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A Histopathological, Immunohistochemical and Biochemical Investigation on the *in vitro* Antioxidant, Myeloprotective, Hematoprotective and Hepatoprotective Effects of Hypericum triquetrifolium Seed Extract Against Cyclophosphamide-Induced Toxicity

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HIGHLIGHTS

- The crude seed methanol extract of *Hypericum triquetrifolium* (HT) has strong antioxidant activity when compared with standart antioxidants (BHA and BHT).
- 25 and 50 mg/kg HT+CP, reduced the levels of serum ALT, ALP and LDH.
- In the livers of the rats that were treated with 25, 50 or 100 mg/kg HT dose-dependent, Bax decrease, caspase-3 density and Bcl-2 increase was observed when compared with the rats that received 25, 50 or 100 mg/kg HT+CP
- In conclusion, HT seed methanol extracts show high antioxidant activity and protective effects on blood, bone marrow nucleated cells and liver tissue despite high doses of CP without limiting its chemotheputic effectiveness.

Abstract: *The aim of this study was to investigate in vitro antioxidant properties and in vivo protective effects of the methanol extract of the Hypericum triquetrifolium Turra (HT) seed against acute hepatotoxicity, myelotoxicity and hematotoxicity in rats induced by cyclophosphamide (CP). In order to investigate in vivo protective effects of the HT extract on rat tissues, the rats were divided into nine groups. The toxic effects of CP and the protective effects of HT extract on nucleated cells that are produced by bone marrow, serum alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and oxidative stress index (OSI) levels were investigated biochemically. Additionally, liver tissue samples were examined for histopathological changes and apoptosis by Bcl-2, Bax and caspase-3 immunohistochemistry. The results of this study show that HT seed methanol extract has high total phenolic content (179.52 µg GAE/mg) and antioxidant activity (87.48% in 500 µg/mL concentration). CP administration caused hepatotoxicity, myelotoxicity and hematotoxicity in the rats. Whereas, the groups of rats that were injected with different concentrations of HT (25, 50 and 100 mg/kg) and CP (150 mg/kg) showed significant protective effects on bone marrow nucleated cells and important decreases on serum ALT, ALP, LDH and OSI levels were observed when compared with the CP injected group.*

Keywords: cyclophosphamide; hematoprotective; hepatoprotective; *Hypericum triquetrifolium*; myeloprotective; pharmacological activity.

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INTRODUCTION

Cancer is a common term for a class of diseases and has become the most common cause of death in the world in recent years [1]. Chemotherapy is the most frequently used method to treat malignant cells. It is a known fact that the 80-96% of cancer patients receive chemotherapy [2] Chemotherapy affects both active cells such as cancer cells and some healthy blood cells. Side effects of chemotherapy occur when the chemotherapy damages healthy cells. Chemotherapeutic agents are generally toxic to healthy cells and can cause critical side effects such as myelosuppression and multiple organ failure [3]. Thus, new and more effective ways must be developed to minimize the side effects of chemotherapeutic agents without losing chemotherapeutic effectiveness. Various clinical and preclinical studies have suggested that a combination of chemotherapeutic and chemopreventive

drugs can be helpful in reducing the systemic toxicity of chemotherapeutic drugs [4,5]. In spite of the wide spectrum of application, the usage of cyclophosphamide (CP) is generally limited due to its undesirable toxic side effects, including hepatotoxicity and myelosuppression/immunotoxicity, which limit its usefulness [6,7]. Moreover, CP treatment significantly decreases erythrocyte, leucocyte, thrombocyte and bone marrow nucleated cells [8].

CP, an alkylating chemotherapeutic prodrug, is metabolized by liver cytochrome P450 enzymes, namely CYP3A4 and CYP2B6 which demontsrate active therapeutic and cytotoxic metabolites and diffuse out of the hepatocytes into the plasma [9]. CP spreads throughout the body and generates two active metabolites: phosphoramide mustard (PAM) and acrolein (ACR). While CP's immunosuppressive and antineoplastic effects are related with PAM, ACR is responsible for its undesirable toxicity [10] ACR produces highly reactive oxygen species (ROS) and oxidative stress in hepatocytes [11,12]. Thus, it interacts with protein and causes changes in the structure and function of enzymes, and harms the tissue antioxidant defense mechanism [13]. CP-induced toxicity is a consequence of a mitochondrial dysfunction and ends with a decrease of adenosine triphosphate owing to nitrosative and oxidative stress [5].

CP is used in combination with different detoxifying and protective agents with the aim of decreasing or removing its adverse toxic effects [14]. Extracts of herbal medicines are shown to have beneficial therapeutic effects such as antioxidant, anticancer, anti-inflammatory, antimicrobial effects [15,16]. The antioxidants can minimize the toxic side effects of chemotherapeutic agents and allow the use of higher doses of anticancer drugs [5,17]. The *Hypericum* species known as ''Clusioid clade'' are a member of the Hypericaceae family, belonging to a large clade of generally tropical plants [18]. The methanolic extract of the *Hypericum* species is particularly rich in bioactive compounds, such as naphthodianthrones (pseudohypericin, hypericin), phloroglucinol derivatives (adhyperforin, hyperforin), flavonoids, tannins, essential oils, xanthones, procyanidins, and some other water-soluble components that possess a broad range of biological properties [19,20].

Hypericum triquetrifolium Turra. (HT) is traditionally known as "kantaron, kantarum, botav, batof, bahtof, gulazer, kıppen, koyun kıran and binbirdelik otu". HT has been used for many years in the Southeast of Turkey to treat stomach disorders (ulcer, gastritis), jaundice, wounds, gingivitis, anemia, reduce fevers and also as an appetative [21,22]. Moreover, HT includes hyperoside, chlorogenic acid, quercetin, quercitrin, rutin, kaempferol (phenolic and flavonoid compounds) which are important for antioxidant capacities [23]. Previous studies have shown us that the seed extracts of *Hypericum* species have strong antioxidant activities [24,25].This significant antioxidant capacity is what may be beneficial in inhibiting or decreasing the progress of various oxidative stress-induced diseases [23]. However, its effects against hepatotoxicity, myelotoxicity and hematotoxicity have not been clarified in detail. Therefore this study aimed to present the *in vitro* antioxidant and protective effects of HT seed methanol extract on the CP-induced toxicity of the hepatic cells, bone marrow nucleated cells and peripheral blood cells of rats.

MATERIAL AND METHODS

Drugs and chemicals

CP (Endoxan, Cyclophosphamide Monohydrate, C0768) was acquired commercially from Sigma-Aldrich, Taufkirchen, Germany. The CP (500 mg) was dissolved in 25 mL bidistilled water to its own appropriate concentration prior to injection, sequentially, and was stocked at 4°C before use.

Herbal material

The plant materials were collected during the seeding stages in August and September, 2015 from wild population, growing in the district of Zınar in Mardin, Turkey. Voucher specimens were deposited at the Herbarium of Mardin Artuklu University (2015-3-MAU). The seeds (20g) were ground into powder. The seed powder was preserved in a glass flask with 200 mL (99%) methanol at room temperature. The extraction process was repeated 3 times under magnetic stirrer. Before the solvent was removed onto a rotary evaporator it was filtrated under vacuum. Approximately 4g of the crude methanol extracts of HT seeds were obtained and saved in light-hindered glass vials at -20°C prior to the experiments.

Determination of antioxidant capacity

Total phenolic assay

The amount of total phenolic content in seed methanol extract was determined according to the Folin-Ciocalteu method [26]. In brief, 0.2 mL of sample solution (2 mg/mL) was added into a test tube containing 1 mL of Folin–Ciocalteu reagent and 2 mL of Na2CO3 (7.5%). The final volume was brought up to 7 mL with deionized water. After 2 hours of incubation at room temperature, the absorbance was measured at 765nm with a spectrophotometer (Perkin Elmer lambda 25 UV/Vis, USA). The results were expressed as gallic acid (standard) equivalents (GAE). y = 0.002X-0.051(R2 = 0.995) was used as the equation according to linear calibration curve.

Scavenging activity on 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical

The free radical scavenging activities were quantitatively tested using a DPPH based on the method of Shimada et al. (1992) [27]. In brief, 0.1 mM solution of DPPH in ethanol was prepared. Then, 1 mL of this solution was added to 3 mL of each extract solution at different concentrations (25–500 µg). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. The absorbance was then measured at 517 nm with a spectrophotometer (Perkin Elmer lambda 25 UV/Vis, USA). The lower absorbance of the reaction mixture was indicated as higher free radical scavenging activity. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) were used as the standard antioxidants (positive controls) for free radical scavenging activity test. The radical scavenging activity was calculated with following formula:

DPPH scavenging activity (%) = (A_{517} of control−A_{517} of sample/A_{517} of control)×100 [28].

Experimental design

Healthy male Wistar albino rats (3-4 months old, weighing 200–240 g) were commercially purchased from Kobay Experimental Animals Lab. San. Tic. AS., Ankara, Turkey. This study was conducted with the approval of the Local Ethics Committee of Animal Experiments of Eskişehir Osmangazi University (Ethical approval code: 444-1/2015). The rats were housed in an appropriate environment (12 hours light/dark cycle and 24±2°C) and had free access to water and pellet feed. After a 2 week acclimatization period, the rats were randomly divided into 9 groups (n=7 per group). All drug or chemicals were intraperitoneally (i.p.) injected into the rats. The test samples (HT crude seed methanol extract) were prepared by dissolving them in 0.2% dimethyl sulfoxide (20 mL DMSO in 10 mL 0.9% NaCl, Sigma-Aldrich, Germany) solution to obtain concentration of 25, 50 and 100 mg/kg in a flask. The flask was partially immersed into an ultrasonic bath for 3 minutes at 30°C for effective dissolving. 0.5 mL 0.2% DMSO and serum physiologic (SP) were used as positive controls.

The rats in Group 1 (the control group) were injected with a 0.5 mL saline solution. The rats in Group 2 were injected with CP (150 mg/kg) on the sixth day of experiment. The rats in the Groups 3, 4 and 5 were dosed with 25, 50 and 100 mg/kg HT extracts, respectively, for 6

days. The rats in Groups 6, 7 and 8 were injected with 25, 50 or 100 mg/kg HT extracts, respectively, for 6 days and a single dose of CP was injected on the sixth day. The rats in Group 9 were injected with 0.5mL of 0.2% DMSO. The rats were weighed before the injections and dissection. At the end of the experiments, the rats were anesthetized with ketamine (50 mg/kg) intraperitoneally. The liver of the rats was excised and cut into small pieces and placed into tissue containers which contained 10% neutral buffered formaldehyde.

Counting of blood and bone marrow nucleated cells

On the final day (day 7) of the experiment all the rats were dissected and blood samples were collected into test tubes by cardiac puncture. After dissection, bone marrow samples from femurs were flushed into phosphate buffered saline containing 2% bovine calf serum and homogenized. Then, bone marrow nucleated cells and peripheric blood cells were counted with a cell counter (Indiamart, MS4S, India).

Measurement of hepatic marker enzymes level

Blood samples were centrifuged at 3000 rpm for 10 min to separate serum and stored at -20°C for subsequent biochemical measurements of alanine transaminase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) hepatic enzymes. The serum levels of ALT, ALP and LDH were measured by commercial kits provided by Spectrum Diagnostic (Cairo, Egypt), seriatim.

Measurement of oxidative stress index (OSI)

Serum samples were analyzed for total antioxidant level (TAL) and total oxidant level (TOL). Thereafter, measurements were performed by using Hitachi-917 autoanalyzer (Human Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) together with commercial kits (RelAssay, Ref. RL27, Turkey). Oxidative stress index (OSI) was calculated by dividing the TOL by the TAL. For this, the unit of the TAL value was changed from the mmol Trolox equivalent/L type to µmol Trolox equivalent/L type. OSI was calculated using the following formula [29].

OSI= [(TOL, µmol H2O2 equivalent/L) / (TAL, µmol Trolox equivalent/L) x 100]

Histopathological examination

After fixation with 10% neutral buffered formaldehyde, hepatic tissues were processed routinely and embedded in paraffin. Then, 5 µm-thick serial sections were cut and stained with hematoxylin-eosin. Microscopical examination was performed in a blind manner by an expert histologist.

Immunohistochemistry staining

Sections of liver tissues were deparaffinized and rehydrated routinely. Antigen retrieval by citrate buffer (pH 6.0) was done by heating the sections in a microwave at 700W for 10 min. After blocking with 3 ml/L H_2O_2 and swine serum, the sections were incubated with the primary antibodies, directed against Bcl-2 (Abcam), Bax (Abcam) and caspase-3 (Thermo) at dilutions of Ultravisionquanto detection system (Thermo Scientific), respectively.

Statistical analysis

A package software version of SPSS 12.0 for windows was used in assessing the data obtained in this study. The antioxidant test data were expressed as means±standard deviation (SD). The results of the biochemical and histological test were signified as

means±SD. The difference observed for Bcl-2, Bax and Caspase-3 in the groups was evaluated with a Kruskal–Wallis One Way. The numerical value (p) for the difference was accepted as significant if it was $p < 0.05$.

RESULTS

Total Phenolic Content and Antioxidant Findings

In this study, the total phenolic content of seed methanol extract of HT was found as 179.52±0.52 µg GAE/mg extract. The inhibition percentage of DPPH free radical scavenging activity of seed methanol extract of HT (25 to 500 µg/mL concentrations) and positive controls (BHA and BHT) are shown in Table 1. The decrease in the concentration of DPPH radicals was due to the scavenging ability of the rich antioxidant contents of the HT seed methanol extract. Linear increase in activities was observed with high concentrations (250 and 500 µg/mL). These values were found to be very close to the BHT and higher than BHA which are the two most known synthetic antioxidants. The results show that the crude seed methanol extract of HT has strong antioxidant activity when compared with BHA and BHT. These activities of plant extracts are mainly attributed to the presence of rich phenolic compounds [30].

Table 1: Effect of seed methanol extract of *Hypericum triquetrifolium* on the inhibition of DPPH free radical.

	% Inhibition of DPPH free radical			
		Standards		**Sample**
	Concentrations	**BHA*** **	**BHT**	**HT methanol**
(µg/mL)	25	*67.60±0.08[a**]	53.36±0.06[a]	14.71±0.05[a]
	50	79.34±0.09[b]	78.71±0.09[b]	27.23±0.06[b]
	100	85.13±0.13[c]	85.13±0.11[c]	65.10±0.12[c]
	250	85.91±0.06[d]	87.01±0.10[d]	83.72±0.14[d]
	500	85.91±0.06[d]	87.94±0.09[e]	87.48±0.11[e]

*Means are the averages of 3 replicates. **Values reported are means±standard deviation; means followed by different letters in same columns are significantly different ($p<0.05$) ***BHA: butylated hydroxyl anisole; BHT: butylated hydroxyl toluene; DPPH: 2,2-diphenyl-1- picrylhydrazyl.

Biochemical Findings

The results of the present study showed a significant ($p<0.001$) decrease in the count of bone marrow nucleated cells in the 150 mg/kg CP-injected group when compared with the control group. A significant increase ($p<0.001$) in the count of bone marrow nucleated cells was observed in the HT+CP-injected groups (especially 50 mg/kg HT+CP group) in comparison with CP group. The count of bone marrow nucleated cells in the DMSO group showed no significant differences ($p>0.05$) from the control group. In the present study, a decrease in the count of erythrocyte cells was observed in the CP group when compared with the control group. The number of erythrocytes in the 50 mg/kg HT+CP-injected group increased and, despite CP toxicity, came closer to the number of erythrocytes in the control group. There was a statistical significant decrease in the number of leucocyte cells in the

CP-injected groups, which was thought to have been caused by the high dose of CP. The number of leucocyte cells showed a significant increase in the 25, 50, 100 mg/kg HT+CP-injected groups when compared with 150 mg/kg CP-injected group ($p<0.001$). The number of thrombocytes cells decreased ($p<0.001$) in the CP-injected group in comparison with control group and the 25, 50, 100 mg/kg HT-injected groups. The current study made it clear that CP decreased the number of leucocyte and thrombocyte cells more than erythrocyte cells. The number of thrombocyte cells increased in the 25, 50 and 100 mg/kg HT+CP-injected and was especially high in the 50 mg/kg HT+CP-injected group. These results were found to be statistically significant ($p<0.001$). In the DMSO group, the number of erythrocyte, leucocyte and thrombocyte cells showed no significant change from the control group ($p>0.05$) (Table 2).

Table 2. The effects of 150 mg/kg cyclophosphamide (CP) and 25, 50 and 100 mg/kg doses of *Hypericum triquetrifolium* (HT) in the numbers of peripheral blood cells (erythrocytes, leucocytes, thrombocytes) and bone marrow nucleated cells.

Groups	Erythrocytes ($\times10^3$/ml)	Leucocytes ($\times10^3$/ml)	Thrombocytes ($\times10^3$/ml)	Bone marrow nucleated cells ($\times10^3$/ml)
1-Control (saline)	*8.42 ±0.51^{a**}	6.55±0.80^{a}	718.57±57.71^{a}	33.91±2.50^{a}
2- CP (150 mg/kg)	7.87±0.32^{a}	2.02±0.22^{b}	340.00±17.36^{b}	5.59±0.44^{b}
3- HT (25 mg/kg)	8.04±0.33^{a}	10.98±1.33^{c}	666.29±38.42^{c}	37.65±1.20^{c}
4- HT (50 mg/kg)	7.73±0.38^{b}	13.86±1.03^{d}	666.57±52.68^{c}	38.44±1.39^{c}
5- HT (100 mg/kg)	7.62±0.36^{b}	10.99±1.18^{c}	693.29±123.80^{c}	33.01±1.63^{c}
6- CP+HT (25 mg/kg)	7.43±0.30^{b}	4.29±0.57^{e}	546.00±47.43^{d}	10.99±1.34^{d}
7- CP+HT (50 mg/kg)	7.97±0.40^{a}	5.23±0.64^{e}	671.71±48.58^{e}	12.35±1.27^{d}
8- CP+HT (100mg/kg)	7.21±0.30^{b}	4.87±0.64^{e}	576.71±58.41^{f}	9.95±0.34^{d}
9-Control (DMSO)	7.74±0.34^{b}	7.53±0.53^{f}	741.00±68.34^{g}	35.40±0.99^{c}

*Means are the averages of 3 replicates. **Values reported are means±standard deviation; means followed by different letters in same columns are significantly different ($p<0.001$: statistically significant differences).

Serum ALT, ALP and LDH levels were found to be increased in the CP group when compared with the control group. In this study, an increase in ALT, ALP and LDH in CP-injected hepatocyte injury was also demonstrated. This was evident by the significant ($p<0.001$) increase in serum ALT and ALP in comparison with the control group. On the other hand, 25, 50, 100 mg/kg HT+CP, especially 25 and 50 mg/kg HT+CP, reduced the levels of serum ALT, ALP and LDH (Table 3). In the CP group, the level of oxidative stress index (OSI) increased when compared with the control group. HT in doses of 25, 50 and 100 mg/kg prevented the CP-injected toxicity, which can be explained with the decrease in the level of (OSI) (Table 3).

Table 3. The serum levels of hepatic enzymes (ALT, ALP, LDH) and OSI of the rat groups. Data were expressed as mean ± standard deviation. Cylophosphamide (CP) in 150 mg/kg and *Hypericum triquetrifolium* (HT) in 25, 50 and 100 mg/kg doses were used.

	ALT (U/L)	ALP (U/L)	LDH (U/L)	OSI(TOS/TASx100)
1-Control (saline)	*12.77±0.98^{a**}	49.02±5.35^{a}	349.67±23.26^{a}	0.0156±0.0009^{a}
2- CP (150 mg/kg)	26.17±2.28^{b}	89.85±5.32^{b}	514.29±15.04^{b}	0.2431±0.0155^{b}
3- HT (25 mg/kg)	13.09±1.54^{a}	50.64±4.19^{a}	397.79±44.04^{a}	0.0165±0.0014^{a}
4-HT (50 mg/kg)	11.94±1.99^{a}	67.87±6.00^{c}	393.01±27.44^{a}	0.0161±0.0006^{a}
5-HT (100 mg/kg)	9.21±0.91^{c}	28.61±2.72^{d}	293.42±17.34^{c}	0.0152±0.0006^{a}
6-CP+HT (25 mg/kg)	25.34±2.32^{d}	83.92±3.75^{e}	472.69±31.03^{b}	0.1900±0.0074^{c}
7-CP+HT (50 mg/kg)	17.65±1.38^{e}	72.61±4.50^{f}	382.91±31.78^{d}	0.1321±0.0095^{d}
8-CP+HT(100 mg/kg)	11.72±1.62^{a}	58.65±3.77^{g}	350.66±21.37^{d}	0.0899±0.0040^{e}
9-Control (DMSO)	12.35±1.71^{a}	52.76±3.37^{g}	353.49±29.87^{d}	0.0155±0.0009^{a}

*Means are the averages of 3 replicates. **Values reported are means±standard deviation; means followed by different letters in same columns are significantly different ($p<0.001$: statistically significant differences).

Histopatholgical Findings

The histopathological assessments of the study show that the hepatic tissues of the 25, 50 and 100 mg/kg HT and 0.2% DMSO groups have normal histology, like the control group. While no major deffects were seen in the livers of the 150 mg/kg CP-injected rats, in some regions of their liver tissue there were small haemorrhage and edema sites, ballooning, vacuolization and hepatocellular necrosis (Fig. 1- 4). No obvious disturbances were seen in the liver tissues of the 25, 50 or 100 mg/kg HT plus 150 mg/kg CP-injected rat groups. However, when the co-administered HT dose increased, the liver histology improved (Fig. 4).

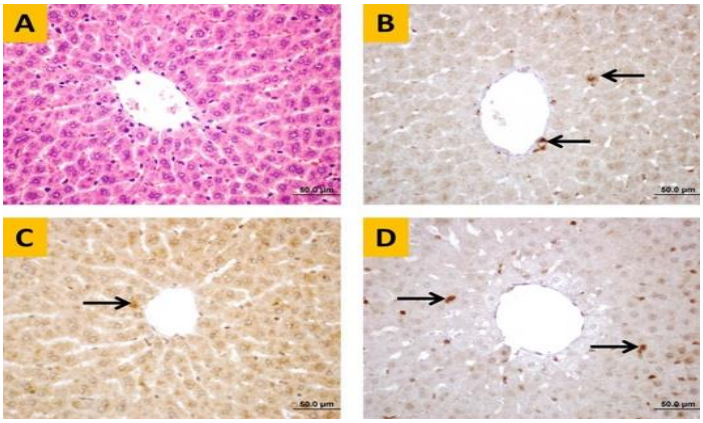

Figure 1 (A-D): Microscopic images of hepatic sections of control (SF) group. The arrows show some of the strong positive immunoreactive cells. **A:** In the middle of the hematoxylin-eosin stained section vena centralis and radiating hepatocyte cords that are around of vena centralis show typical normal appearance. **B:** In the caspase-3 stained section is seen that the number of apoptotic cells is low. **C:** In the anti-apoptotic marker Bcl-2 stained section, cells are seen positively stained at different densities. **D:** In the Bax-stained section is seen that the number of apoptotic cells is low. The bars are all 50 µm.

Immunohistochemical Findings

Apoptosis assessments show that Bcl-2, caspase-3 and bax immunohistochemistry results all differ in accordance with the liver tissue section of the rat groups. Caspase-3 activity and

Bax increase and Bcl-2 decline were observed in the CP-injected rat livers when compared with the control group (Fig. 2B,C,D). In the livers of the rats that were treated with only 25, 50 or 100 mg/kg HT dose-dependent, Bax decrease, caspase-3 density and Bcl-2 increase was observed when compared with the rats that received 25, 50 or 100 mg/kg HT+CP (Fig. 3B,C,D; 4B,C,D). Also, in the HT+CP-injected rats, apoptotic cells were more commonly observed than in the other groups (Fig. 1,2,3,4).

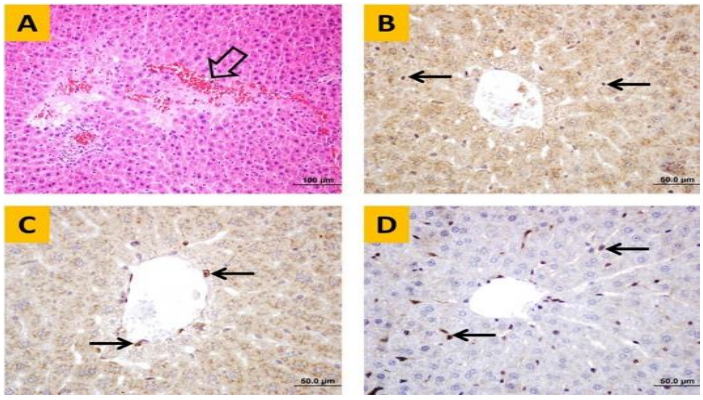

Figure 2 (A-D): Microscopic images of hepatic sections of Cyclophosphamide (CP) group. The thin arrows show some of the strong positive immunoreactive cells. **A:** The hollow arrow on the section painted with H-E shows the area of bleeding in the middle. **B:** In the caspase-3 stained section, positively reacting apoptotic cells are seen at different densities. **C:** In the anti-apoptotic marker Bcl-2-stained section cells are seen positively stained at different densities. **D:** In the Bax-stained section is seen that the number of apoptotic cells is lower. The bars are; 100 µm in A, and 50 µm in the others.

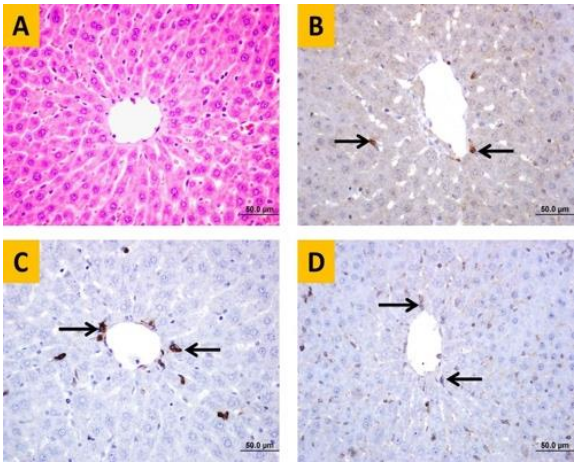

Figure 3: Microscopic images of hepatic sections of 100 mg/kg *Hypericum triquetrifolium* Turra. (HT) group. The arrows show some of the strong positive immunoreactive cells. **A:** In the middle of the hematoxylin-eosin stained section vena centralis and radiating hepatocyte cords that are around of vena centralis show typical normal appearance. **B:** In the caspase-3 stained section is seen that the number of apoptotic cells is low. **C:** In the anti-apoptotic marker Bcl-2 stained section, the number of positively stained cells is seen low. **D:** In the Bax-stained section is seen that the number of apoptotic cells is low. The bars are all 50 µm.

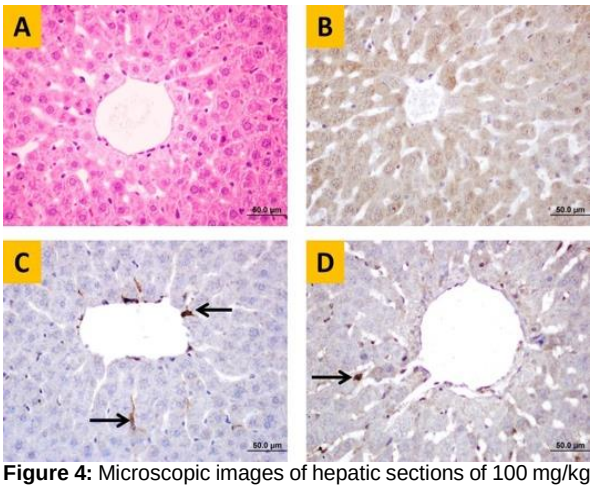

Figure 4: Microscopic images of hepatic sections of 100 mg/kg *Hypericum triquetrifolium* Turra. (HT)+Cyclophosphamide (CP) group. The arrows show some of the strong positive immunoreactive cells. **A:** In the middle of the hematoxylin-eosin stained section vena centralis and radiating hepatocyte cords that are around of vena centralis show typical normal appearance. **B:** In the caspase-3 stained section is seen that the most of the cells have low density reaction. **C:** In the anti-apoptotic marker Bcl-2 stained section, the number of positively stained cells is seen low. **D:** In the Bax-stained section is seen that the number of apoptotic cells is low. The bars are all 50 µm.

DISCUSSIONS

CP is used widely in clinical settings as a chemotherapeutic agent. However, its side effects such as hepatotoxicity and myelosuppression limit its usage [31]. CP is a cytotoxic drug which non-specifically affects not only cancer cells but also normal healthy cells with high proliferation capacity by binding onto DNA and interfering with cell cycle [32]. Damage to the immune system is a major side effect of chemotherapeutic agents [3,8]. Bone marrow stem cells that get damaged or die cannot produce new blood cells and this leads to thrombocytopenia and leukopenia. Cellular antioxidant system is a major defence against CP-induced oxidative stress [33,34]. ACR, which leads to a breach in the normal antioxidative defence system and can result in uninterrupted ROS accumulation, attacks to the tissue antioxidant enzymes.

Myelosupression is an undesirable side effect of CP chemotherapy. Correspondingly the present study showed that CP decreased the number of bone marrow nucleated cells while HT+CP increased the number of bone marrow nucleated cells. It can be said that HT has a protective effect on bone marrow nucleated cells. According to these results, HT seed extract, especially its biologically active compounds, may be great candidates for alternative adjuvant chemotherapy for decreasing the CP toxicity. The results conclude that a 25 mg/kg dose of HT was not enough to prevent CP toxicity on erythrocyte cells when compared with the CP group. On the other hand a 100 mg/kg dose of HT is toxic for erythrocyte cells as it shows no cytoprotective effects. It can be conclude from the results that 50 mg/kg dose of HT+CP is the optimum protective dose on erythrocyte cells. In agreement with the results, a significant ($p<0.001$) decrease in the number of leucocyte cells in the CP administered group was observed.31 The number of leucocyte cells showed a significant increase in the 25, 50, 100 mg/kg HT+CP-injected groups when compared with the 150 mg/kg CP-injected group ($p<0.001$). From these results it can be inferred that all doses of CP+HT increased the number of leucocyte cells and the 50 mg/kg HT+CP is the optimum dose for leucocyte cells.

The number of thrombocyte cells decreased ($p<0.001$) in the CP group in comparison with the control group and the 25, 50, 100 mg/kg HT groups. Similar to the present study, Merwid et al. (2011) [35] reported that CP, which is an anticancer or immunosuppressive drug, significantly decreased thrombocyte and leucocyte cells, however the usage of high doses of CP is limited due to its toxicity [35]. The present study made it clear that CP decreased the number of leucocyte and thrombocyte cells more than erythrocyte cells. The effect of CP on the erythrocyte cells was not so clear as in the case of leucocyte and thrombocyte cells (Table 2).

The values of serum ALT, ALP and LDH are the most commonly used clinical markers of hepatocellular toxicity. In this study, it was seen that CP-induced hepatotoxicity caused damage to liver tissues and, as a result, the cytosolic enzymes (ALT, ALP, LDH) were transferred to blood increasing the serum levels (Table 3). In previous studies it was demonstrated that CP-induced hepatotoxicity was associated with elevated ALT levels in mice and rats [7,36,37]. The increase in OSI levels may be a result of the decrease in antioxidant status, which was evident by the substantial decline in the CP-injected group. Thus, the prevention of OSI increment and enhancement of the antioxidant enzymes play a central role in the cytoprotective activity of HT against CP. In the same way the present study is supported by numerous studies and in all of these studies the herbs play a crucial role with their antioxidant and antitumoral properties [5,13].

In some parts of the CP-injected liver tissues there were small haemorrhage and edema sites, ballooning, vacuolization and hepatocellular necrosis. Similar results were demonstrated in former studies [38,39]. It could be inferred that HT improved the CP-injected cellular damage and inflammation in the livers of rats with its antioxidant and cytoprotective properties. In other studies, experimental evidence shows that oxidative stress is the reason for CP-induced hepatotoxicity and also histopathological examinations demonstrated that CP causes serious damage to liver tissue and increases liver injury score [34,40].

CP increases caspase-3 and bax expression and decreases bcl-2, indicating histolological damage while CP+HT decreases caspase-3 and bax expression and increases bcl-2, indicating protectivity. In earlier investigations, parallel to the present results, it was demonstrated that there is CP induced apoptosis in the liver tissue [39,41]. In the livers of the rats that were treated only with HT in different doses dose-dependent Bax decrease, caspase-3 density and Bcl-2 increase was observed when compared with the rats that received CP+HT. Also, in the rats injected with CP plus HT, apoptotic cells were less than in the CP group. It could be inferred that the antiapoptotic activity of HT is generally a reflection of its anti-inflammatory and antioxidant properties.

CONCLUSION

The therapeutic effects of anticancer agents are associated with adverse side effects due to the toxicity they cause. Even though CP is very commonly preferred as a

chemotherapeutic agent, its toxic side effects limit high-dose use. Thus, new effective agents are required in order to protect the normal tissue from chemotherapy-related toxicity without protecting the tumour and tumour growth stimulation properties. Therefore, the combination of the treatment regimens with antioxidant and cytoprotective properties can be useful in the protection of healthy cells and tissue against CP-induced oxidative damage. From the results of this study, it can be concluded that HT seed methanol extracts show high antioxidant activity and protective effects on blood, bone marrow nucleated cells and liver tissue despite high doses of CP without limiting its chemotherapeutic effectiveness. Based on the findings of this study, it can be proposed that all doses of HT especially 50 mg/kg HT plus CP are strong candidates in preventing the CP-induced myelotoxicity, hemotoxicity and hepatotoxicity.

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