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Cardioprotective effects of *Hypericum triquetrifolium* Turra. against cyclophosphamide related cardiotoxicity in rats

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ABSTRACT: Cyclophosphamide (CYP) is commonly used as anticancer agent but its usage is limited by cardiotoxic side effects such as dose-dependent cardiac damage, morphologically defined necrosis and bleeding. *Hypericum triquetrifolium* Turra. (HT) shows anti-oxidative and anticarciogenic properties with its rich phenolic contents. The current study was designed to investigate the possible protective effect of HT on CYP-induced cardiotoxicity. Albino rats were randomly divided into 9 groups, each included 7 animals. Serum creatine kinase-MB (CK-MB), malondialdehyde (MDA), aspartate transaminase (AST), glutathione (GSH), total antioxidant (TAC) and total oxidant capacity (TOC) levels were investigated. Furthermore, the cardiac tissue samples were investigated histopatologically. While the levels of serum CK-MB, MDA, AST and TOC were high, the levels of serum GSH and TAC levels were low in the CYP groups. It was also observed that CYP-induced cardiotoxicity was dose dependent. In the treatment with CYP plus HT doses there was observed an essential decrease in the CYP cardiotoxicity; decreased cell damage and oxidative stress parameters and also increased GSH and TAC levels. Based on our findings, it can be proposed that HT seed methanol extract was a strong candidate in preventing the CYP-induced cardiotoxicity.

KEYWORDS: Cardiotoxicity; cyclophosphamide; cardioprotective effects; *Hypericum triquetrifolium* Turra..

1. INTRODUCTION

Cyclophosphamide (CYP) has an extensive usage of clinical treatments, moreover it has been proved to be influential for therapy of malignant and non-malignant disease.

CYP is an antineoplastic prodrug that is dependent on hepatic cytochrome P450 metabolism for its anti-tumoral effectiveness then form 4-hydroxycyclophosphamide which generates the chemically responsive metabolit such as phosphoramide mustard and acrolein that alkylate protein, DNA and cause to cross link [1]. Phosphoramide mustard is mostly responsible with CYP' s antineoplastic effects, while acrolein is responsible with the adverse effects. Acrolein, a highly reactive aldehyde pollutant, [2] interacts with the body's antioxidant defense system and produces reactive oxygen species [3, 4] while phosphoramide mustard demonstrates anti-tumoral effects. Acrolein leads to the damage of the cells after binding with the glutathione (GSH) and reduces its level in the cells [2, 5].

Membrane lipids are very susceptible to free-radical damage. Lipids after reacting with free radicals show lipid peroxidation [6]. Lipid peroxidation' s products, such as MDA, are toxic for tissue cell which cause terrible impact on the function of status and they are effective gauges for tissue injury [7, 8]. Cell mechanism of CYP-related cardiotoxicity are seen as a mediation by the rise in reactive oxygen radicals, inflammation and apoptosis [9-11]. It is demonstrated that high doses CYP chemotherapy can lead to fatal cardiac toxicity and letal disorders such as depression of myocardium, congestive cardiac deficiency, heart cushioning and arrhythmias [12]. Myocardial damage is one of the most significant adverse effect of CYP that generally result

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in death of the patients. CYP-related cardiotoxicity was related with depression of the antioxidant defense mechanism [13, 14]. Antioxidant' s protective roles become very crucial to avoid from toxicity of drugs [15, 16]. Antioxidant agents can detoxify the toxic effects of undesirable agents.

Hypericum species (belongs to the Hypericaceae family) have been traditionally used in varied part of the world for its antioxidative, antiseptic, sedative, anticholinesterase, cytotoxic, antigenotoxic and antimicrobial features [15, 17-20]. *Hypericum triquetrifolium* Turra. (HT) has get quite a lot of scientific attention recently as the source of a various biologic active compounds such as polyphenols [15], hyperoside, quercetin, quercitrin, chlorogenic acid, rutin [17], kaempferol and flavonoids [18]. Aerial parts of HT with methanolic extract possesses highly antioxidant activities [18] and this feature might be useful in protecting or decelerating the continue of diverse oxidative stress-induced disorders [20]. Current study intend to research the possible cytoprotective effects of HT on CYP-related cardiac toxicity.

2. RESULTS

2.1. Histopathological assessment

In the 25, 50,100 mg/kg HT and 0.2% DMSO groups, cardiac tissues had normal histology like control (0.5 ml saline) group. Degenerations were seen in some heart cells of 150 mg /kg CYP alone treated rats. Besides, slight edema, hemorrhage and inflammation were seen. Although no significant degenerations were observed in heart tissues of 25, 50, 100 mg/kg HT plus 150 mg/kg CYP treated rats, heart histology was better as HT dose increases (Figure 1A-C).

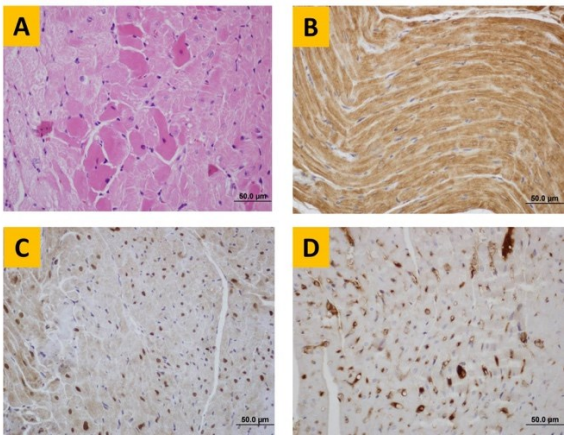

Figure 1. 150mg/kg CYP Group **A:** H&E, **B:** Caspase-3, **C:** Bcl-2, **D:** Bax

2.2. Apoptosis assessment

Bcl-2 (anti-apoptotic protein) immunohistochemistry results showed no conspicious difference in heart tissue sections. However, pro-apoptotic caspase-3 and bax immunohistochemistry were displayed difference among the groups' heart tissue sections. In the 150 mg/kg CYP and 25, 50, 100 mg/kg HT plus 150 mg/kg CYP treated rats, apoptotic cells were observed more common than in other groups. Bax and caspase-3 expression was reduced by CYP+HT doses when compared with single dose of CYP. Similarly, reduced Bcl-2 expression due to CYP toxicity was significantly improved. As the HT doses (25, 50, 100) given with CYP increased, the density of apoptosis decreased in cardiac tissue cells (Figure 1, 2, 3).

2.3. Biochemical assessment

Group treated with 150 mg/kg Cyclophosphamide (CYP) showed an important decline ($p<0.001$) in the serum activities of CK-MB compared with control group. Administration with 25, 50 mg/kg HT there is no statistical significance compared the control. But CYP plus 25, 50, 100 mg/kg HT specially 100 mg/kg HT treatment induced important decrease in the level of serum CK-MB ($p<0.001$) compared to the single dose CYP treated group. The DMSO group showed no significant ($p>0.05$) difference from control (Figure 4, table 1).

150 mg/kg CYP administration induced an important increment in the serum AST level ($p<0.001$) compared with control. Administration of 25, 50, mg/kg HT alone induced no difference in the serum AST from the

control group. CYP plus 50 and 100 mg/kg HT showed an important decrease in the AST level compared to the single dose of CYP group ($p<0.001$). When we compared groups 25, 50 and 100 mg/kg HT with each other, a more significant decrease was observed especially in group 100 mg/kg HT. The 0.2% DMSO group showed no significant ($p>0.05$) difference from the control group (Figure 5, Table 1).

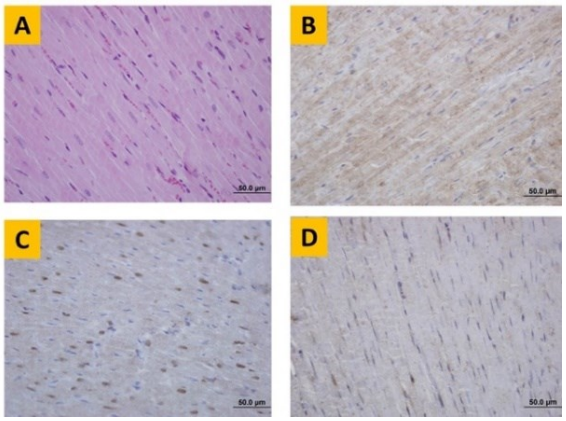

Figure 2. 100 mg/kg HT group **A**: H&E, **B**: Caspase-3, **C**: Bcl-2, **D**: Bax

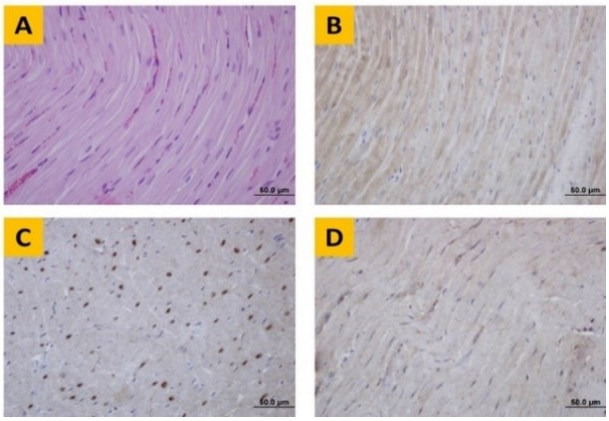

Figure 3. 150 mg/kg CYP+100 mg/kg HT Group **A:** H&E, **B:** Caspase-3, **C:** Bcl-2, **D:** Bax.

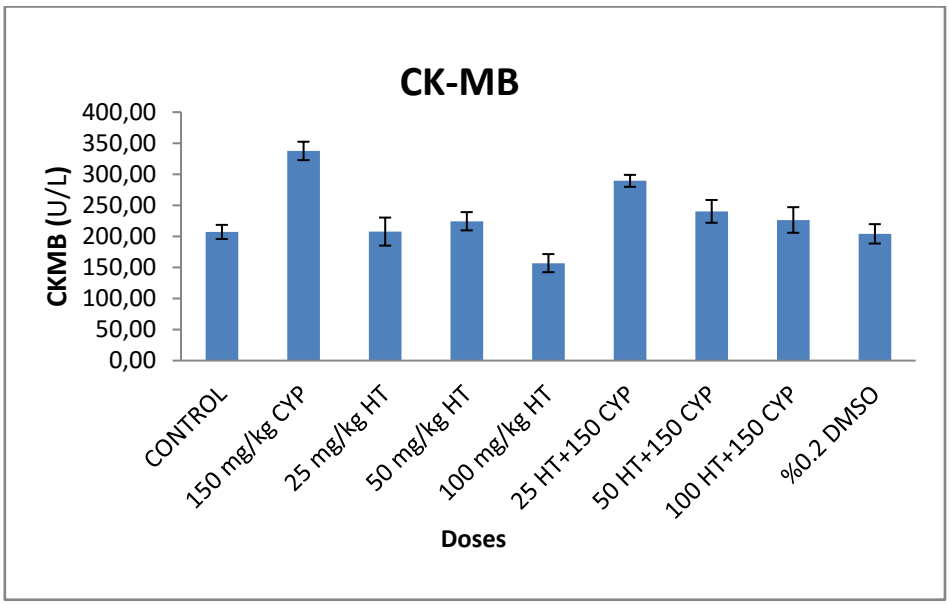

Figure 4. CK-MB levels of 150 mg/kg CYP, 150 CYP+ 25, 50,100 mg/kg HT and 0.2 % DMSO applied experimental groups.

An important increase was observed on the level MDA ($p<0.001$) after 150 mg/kg CYP treatment compared to the control group. Only 25, 50, 100 mg/kg HT induced groups showed no important difference in the serum MDA from the control group. When administrating with 25, 50, 100 mg/kg HT plus 150 mg/kg CYP there was seen an important decrease in the level of MDA compared with single dose CYP treated group ($p<0.001$). However, the MDA level in the group treated with 100 mg/kg HT plus CYP was significantly lower than 25, 50 mg/kg HT plus CYP. In the DMSO group, MDA level was close to the control level and showed no significant ($p>0.05$) difference (Figure 6, Table 1).

CYP-induced (150 mg/kg) group showed an important decrease ($p<0.001$) in the GSH level compared with the control group. Administration of only 25, 50, 100 mg/kg HT induced groups showed no difference in the GSH level from the control group ($p>0.05$). Administration with 50, 100 mg/kg HT plus 150 mg/kg CYP showed an important increase in the level of GSH compared with the single dose CYP-administered group ($p<0.001$). In the 0.2% DMSO group, GSH level was close to the control and showed no significant ($p>0.05$) differences (Figure 7, Table 1).

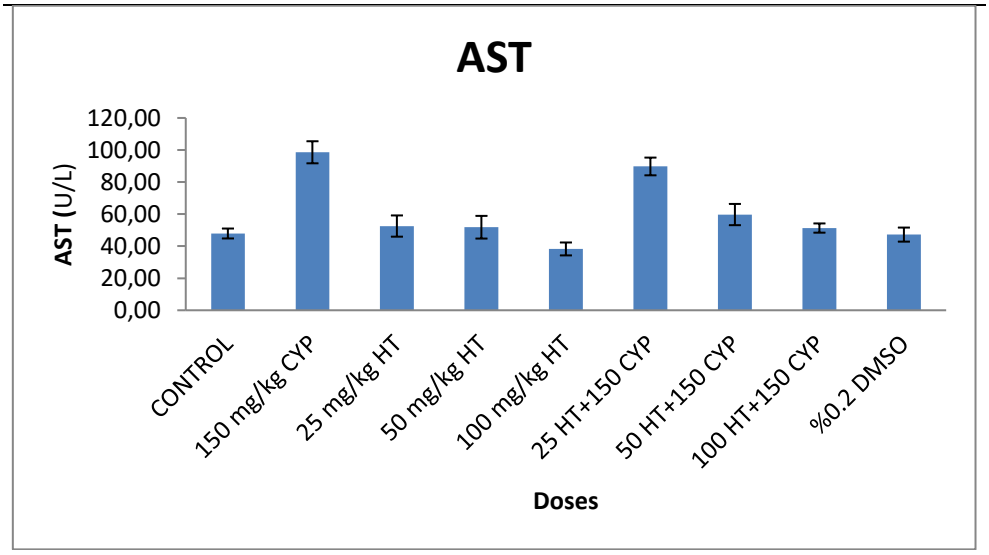

Figure 5 - AST levels of 150 mg/kg CYP, 150 CYP+ 25, 50, 100 mg/kg HT and 0.2 % DMSO applied experimental groups.

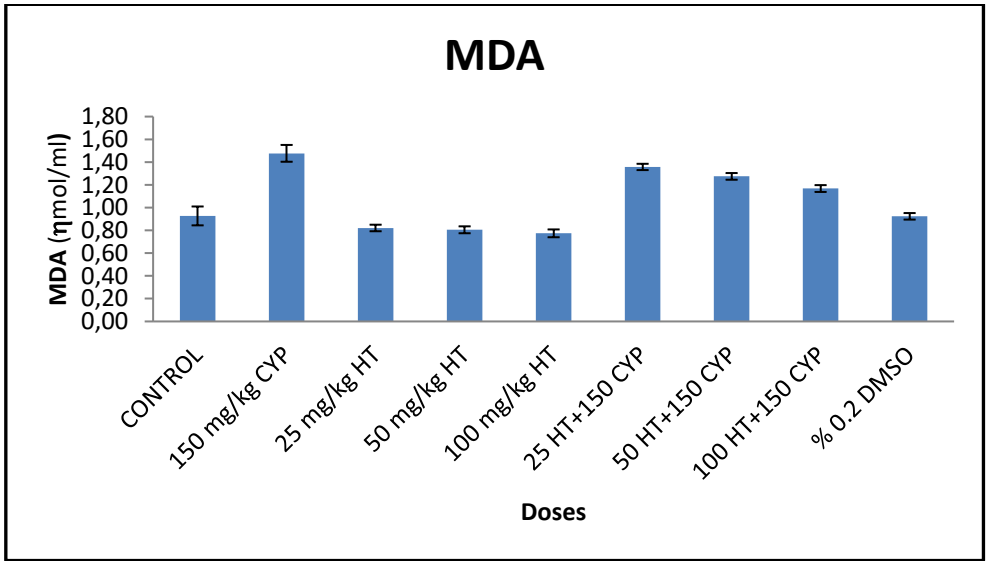

Figure 6 -MDA levels of 150 mg/kg CYP, 150 CYP+25, 50, 100 HT and 0.2 % DMSO applied experimental groups.

TAC level was found considerably lower in the 150 mg/kg CYP-induced rat group compared with the control ($p<0.001$). No change was seen in the only 25, 50, 100 mg/kg HT dose administration groups compared to control group in the TAC level ($p>0.05$). Administration of rats with 50, 100 mg/kg HT plus 150 mg/kg CYP showed an important increase ($p<0.001$) in the TAC level when compared with the single dose CYP-induced group. More significant increase was seen, especially in group 100 mg/kg HT when we compared the groups of 25, 50 and 100 mg/kg HT with each other. In the 0.2% DMSO group, TAC level was close to the control and showed no significant ($p>0.05$) differences (Figure 8, Table 1).

Table 1: The demonstration of the effects of 25, 50,100 mg/kg doses of *Hypericum triquetrifolium* Turra.' s (HT) biochemical assessments on 150 mg/ kg CYP toxicity in rats in multiple comparision

	CK-MB	p<0.001*	MDA	p<0.001	AST	p<0.001	GSH	p<0.001	TAC	p<0.001	TOC	p<0.001
1.Control	207.1±11.4	1-2, 1-5, 1-6, 1-7	0.93±0.08	1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8	47.9±3.1	1-2, 1-5, 1-6, 1-7	4.85±0.47	1-2, 1-6, 1-7, 1-8	1.36±0.01	1-2, 1-6, 1-7	0.21±0.01	1-2, 1-6, 1-7, 1-8
2. 150 mg/kg CYP	337.6±14.8	2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9	1.48±0.07	2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9	98.6±6.9	2-3, 2-4, 2-5, 2-7, 2-8, 2-9	2.30±0.10	2-3, 2-4, 2-5, 2-7, 2-8, 2-9	1.08±0.05	2-3, 2-4, 2-5, 2-7, 2-8, 2-9	2.63±0.11	2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9
3. 25 mg/kg HT	207.7±22.6	3-5, 3-6, 3-7	0.8±0.03	3-6, 3-7, 3-8, 3-9	52.6±6.6	3-5, 3-6	5.00±0.40	3-6, 3-7, 3-8	1.35±0.08	3-6, 3-7	0.22±0.02	3-6, 3-7, 3-8
4. 50 mg/kg HT	224.3±14.7	4-5, 4-6	0.81±0.03	4-6, 4-7, 4-8, 4-9	51.8±7.1	4-5, 4-6	5.11±0.01	4-6, 4-7, 4-8	1.38±0.07	4-6, 4-7	0.22±0.01	4-6, 4-7, 4-8
5. 100 mg/kg HT	156.9±14.6	5-6, 5-7, 5-8, 5-9	0.77±0.03	5-6, 5-7, 5-8, 5-9	38.3±4.0	5-6, 5-7, 5-8	5.30±0.25	5-6, 5-7, 5-8	1.42±0.05	5-6, 5-7, 5-8	0.22±0.06	5-6, 5-7, 5-8
6. 25 mg/kg CYP+HT	289.4±9.6	6-7, 6-8, 6-9,	1.36±0.03	6-7, 6-8, 6-9	89.7±5.5	6-7, 6-8, 6-9	2.45±0.11	6-8, 6-9	1.14±0.02	6-8	2.17±0.08	6-7, 6-8, 6-9
7. 50 mg/kg CYP+HT	240.2±18.3	7-9	1.27±0.03	7-8, 7-9	59.7±6.6	7-9	2.80±0.16	7-8, 7-9	1.20±0.02	7-9	1.59±0.11	7-8, 7-9
8.100 mg/kg CYP+HT	226.4±20.6		1.17±0.03	8-9	51.3±2.9		2.82±0.23	8-9	1.30±0.02		1.17±0.06	8-9
9. DMSO	204.1±15.6		0.92±0.03		47.2±4.4		4.90±0.24		1.38±0.06		0.22±0.01	

*(p<0.001: statistically significant differences, (p>0.05): no statistically significant differences).

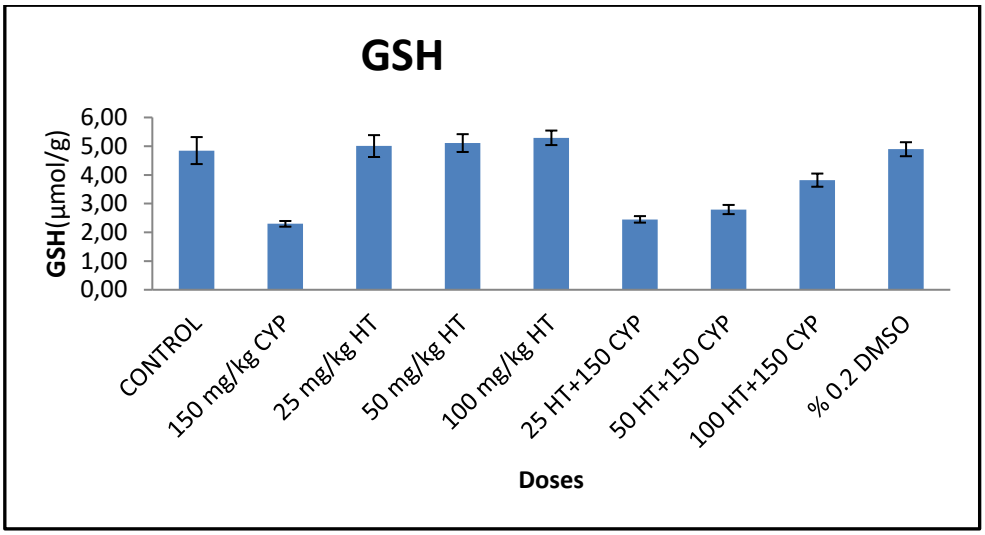

Figure 7. GSH levels of 150 mg/kg CYP, 150 CYP+ 25, 50, 100 mg/kg HT and 0.2% DMSO applied experimental groups.

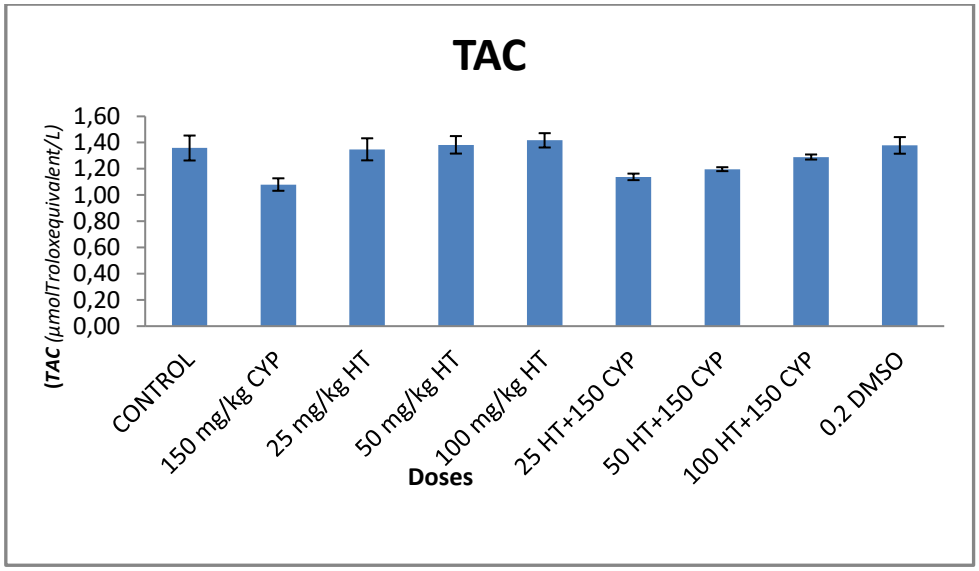

Figure 8. TAC levels of 150 mg/kg CYP, 150CYP+ 25, 50, 100 mg/kg HT and 0.2% DMSO applied experimental groups.

Results showed no important difference in TOC level in the cardiac tissue when groups of 25, 50 and 100 mg/kg HT were compared with control ($p>0.05$). 150 mg/kg CYP treatment caused a significant increase in TOC level compared with the control ($p<0.001$). This situation was strongly reversed by 25, 50, 100 mg/kg HT and especially by 100 mg/kg HT in the combination with CYP ($p<0.001$). As in all the other groups, there was no significant ($p>0.05$) difference in 0.2% DMSO group compared to control (Figure 9, table 1).

3. DISCUSSION

Cardiovascular disorders were proven by a wide range of anti-tumoral approaches, such as chemotherapy, radiotherapy, targeted therapies, hormone therapy or anti-angiogenic agents. As an important part of the cardiovascular complications are thought to be the result of effects of the anticancer therapy on the myocardium and cardiotoxicity induced by chemotherapeutic agents is a growing problem. Cyclophosphamide (CYP) demonstrates a powerful anti-tumor and immunosuppressant properties on the other hand its cardiac toxicity is one of the most terrible side effect and restrict its usage specially in high-dose therapies.

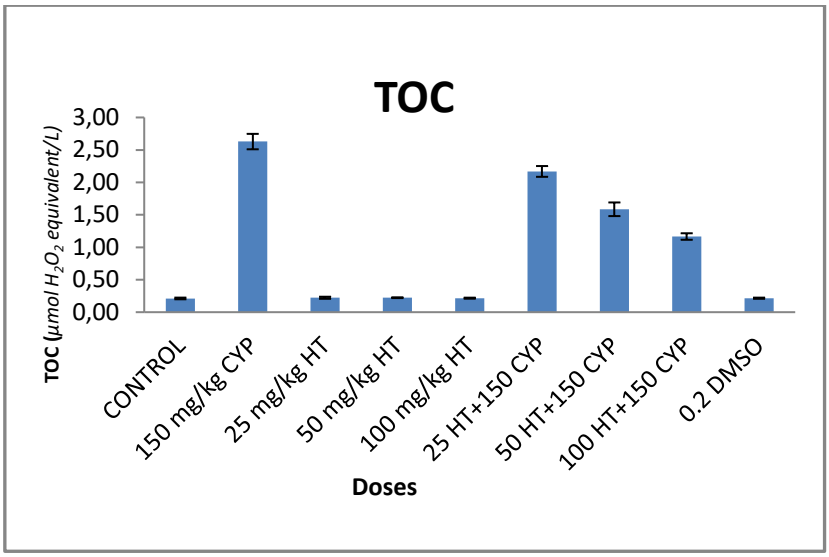

Figure 9. TOC levels of 150 mg/kg CYP, 150 CYP+ 25, 50, 100 mg/kg HT and 0.2% DMSO applied experimental groups.

Major limitation of CYP usages is the injury of normal tissue and leads to multiple organ toxicity. The injury of normal tissue cells is one of the biggest limitation for the usage of CYP, which increases various adverse effect. CYP' s active metabolites are transported from systemic circulation into the normal or tumor tissues, and induce cytotoxicity but also cause various toxicity for healthy cells in patients and experimental animals. CYP generates biochemical and physiological disturbances. Preventation or minimisation of the adverse effects CYP may supply better tolerance of the drugs and more effective therapies for patients in need of CYP treatment. In the numerous researches it is demonstrated that antioxidants show advantageous properties to decrease the cardiac disorders. Therefore, there is a need for strong and more effective agents that can protect the healthy cells and tissues from chemotheraputic toxicities but without tumour protection. In the numerous researches [2, 16, 25] it is reported that medicinal plant-based natural compounds might supply protective effects against CYP induced toxicity. Moreover it was demonstrated that medicinal plants contain large amounts of antioxidant properties and prevent adverse effect of free radicals-induced oxidative stress [20].

Our histopatological results showed that CYP induces a significant myocardial cell injury. Reduced anti-apoptotic protein Bcl-2 expression and increased pro-apoptotic protein Bax, caspase-3 is due to CYP toxicity. Caspase-3, the main apoptotic executioner, causes chromatin condensation, protein breakdown, and DNA fragmentation. We may conclude that high-dose chemotherapy regimens together with using CYP may frequently associated with cardiotoxicity that could lead to myocyte devastation and congestive heart failure and CYP toxicity might be the result of increased permeability of cardiac mitochondrial membrane. As a matter of fact, this result has also been expressed by similar studies [16, 26]. Overproduction of reactive oxygen species leads to the oxidative stress, DNA injury, cellular membrane impairment and necrosis in cells and tissues [27]. Also, in some other researches it was observed that CYP quickly damages membranes by lipid peroxidation and results in the loss of myocardial membrane integrity and function [28, 29]. CYP induced cardiotoxicity has been implicated to an increase in the generation of reactive oxygen species, which impair the cardiac tissue by exceeding the oxygen radical detoxifying capacity of cardiac mitochondrial cells [30]. Besides, in a clinical study it was demonstrated that patients with fulminant cardiotoxicity following CYP therapy showed myocardial hemorrhage, pericardial effusion and fibrinous pericarditis [31]. The pathogenesis of CYP-induced acute cardiotoxicity caused to the increase in ROS and the decrease in the antioxidant defense mechanism [9].

HT is an important source of various bioactive compounds. The amounts of these compounds, such as phenols, in plants vary among different aboveground parts (leaf, flower, fruit and seed) during the seasonal period. Our results clearly demonstrated that there is a conspicuous relationship between the use of HT and reductions in the CYP-induced lipid peroxidation, hemorrhage and histological damage in myocardial tissue. Also, our results showed that reduced Bcl-2, increased Bax and caspase-3 expression due to CYP toxicity was significantly improved by HT.

Cardiotoxic drug CYP directly causes an injury of myocardium cells then enzymes are released and passed to the blood stream. AST and CKMB are act as the diagnostic marker of myocardium injuries and are released into the flow of blood. We can infer from this overproduction of reactive oxygen species initiates lipid peroxidation by CYP-induced membrane damage and results in loss of functions and integrity of myocardium membrane. In fact, in our study CK-MB and AST levels increased importantly in the CYP group, seriatim, when compared to control group. This increase showed heart tissue damage, which are related with myocardial infarction, cardiac failure and myocarditis (Figure 2, 3). Similar to our study in many other studies [11, 16, 26] there was seen an increasing level of CK-MB when treated with CYP. Our biochemical data has been expressed in the same line with other studies which observed a significant increase in AST and CK-MB levels [25, 29, 32]. After using the dose of 25, 50, 100 mg/kg HT plus CYP; it was resulted in a clear decrease in AST and CK-MB levels compared to the CYP group (Figure 4, 5, table 1).

Significant complications in the development of tissue injuries are lipid peroxidation such as MDA which is one of the end-products of lipid peroxidations. Furthermore, MDA is a significant determinant in the evaluation for lipids peroxidation, oxidative stress and antioxidant status in tissues. As it was represented in the figure 4, CYP-related cardiotoxicity was obviously seen by the increasement in serum MDA level. MDA levels showed an important increase in the CYP-induced group compared with control rats. In the only of 25, 50,100 HT administered groups observed an important decrease in the MDA levels while compared with CYP-treated group (Figure 6, table 1). It was demonstrated in the experimental study that CYP administration led to oxidative stress measured as MDA concentration as the indicator of lipid peroxidation [33]. As it's seen in the figure 4, MDA level was remarkably high in the CYP-treated group compared to control [34, 25]. HT doses, specially in the 50 mg/kg CYP+HT significantly inhibited the increase in MDA level.

GSH is an important natural non-enzymatic biomarker of cellular antioxidative status to defense against the reactive oxygen species. GSH shows a significant activity in the drug detoxify but active metabolite of CYP depletes cellular GSH levels. In the figure 7, serum GSH level was represented a remarkable decrease in CYP-treated group compared with control rats. In our result the decrease of GSH level is to be identified with an increment toxicity by CYP. Levels of free radical detoxifying enzymes like GSH were decreased in the heart during CYP intoxication. Reduced GSH has low molecule weight scavenger for free radicals and a significant agent to inhibit the formation free radicals (Figure 7, table 1). The results obtained in the research of Machida et al [35] showed that high dose CYP (200 mg/kg)-caused cardiac toxicity was associated with increment in the reactive species of oxygen while there was a decline in the antioxidative defense systems in myocardium that antioxidative compounds lightened CYP-caused cardiac toxicity. The GSH level was found significantly increased in the 25, 50, 100 mg/kg HT-administered groups compared with group of CYP. In the same line it was observed that HT following CYP treatment protected the normal GSH level [34]. Paralel to our results, Liu et al [25] demonstrated the highest MDA level and the lowest GSH level in the CYP group compared to control. In brief, according to these results, we could say that, levels of AST, CK-MB and MDA were significantly increased while GSH level decreased in the CYP group compared with control. In the HT+CYP administered groups, a dose-dependent (specially in the 100 mg/kg HT+CYP group) decline in the cardiac biomarker's injuries (AST, CK-MB, MDA) and a signifiant increase in GSH level were observed. Paralel to the current study, Asiri et al [11] demonstrated that CYP significantly increased serum cardiac enzymes, CK-MB and AST while demonstrating an important decline in GSH. Similarly, Wei et al [36] resulted that CYP induces oxidative injuries in vital organs by increasing the MDA and declining GSH. CYP-related reducement in the activity of the antioxidantive enzymes in the myocardial cells was because of the disorders of these enzymes by reactive oxygen species.

Our findings showed that level of TAC decreased with dose of CYP (Figure 8, table 1). It was observed that there is an significant rise in the level of TAC with administered CYP plus HT. These may imply that overproduction of reactive oxygen species could be reduced by the HT, in the same line, other studies indicated a decrease in the antioxidantive enzyme activity is a consequence of raised oxidative stress in the heart tissue [34, 37]. This results are consistent with numerous researches [11, 16, 38]. In 150 mg/kg CYP treated group the level of TOC increased, which indicated CYP-related oxidative stress and cardiac toxicity. The improvement of TOC levels was found close to control group after being treated with dose of 25, 50, 100 mg/kg HT, specially dose of 50 mg/kg HT. As a result; these experimental results showed that HT may be helful to reduce oxidative stress-induced tissue failure (Figure 9, table 1).

The elevated levels of serum enzymes might be due to the overproduction of reactive oxygen species by CYP-induced chemotherapy which led to injuries of membrane by starting lipids peroxidation. These resulted in loss of myocardial membranes, integrity and functions. Also, reactive oxygen species or free

radicals attack the diverse component of cells containing DNA, proteins and membrane lipids, thereby this causes to important cell failures. Moreover, these bioactive compound's rations play a significant role in the drug' s efficacy.

4. CONCLUSION

Cardiotoxicity is becoming one of the most important complication of cancer chemotherapy. High dose CYP-therapy has an anti-angiogenic effect in tumor cells but we need to use high dose to be effective, and also we need to eleminate the side effect of CYP on the cardiac tissue cell. Treatment with antioxidantive HT showed a significant protection against these abnormalities. Based on our data, it can be proposed that HT was a strong candidate to protect the healthy cell from the CYP' s cardiotoxic side effects but more clinical reseraches need to be done in order to verify this research.

5. MATERIALS AND METHODS

5.1. Drug and chemicals

Endoxan, Cyclophosphamide Monohydrate, C0768 (CYP), Serum creatine kinase-MB (CK-MB), malondialdehyde (MDA), aspartate transaminase (AST), glutathione (GSH), total antioxidant (TAC) and total oxidant capacity (TOC) assay kits were commercially purchased from Sigma-Aldrich, Taufkirchen, Germany. CYP (500 mg) was dissolved in 25 mL bidistilled water to its own appropriate concentration prior to its injection to animals, sequentially, and stocked at 4 °C before use. Single dose CYP (150 mg/kg b.w. - i.p) administered rats. In human serum the biological half-life (t ½) of CYP is about 6.5 hours [21].

5.2. Preperation of plant material

Hypericum triquetrifolium Turra. (HT) plant materials were collected at seeding stages from August to September, 2015. Voucher specimens were deposited at the Mardin Artuklu University Herbarium (C.KESKİN 2015-14), Mardin, Turkey. Taxonomic identification of plant materials was confirmed by Dr Cumali Keskin from the same institution. 20 g of seed were ground into powder. Seed powder were extracted 3 times with 200 ml absolute methanol under magnetic stirrer. After the all extractions processes aproximately 4 g of the crude methanol extracts were obtained and stored at -20°C before the experiments. Obtained crude methanol extract was dissolved with 0.2% dimethyl sulfoxide (DMSO) to obtain different concentrations (25, 50 and 100 mg/kg) into the ultrasonic bath.

5.3. Animals

Wistar Albino rats provided from Kobay Experimental Animals Lab. San. Tic. A.SS., and this research performed according to the confirmation the ESOGU Experimental Animals Ethic Committee prior to the experiment. 220±20 g healthy, 3-4 mont, male rats were kept in transparent polypropylene cages under standard enviromental conditions and at 25±2°C room temperature, humidity 60-70%, supplied 12 h light-dark cycles under the standart enviromental condition at room temperature and suplied 12 h light / 12 h dark cycle and were allowed freely to access standart pellet food with regular tap water. Rats were housed in the rat cages for 2 weeks in order to adapt the enviroment. Local institutional animal caring and using committee confirmed the protocol of experiment.

5.4. Experiment

63 rats were seperated into 9 groups, every one inclued 7 rats. Group 1 (control) treated with saline (0.5 ml); group 2 treated with single dose CYP (150 mg/kg), respectively; groups 3, 4 and 5 treated with 25, 50 and 100 mg/kg HT; groups 6, 7 and 8 treated with 25, 50 and 100 mg/kg HT + CYP, Group 9 treated with 0.5ml - % 0.2 DMSO. Doses of medicines and chemicals were determined and prepared for use in the injection. All injections were made intraperitoneally (i.p). Group1: control rats were treated with 0.5 ml serum physiologic (SF) so-called normal saline for 6 days. Group 2; rats were treated with SF for 5 days then 6th day treated with single dose of CYP (150 mg/kg). Groups 3,4,5; rats were respectively treated with 25, 50,100 mg/kg HT-dose for 6 days. Groups 6, 7, 8; rats were respectively treated with 25, 50,100 mg/kg HT dose. Then 6th day single dose of 150 mg/kg CYP was administered. Group 9; treated with % 0.2 DMSO (dimethyle sulfoxide) for 6 days. Before the animals were sacrificed on 7th day, the samples of blood were taken with cardiac puncture

via the ketamine/xylazine anesthesia to using for measurement serum parameters. Then the animals sacrificed at the 7th day.

5.5. Measurement of cardiac marker enzymes level

Rats blood samples were centrifuged at 3000 rpm for 10 min and examined for creatinekinase-MB (CK-MB) and aspartate transaminase (AST) enzymes [22].

5.6. Measurement of malondialdehyde (MDA) levels

Serum samples including lipid peroxidation products namely MDA were examined by spectrophotometer at 520 nm by the tiobarbituric acid (TBA) reactive substance method according to Yagi (1984) [23].

5.7. Measurement of serum glutatyon (GSH) levels

Levels of GSH measured at 412 nm (v/v) according to Sedlak and Lindsay (1968) [24] method. Serum samples were precipitated with % 50 trichloroacetic acid (TCA) and centrifuged at 1000 rpm for 5 min. 0.5 mL sample was taken from the upper phase, then 2.0 mL Tris-EDTA buffer (0.2 M, pH 8.9) and 0.1 mL 0.01 M 5,5'-ditiyo-bis-2-nitrobenzoic acid were added. This mixture was incubated at room temperature for 5 min. and its absorbance was measured by UV-1700 Shimadzu spectrophotometer.

5.8. Measurement of total oxidant capacity (TOC) and total antioxidant capacity (TAC) levels

Serum samples were analyzed for TAC and TOC, after blood samples were centrifuged at 3000 rpm to 10 min. Afterwards, measurements were performed by using HITACHI - 917 oto analyzer (Human Gesellschaft für Biochemica und Diagnostica GmbH, Wiesbaden, Germany) together with commercial kits.

5.9. Histological measurement

Cardiac tissues were stained in % 10 formaldehyde solution. Through routine histologic preperation, tissue samples were embedded in paraffin then 5.0 micron thick serial sections were done, which were kept with Hematoxylin - Eosin. After all, histopathological properties were measured. The consequences were assayed with Variance of One Way Analysis and Kruskal-Wallis test to scores varying with unnatural distribution. Differences between the groups of experiment were important if $p<0.05$.

5.10. Immunohistochemistry

Sections of cardiac tissues were deparaffinized and rehydrated routinely. Antigen retrieval by citrate buffer (pH 6.0) was done by heating the sections in a microwave at 700 W for 10 min. After blocking with 3 mL/L H_2O_2 and swine serum, 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.

5.11. Statistical analysis

The result of the test was signified as means ± S.E.M. Statistical analysing was performed by One Way Analysis of Variance and Kruskal-Wallis One Way Analysis of Variance on Ranks Test. Then $p<0.05$ received as considerable statistical importance. Each experiment was repeated at least three times.

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