance and inhibiting tumor growth in lung cancer.

A serine/threonine kinase known as the mammalian target of rapamycin (mTOR) regulates cell growth, proliferation, and metabolism by forming two distinct subcomplexes, mTORC2 and mTORC1. mTORC1 augments the biosynthesis of proteins and lipids by incorporating signals from growth factors, nutrients, and energy.The P13K/AKT/mTOR pathway's feedback is enhanced by mTORC2's phosphorylation of AKT. Deregulation of this pathway, which is frequently the consequence of mutations in, AKT, PI3K, or KRAS, or the loss of PTEN, is a prevalent phenomenon in non-small cell lung cancer (NSCLC) and contributes to late-stage disease and tumorigenesis [56] [57].

TQ treatment downregulates the mTOR overexpression which is generated by tumor-induced mice possibly through inhibition of phosphorylation of Akt and upregulation of tumor suppressor PTEN which negatively regulates the PI3K/AKT/mTOR pathway. This inhibition leads to mTOR inhibition affecting several processes, such as cell proliferation and survival [58], Likewise, 1,3,4-oxadiazole derivatives are also inhibitors of this pathway where the inhibition of this pathway results in autophagy, which is a cellular death process activated when mTOR is inhibited [59].

Caspase-1, an inflammatory caspase, contributes to lung cancer in two ways, by processing cytokines such as IL-1β and IL-18 and triggering pyroptosis, a type of inflammatory cell death. Overexpression of caspase-1 and other inflammasome components (e.g., NLRP3, AIM2, GSDMD) is observed in NSCLC, where they contribute to tumor growth by creating a pro-inflammatory microenvironment. This is consistent with the results demonstrated in the tumor-induced mice group. However, pyroptosis can also induce cell death, making its role in cancer complex and context-dependent [60-63].

In the TQ-group, treatment with thymoquinone reduces caspase-1 activity possibly due to impaired proteolytic diversion of pro-caspase-1, which leads to decreased secretion of IL-18and IL-1β, key cytokines for tumor growth. TQ also inhibited the activity of the NLRP3 inflammasome through decreasing reactive oxygen species (ROS) [64-66], Similarly, the inhibition of the NLRP3 inflammasome and the reduction of inflammasome-associated protein expression resulted in a significant down-regulation of caspase 1 in 1,3,4-oxadiazole derivatives. further inhibiting caspase-1 activity [67]. The co-treatment group exhibited the most pronounced reduction in Caspase-1 expression, likely attributable to the synergistic interaction between the two therapeutic agents. This enhanced effect may result from their combined ability to target multiple pathways involved in inflammasome activation, such as ROS scavenging and NF-κB inhibition, leading to a more robust suppression of Caspase-1 compared to either treatment alone.

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