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The protective and antiapoptotic effects of *Hypericum triquetrifolium* Turra against cyclophosphamide-induced lung injury in rats: in vitro evaluation

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ABSTRACT

Objectives: *Hypericum triquetrifolium* Turra (HTT) has been traditionally used in medical treatments due to its sedative, antiseptic, antiinflammatory, and anthelmintic properties. The present study aims to investigate the lung-protective and antiapoptotic effects of HTT against cyclophosphamide (CP)-induced lung injury in rats.

Methods: Thirty-five Sprague Dawley rats were categorized into 5 groups, each consisting of seven members. Phenolic acid and flavonoid contents of this plant were determined. The lung tissue samples cultivated from the rats were examined in histopathological and immunohistochemically for the apoptosis markers of Caspase-3, Bax, and Bcl-2.

Results: Histopathological results indicated that structural defects, bleeding areas, and edema had occurred in the lungs of the CP-Alone Group. Besides, Caspase-3 and Bax positivity of the lung cells had also increased while Bcl-2 positivity had decreased. On the other hand, in the HTT+CP Group, HTT was shown to have reversed the aforementioned changes positively.

Conclusion: Based on in vivo results, HTT could be a strong protective candidate for CP-induced lung injury and apoptosis

Keywords: Cyclophosphamide, *Hypericum triquetrifolium*, immunohistochemical, apoptosis, rats

The lung is vulnerable to the detrimental effects of various xenobiotics, such as drugs, natural toxins, and environmental pollutants [1]. Alkylating antineoplastic groups of drugs are potent drugs that can damage the balance of antioxidant-oxidants [2]. Cyclophosphamide (CP) is an oxazaphosphorine derivative of alkylating nitrogen mustard typically used as a chemotherapeutic drug in treating cancer [3]. Research shows that the damage CP causes to healthy tissues, including the kidney, liver, lung, and testicle, limits the use of this drug in cancer treatment [4, 5].

Inadequacy of detoxifying enzymes in the lung tissue is accepted to be the main reason for lung toxicity caused by CP [6]. It has been reported that CP exposure causes biochemical and physiological distur-

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bances by disrupting the redox balance after oxidative stress [7, 8]. CP is a generally prescribed anticancer drug applied in the treatment of various neoplastic diseases. Cytotoxic effects of CP are due to chemically reactive metabolites that produce cross-linking, which alkylate DNA and protein [9]. Reactive oxygen species (ROS) have essential roles in the pathogenesis of acute and chronic lung damage. Because oxygen is vital for living, the cells that cover the airways and the surface area of the alveolus always need oxygen. Known as pulmonary defense cells, neutrophils, monocytes, and macrophages tend to be effectively inclined to convert molecular oxygen to ROS [10, 11] Studies have shown CP to cause inflammatory reactions in the lungs of rats apart from causing acrolein/ROS formation, increasing lipid peroxidation, and accumulating neutrophils while the drug is being metabolized [3].

Species of *Hypericum*, also known as the Clusioid clade, belong to the Hypericaceae family [12]. There are known to be many bioactive compounds in the methanolic extracts of Hypericum species [13, 14]. A member of the Hypericaceae family, *Hypericum triquetrifolium* Turra (HTT) has been traditionally used in medical treatments due to its sedative, antiseptic, anti-inflammatory, and anthelmintic properties [15]. Furthermore, HTT has flavonoid and phenolic compounds with such properties as chlorogenic acid, hyperoside, quercitrin, quercetin, and routine apart from having antioxidant properties [16, 17]. Besides, the antioxidant property of HTT helps to prevent or reduce the progression of many oxidative stress-mediated diseases [13]. All these taken into account, the present study investigates whether the protective effects of HTT extract can decrease CP-induced toxicity on the lung tissue of rats thanks to in vivo studies.

METHODS

Drug and Chemicals

Endoxan, Cyclophosphamide Monohydrate, and C0768 (CP) were commercially obtained from Sigma-Aldrich, Taufkirchen, Germany. 500 mg CP was dissolved in bi-distilled water (25 mL) before being injected into the rats, each of them was given a single dose of 150 mg/kg of CP intraperitoneally.

Plant Extraction

Samples of HTT were collected during the seeding periods (between August and September 2015), which were then stored in the herbarium of Mardin Artuklu University. Afterward, 20 g of seed powder was extracted three times using 200 ml of absolute methanol, upon which 4.0 g of crude extract was obtained and then stored at -20ºC prior to use the onset of the experiment. Next, this extract was dissolved with 0.2% dimethyl sulfoxide (DMSO) to produce 100 mg/kg HTT. It was filtered for high-performance liquid chromatography (HPLC) via a membrane filter with a pore of 0.22 µm (Carl Roth GmbH, Karlsruhe, Germany). HPLC analyses of phenolic acid and flavonoid were performed using a Waters model 2690 gradient pump with Waters 2487 UV detector and XTerra RP18 column (150 Å~ 3.9 mm, 3.5 µm) [18, 19].

Animals

Male Sprague Dawley rats were provided from the Experimental Animals Lab. Ind. Trade. Corp. Co., which received approval from the Experimental Animal Ethics Committee of Eskişehir Osmangazi University (Protocol No. 444-1/2015). 220±20 g healthy, 3-4 months old male rats were kept in normal environmental conditions, including 25±2 °C room temperature, 60-70%, humidity, and 12 h light-dark cycles. Care was taken to make sure that these animals could reach standard pellet feed and water easily.

Experimental Groups

Five groups of seven rats each were formed from the 35 total animals. Group 1 (the control) received 0.5 mL of saline, Group 2 was given 150 mg/kg of CP, and Group 3 was administrated 100 mg/kg of HTT. Group 4 received HTT+CP, and Group 5 received 0.2 mL of DMSO (0.2%).

Chemical and medication dosages were chosen and prepared for the injection. Every injection was intraperitoneal (i.p). Group 1: Control rats received 0.5 ml of so-called normal saline (SF) over 6 days. Group 2 rats received SF treatment for five days before receiving a single dosage of CP on the sixth day. For six days, rats in Group 3 received the appropriate HTT-dose treatment. Rats in Group 4 received the appropriate dose of HTT. A single dose of CP was given on the sixth day. Group 5 received 0.2% DMSO treatment for 6 days. Blood samples were collected through

heart puncture under ketamine/xylazine anesthesia to evaluate serum parameters before the animals were euthanized on the seventh day (Table 1).

Histological Examination

Lungs from experimental albino rats were processed using an automated tissue processor after being formalin-preserved. Dehydration and fixation served as the processing's first two steps. During fixation, the tissue is submerged for 48 hours in 10 percent buffered formalin before the fixative is washed away with distilled water for 30 minutes. The tissues were then given two cycles of 100 percent alcohol for one hour each to dehydrate them. Initially, the tissues were exposed to 70% alcohol for 120 minutes, then to 90% alcohol for 90 minutes. After dehydration, the samples were cleaned in multiple xylene changes. During the operation, tissue was submerged for one hour in a mixture of 50% xylene and 50% alcohol, then for another 1.5 hours in pure xylene. The samples were then impregnated with molten paraffin wax, blacked out, and embedded [20]. Slices made from 4-5 M paraffin were stained using hematoxylin and eosin. Following conventional HE staining, morphological parameters were evaluated using light microscopy. A pathologist who was unaware of the groups evaluated lung injury using histological abnormalities like alveolar congestion, alveolar wall edema, inflammatory cell infiltration, and bleeding.

The pathologist also recorded the histopathology grade for the tissues in the left lung, which was based on the aforementioned reference [21]. Each benchmark was rated between 0 (normal) and 4 (severe), where 0 indicates no harm or a very minor injury, 1, 2, 3, and 4 are mild, serious, medium, and extremely serious injuries, respectively. The diffuse alveolar injury standard (DAS) score is the name of this approach. The sum of all scores was used to establish the overall score for the pathology of lung tissues.

Table 1. The protocol devised for all the study groups and the chemicals used

Groups	Treatment	Number of animals
Control	Control (saline i.p.)	7
CP	150 mg/kg	7
HTT	100 mg/kg	7
CP + HTT	150 mg/kg + 100 mg/kg	7
DMSO	0.2% (0.2 mL)	7

CP=Cyclophosphamide, HTT= *Hypericum triquetrifolium* Turra, DMSO=dimethyl sulfoxide

Immunohistochemistry Examination

The 5-m sections were deparaffinized, rehydrated, and subjected to antigen retrieval using a variety of newly discovered methods. The samples were cleaned with PBS and then blocked with 10% goat serum. The primary antibody (1:500; anti-Bax, anti-Caspase-3, and anti-Bcl-2 antibody; Abcam, Cambridge, UK) was incubated with the samples for 24 hours at 40°C. After washing the samples, the secondary antibody was incubated with them for 90 minutes at room temperature (1:1500; goat anti-rabbit IgG; Abcam). The slides were examined using the Leica DM500 Biological Microscope after the samples had been cleaned (Caspase-3 (Thermo Fischer), Bcl-2 (Abcam), and Bax (Abcam)

Statistical Analysis

The findings were defined as means±S.E.M. The statistical analyses used were One Way Analysis of Variance and Kruskal-Wallis One Way Analysis of Variance on Ranks Test. The data obtained for the contents of the plant seed material were subjected to ANOVA. All analyses were carried out in triplicates.

RESULTS

Lung tissue samples cultivated from the rats were not only histopathologically but also immunohistochemically analyzed for Bax, Bcl-2, and Caspase-3 on a routine basis.

Content of the Seed of Hypericum triquetrifolium

The levels of all the compounds detected in *H. triquetrifolium* were hyperoside (HT), kaempferol, quercetin, quercitrin, rutin, amentoflavone, chlorogenic acid, and apigenin-7-O-glucoside were examined and presented in Table 2. Considering this table, the compound with the largest amount of *H. triquetrifolium* was determined to be that of Hyperoside (HT) (8.42 mg/g DW).

Histological Evaluations

Table 2. Compounds available in *Hypericum triquetrifolium* (mg/g DW)

Compounds	*H. triquetrifolium*
Hyperoside (HT)	**8.42**
Kaempferol	0.02
Quercetin	1.91
Quercitrin	2.76
Rutin	3.72
Amentoflavone	0.006
Chlorogenic acid	2.07
Apigenin-7-O-glucoside	0.14

Lung tissues of the experimental groups (Control, 0.2% DMSO, and 100 mg/kg HTT) had all normal histology as presented in Table 2, Fig. 1A. Serious damage was observed in the lung tissues of the rats injected CP (150 mg/kg), including structural defects, obstruction, edema foci, bleeding areas, and alveolar cell injuries, as presented in Table 3, Fig. 2A. However, this damage greatly improved in the group injected CP+HTT as presented in Fig. 3A.

Apoptotic Evaluations

The lung tissue samples of Control, CP, HTT, CP+HTT, and DMSO groups were immunohistochemically stained to determine the density and concentration of Bax, Caspase-3, and Bcl-2 apoptotic markers. A comparison of the CP Group with Control, HTT, and DMSO Groups revealed that while the number of the positively-stained cells in Bax and Caspase-3 had increased, those of Bcl-2 had decreased. On the other hand, the number of the positively-stained cells had decreased in Bax and Caspase-3 while they increased in Bcl-2 as shown in Figs. 1-6.

Histological damage scores of the lung tissues are presented in Table 3.

DISCUSSION

This study aims to investigate whether or not HTT can

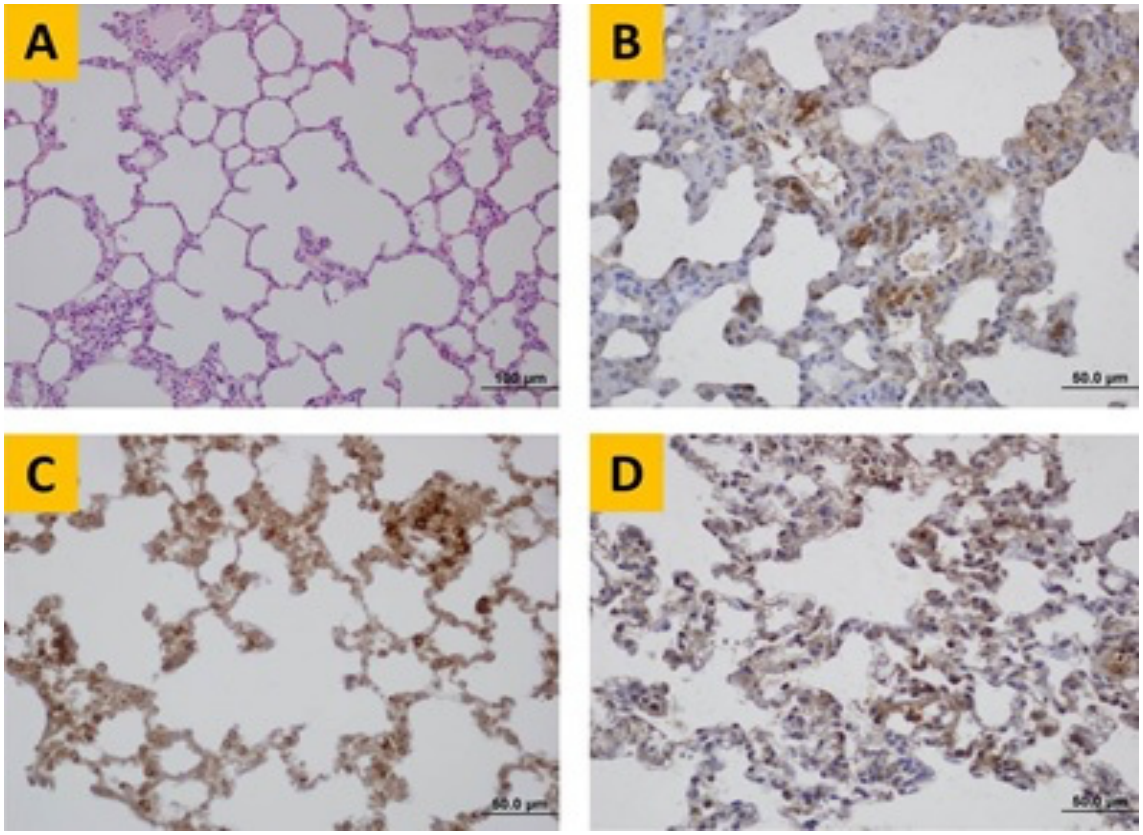

Fig 1. (A) Images of Control, HTT, and DMSO groups (B) Caspase-3 (C) Bcl-2 (D) Bax. Bars are between 50-100 µm.

Table 3. Histological damage scores of the lung tissues

Groups	Hemorrhage	Edema	Congestion
Control	0 (0-0)	0 (0-0)	0 (0-0)
CP	3* (2-3)	3* (2-3)	3* (2-3)
HTT	0 (0-0)	0 (0-0)	0 (0-0)
CP+HTT	0 (0–0)	0 (0-1)	0 (0–1)
DMSO	0 (0-0)	0 (0-0)	0 (0-0)

Data are shown median (minimum-maximum). CP=Cyclophosphamide, HTT= *Hypericum triquetrifolium* Turra, DMSO=dimethyl sulfoxide,

*P<0.001 compared to Control, significant differences

recover CP-induced lung damage thanks to *in vitro* studies. In this study, in addition to apoptosis, severe damage was detected in the lung tissues of the CP Group, including structural defects, obstruction, bleeding areas, edema foci, and alveolar cell injuries, which are in agreement with the literature (Table 3). Suddek *et al*. [3], showed that the rats given CP suffered damage and/or edema, congestion, macrophages infiltration, and neutrophilic in the interalveolar septa. One other study reported alveolar cell injuries, alveolar septa thickness, erythrocytes, and polymorphonuclear cells in the alveolar lumen in histopathological examination of lung tissues in rats given CP [22]. Previous studies have also reported that CP causes apoptosis in the liver, kidney, bladder, and testicles [5, 7, 13]. However, no studies are available in the literature on CP-induced apoptosis. In our CP Group, while the number of the positively-stained cells in Bax and Caspase-3

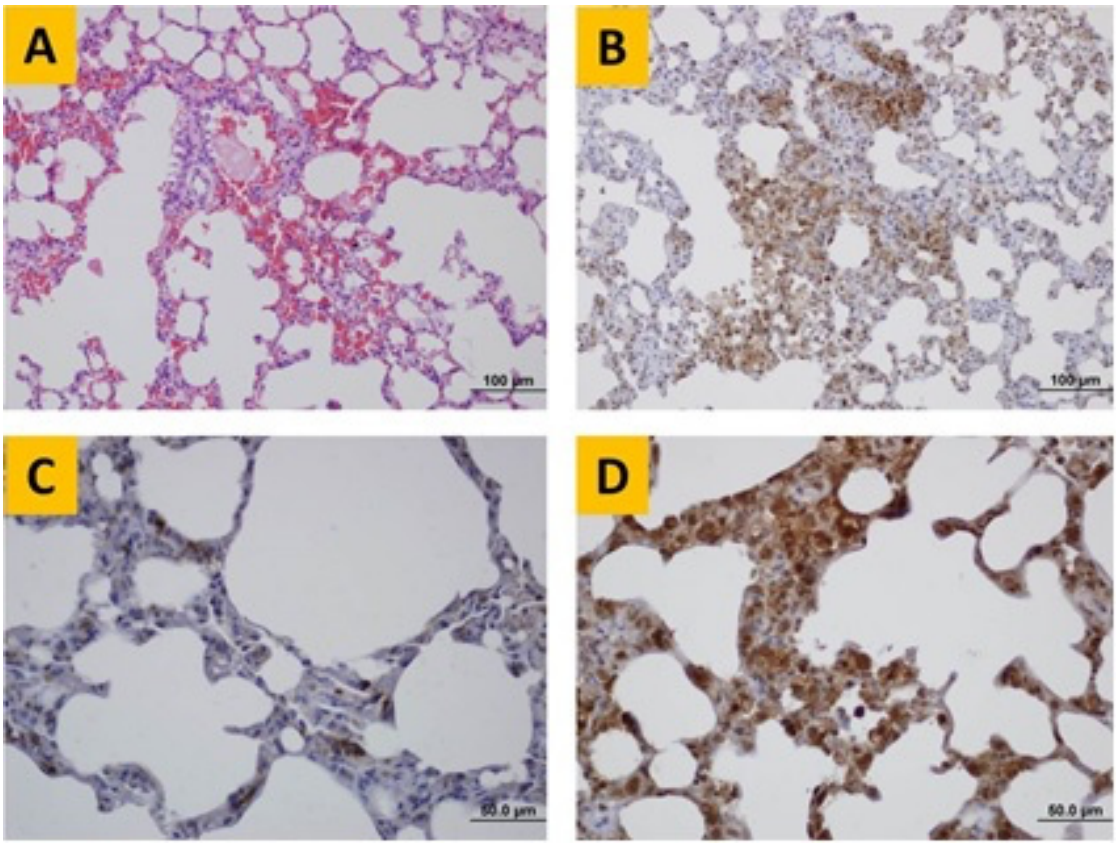

Fig 2. A) Images of CP (B) Caspase-3 (C) Bcl-2 (D) Bax groups. Bars are between 50-100 µm.

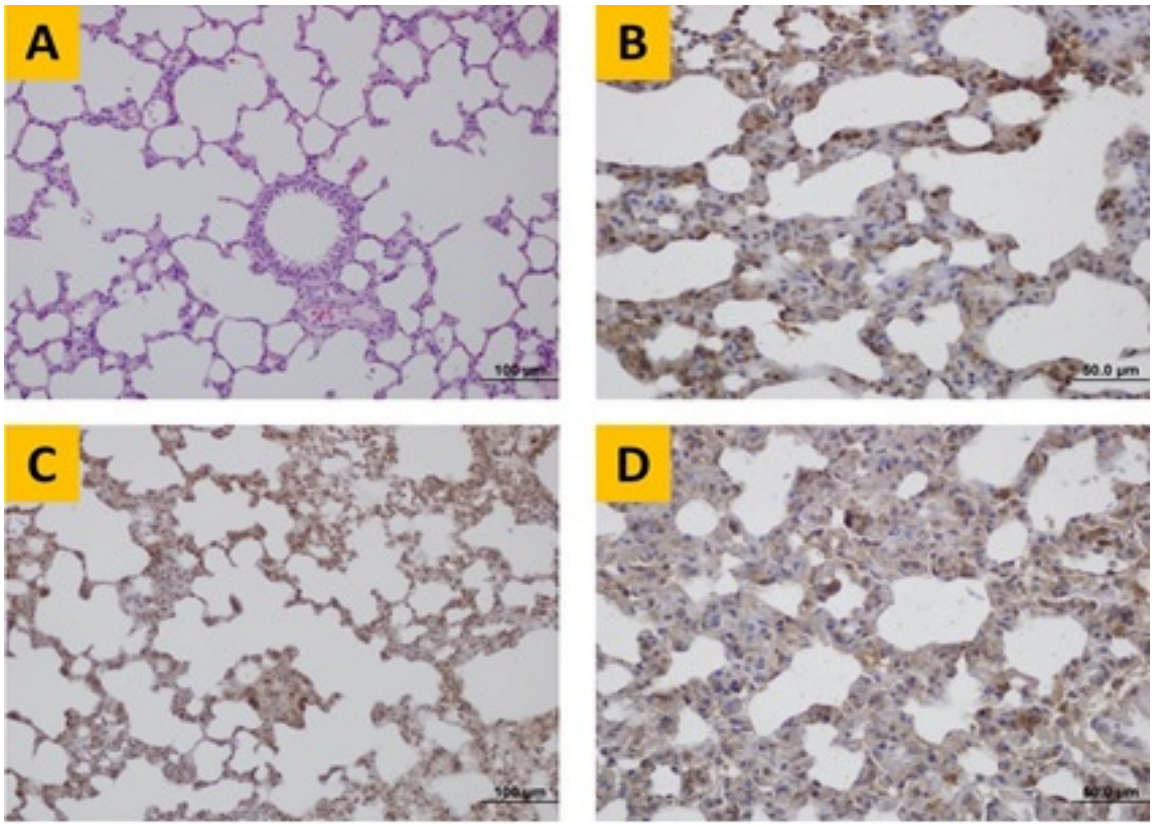

Fig 3. **(A) Images of CP+HTT (B) Caspase-3 (C) Bcl-2 (D) Bax groups. Bars are 50-100 µm.**

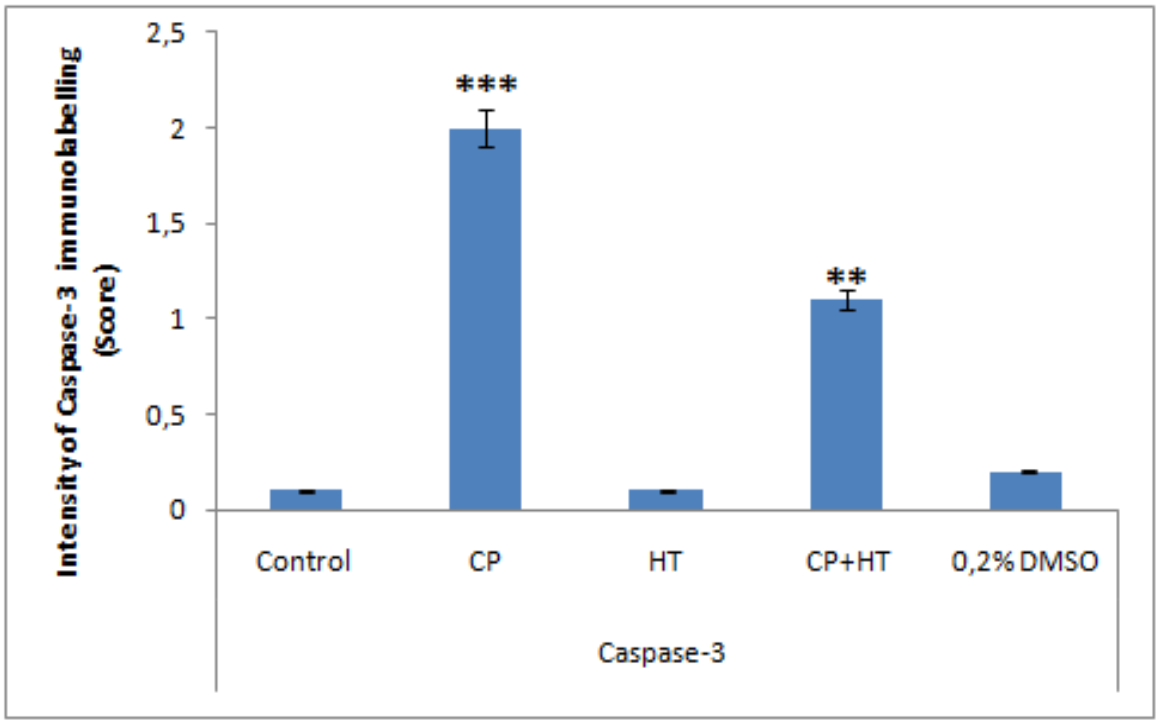

Fig 4. **Intensity of the immunolabeling score of the activated Caspase-3 positive cells in the groups. ***P<0.001 significant difference compared to control group, **P<0.01 different compared to control group.**

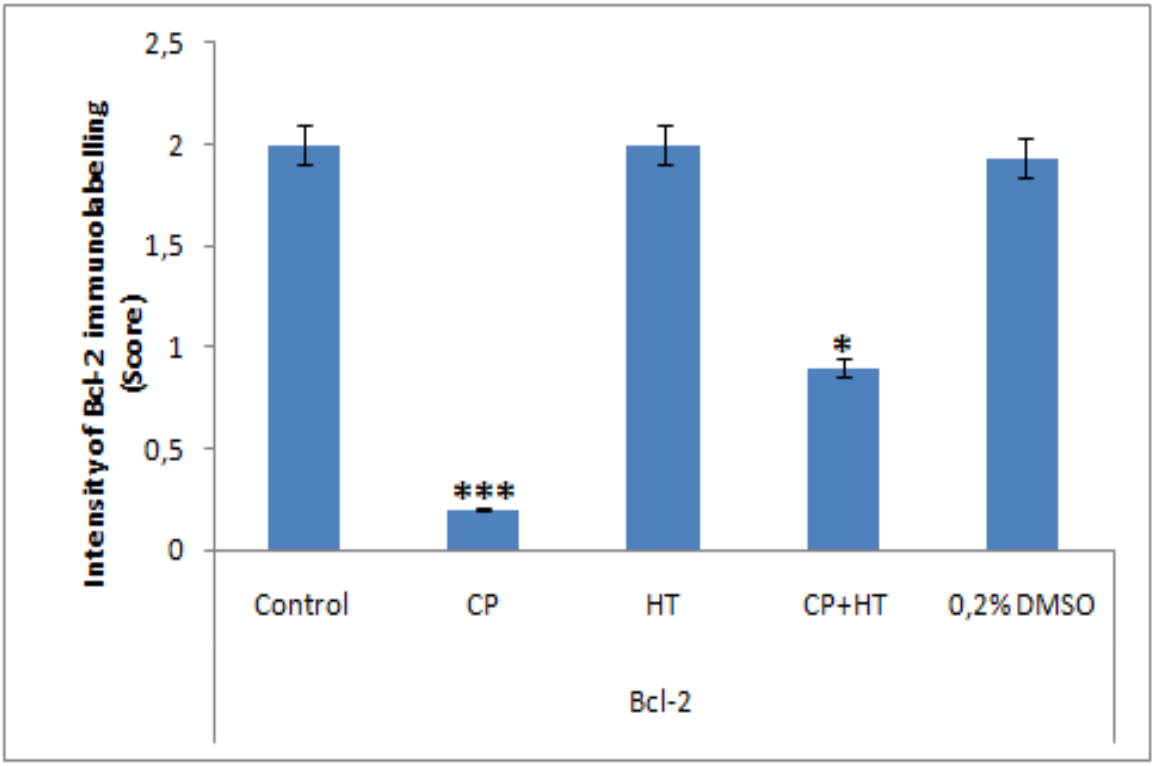

Fig 5. Intensity of the immunolabeling score of the activated Bcl-2 positive cells in the groups. ***P<0.001 significant difference compared to control, *P<0.05 different compared to control group.

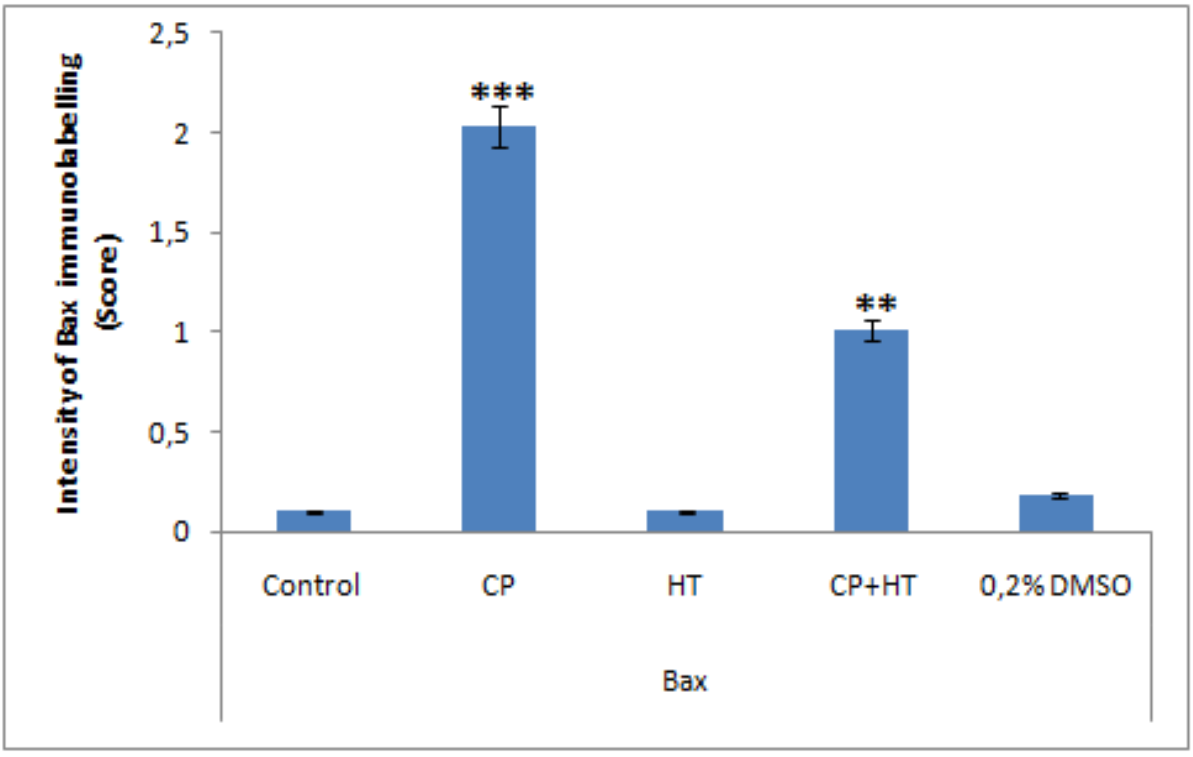

Fig 6. Intensity of the immunolabeling score of the activated Bax positive cells in the groups. ***P<0.001 significant difference compared to control, **P<0.01 different compared to control group.

had increased, those of Bcl-2 had decreased (Figs. 1-6).

High doses of CP are required to stop tumors from becoming resistant to anti-carcinogenic drugs. On the other hand, excessive doses raise the possibility of hazardous side effects while simultaneously extending survival [23]. HTT is known for its phenolic and flavonoid compounds showing antioxidant and anti-apoptotic properties [24]. The anti-oxidative activities of HTT can help suppress or decrease the side effects of many oxidative stress-induced diseases [25]. An experimental study reported that 100 mg/kg HTT achieved significant success in improving CP-induced testicular damage and apoptosis [13]. Also, Yildiz *et al*. [26] reported that CP-related cardiotoxicity, Bax, and Caspase-3 expressions decreased in CP + HTT Group while significantly increasing Bcl-2 expressions, suggesting that not only CP toxicity but also apoptosis intensity must have decreased in the cells. Our study is the first of its kind in the literature in that there are no studies available on the protective or curative effect of HTT upon lung injury.

Severe inflammatory reactions are caused by the acute effect of CYP on the lungs of rats. These reactions include the generation of acrolein during drug metabolism, neutrophil buildup, ROS creation, and increases in lipid peroxidation [27]. HTT and CP were co-administered to the rats, it was observed that this application resulted in a positive effect upon the expressions of Bcl-2. In conclusion, this study suggests that the CP-related increase in Bcl-2 expressions can be prevented by the addition of HTT. These results are consistent with the histopathological results of the present study (Figs. 1-6). The research team has shown in past studies that HTT works through anti-inflammatory and anti-apoptotic pathways to protect against a variety of experimentally produced tissue toxicities [13, 26]. HTT may inadvertently prevent apoptosis. Additionally, by limiting the loss of GSH, it can boost antioxidant levels, reduce inflammatory processes by lowering intracellular Ca++ and ROS levels, and finally, reduced TNF- and ROS levels can inhibit apoptotic cell death [28].

CONCLUSION

Based on our in-vivo results, we suggest that HTT could be a potential candidate for eliminating the toxic side effects of CP upon the lungs. This study, however, also suggests that further scientific research is necessary to better evaluate the clinical applications of HTT on cancer sufferers.

Authors' Contribution

Study Conception: SCY; Study Design: SCY; Supervision: SCY, CK, VŞ, AA; Funding: SCY, CK, VŞ, AA; Materials: SCY, CK, VŞ, AA; Data Collection and/or Processing: SCY, CK, VŞ, AA; Statistical Analysis and/or Data Interpretation: SCY, CK, VŞ, AA; Literature Review: SCY, CK, VŞ, AA; Manuscript Preparation: SCY, CK, VŞ, AA and Critical Review: SCY, CK, VŞ, AA.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financing

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