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Trifolium Pratense Improves Cyclophosphamide-Induced Thrombocytopenia and Leukopenia in a Rat Model of Chemotherapy

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Abstract

Background: Due to the toxicity of chemotherapy drugs in cancer patients, thrombocytopenia can lead to bleeding. *Trifolium pratense* L. is traditionally used as an anti-inflammatory compound for the treatment of various diseases. The aim of the present study was to evaluate the effect of *T. pratense* (red clover) extract (TPE) on thrombocytopenia and the related factors in a rat model of chemotherapy.

Materials and Methods: In this experimental *in vivo* study, 28 rats were randomly divided into four groups of seven members (four males and three females) including Group1 as the control subjects, Group2 as thrombocytopenia cases, and Groups 3 and 4 with thrombocytopenic animals receiving TPE (100 and 200 mg/kg). Thrombocytopenia was induced by intraperitoneal injection of cyclophosphamide (CP) on three consecutive days. Then, the TPE was fed to rats for 14 days. At the end of the study, the rats' weight was measured. Blood samples were collected, complete blood count (CBC) was performed, and PF4 and clotting time were measured. After the dissection of the animals, the bone marrow and spleen were separated, and hisopathological changes were determined. The data were analyzed by one-way ANOVA and a post-hoc Tukey test.

Results: Cyclophosphamide decreased the platelets and the white blood cells (WBCs) and increased PF4 and the clotting time significantly ($P < 0.05$). Also, TPE significantly increased the platelets and the WBC counts but decreased the time of clotting and the PF4 factor ($P < 0.05$). TPE increased megakaryocyte ($P < 0.001$) and enhanced the bone marrow and spleen cellularity.

Conclusion: *T. pratense* can increase the number of platelets and WBCs and improve thrombocytopenia and bone marrow cellularity induced by chemotherapy.

Keywords: Chemotherapy, Cyclophosphamide, Thrombocytopenia, *Trifolium pratense*

Introduction

Thrombocytopenia is one of the most common problems arising in the chemotherapy of cancer patients, and severe types of that occur in approximately 9% of chemotherapy courses. The clinical complications of this disease are purpura, nasal and gingival bleeding, severe hematuria and gastrointestinal bleeding. Furthermore, intracranial hemorrhage has been reported in 45.8% of patients with thrombocytopenia. Its mechanism generally involves bone marrow suppression and platelet destruction, which leads to decreased platelet production (1-3). Chemotherapy often damages normal cells and leads to thrombocytopenia and leukopenia, which limit the doses of cancer therapeutic drugs (4). The primary treatment for this disease is generally corticosteroids, which are recurrent and reversible in most cases (5). Another treatment is intravenous immunoglobulin (IVIG), which unreliably increases the platelets, has transient effects, and requires repeated administration (6). The second stage of treatment is usually splenectomy, whose response to treatment has not been well defined. In this regard, urgent treatments such as platelet reception are

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generally not a long-term therapeutic option. In addition, it is not appropriate for some types of diseases due to heparin (5). Indeed, bone marrow suppression has been a common side effect of chemotherapy (7). Various chemotherapy drugs affect megakaryocytes and platelet production in different ways. Cyclophosphamide (CP) has been the most widely used alkylating agent in chemotherapy with a high therapeutic index, and it has a broad spectrum of activity (8). It does not harm hematopoietic stem cells due to their high levels of aldehyde dehydrogenase, but it affects megakaryocyte precursors (9). In combination with other chemotherapy drugs, CP is used to treat different types of cancers, such as breast, ovarian, endometrial, and lung cancers (10). In the present study, CP was used as a toxic agent due to its ability to cause stable thrombocytopenia (11).

Trifolium pratense L. (red clover) from the leguminous family is a famous medicinal herb that contains isoflavone, phenolic acid, saponin, phytosterol and phytic acid. Isoflavones contain biocyanin A and formononetin, which are precursors of diodesin and genistein, respectively (12, 13). Formononetin has antimicrobial, antioxidant, and neuroprotective properties and stimulates angiogenesis in wound healing and cardiovascular diseases (14). In addition, isoflavone has antioxidant properties and may be beneficial for vessel protection. This compound can inhibit blood coagulation and improve the blood flow (12). *T. pratense* extract (TPE) has shown to have antitumor effects in *in vitro* studies on breast (15), prostate (16), and glioblastoma (17) cell lines as well as an anti-diabetic effect on the pancreatic beta cell (RIN-5) line (18). It also has synergistic antitumor effects with doxorubicin, delays the formation of breast cancer, and increases apoptosis in 4T1 cells (19). Herbal remedies are relatively free of side effects and toxicity and have long been welcomed by the majority. Considering the potential antioxidant effects of *T. pratense* on the oxidative stress of platelets and the possible effect of its compounds on megakaryocyte function, this plant is of interest. The aim of the present study was to investigate the effect of *T. pratense* (red clover) on the blood cell count and the corresponding factors in chemotherapy for CP-induced thrombo-cytopenia in a rat model.

Materials and Methods

Animal subjects

This experimental animal study was conducted on 28 Wistar rats (four males and three females, 200 to 230 g). The animals were housed in plastic cages under the standard conditions of 12 h of light and 12 h of darkness, 20-22°C, and free access to food and water *ad libitum*.

Extract preparation

Trifolium pratense L. seeds were obtained from Karaj Seed and Plant Improvement Research Institute (20) and cultivated in spring. The leaves and areal parts were harvested, dried, and milled at laboratory temperature. Then, 100 g of the powder was mixed with 500 ml of 70% ethanol, and the solution was kept in dark at room temperature for 48 hours. After passing through a filter paper, the solution was dried and stored at 4°C until use. Sterile distilled water was used to prepare different doses of the extract (100 and 200 mg/kg body weight), which were then given to the thrombocytopenic rats. Each rat received 1 ml of the extract orally (12).

Study design

Based on similar animal studies (12, 19, 21), 28 rats were randomly divided into four groups of seven members (four male and three female rats). The control group received a daily dose of 1 ml of a normal saline orally. The thrombocytopenic group received CP (100, 50 and 50 mg/kg intraperitoneally on three consecutive days, respectively) (22) and an oral normal

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saline until the end of the study. In the treated groups, the thrombocytopenic rats received 1 ml of a saline orally on days 4 and 5 as well as one ml of the extract at a dose of 100 mg/kg/day (CP-T100) or 200 mg/kg/day (CP-T200) for 14 days orally.

The induced thrombocytopenia in the rats was assessed on day 6 through counting the platelets using a Neobar homocytometer, according to the standard protocol. Briefly, the blood samples from the retro-barbital network were added to a tube containing EDTA. Next, 20 µl of the blood was mixed with 0.38 ml of freshly prepared ammonium oxalate (1%) as a dilution solution in 10 minutes (23). The homocytometer was filled with the compound and placed in a humid chamber for 10 minutes to let the platelets settle. The platelets were then counted in a Neobar slide (mid-25-squared) by light microscopy.

Histopathology, hematology and blood biochemical evaluation

At the end of the study, the blood samples were taken from the hearts of all the rats, a part of the blood was added to the EDTA tube, and complete blood count (CBC) was performed for the platelets, white blood cells (WBC) and red blood cells (RBC). The other parts of the blood samples were centrifuged at 2500 rpm for 15 minutes, and the serum was used to assay platelet factor 4 (PF4) with an Elisa Kit (BT-Labs. Shanghai, China). A portion of the blood sample was also taken to measure the clotting time through the Lee-White method. The spleen and femoral bone marrow were removed and fixed in 10% buffer formalin. After tissue processing, 5-µm sections were prepared, five slices were used from each rat, and hematoxylin and eosin staining were performed. The megakaryocytes in ten microscopic fields were counted, and their average number was calculated.

Statistical analysis

After the normal distribution of the data was ensured with the Kolomogrov-Spirinov test, a t-test was used to compare the baseline and final weights of the groups, and one-way ANOVA was applied for the other quantitative data. The data were reported as mean values and standard deviations, and the P levels less than 0.05 were considered significant.

Results

Blood cells count

In the CP group, the number of platelets was significantly decreased compared to that in the control group ($P < 0.001$). Also, as Figure 1A and Table I suggest, in the thrombocytopenic-treated rats which received doses of 100 or 200, the platelets significantly increased compared to those in the CP group ($P < 0.001$). The WBCs in the CP group were significantly fewer than in the control group ($P = 0.001$). In the thrombocytopenic-treated groups, the mean WBC count was significantly higher than that in the CP group ($P = 0.038$ and $P = 0.02$, respectively) (Figure 1B, Table I). As the results showed, the mean reduction of the RBC count was not significant in the CP group, ($P = 0.267$). The mean RBC count increased in the thrombocytopenic groups treated with the extracts compared to the CP group, but it was not significant ($P > 0.05$) (Figure 1C, Table I).

Clotting time and PF4 assay

The measurement of the clotting time in the study groups showed a significant increase in the CP group compared to the control group ($P < 0.001$). In the thrombocytopenia groups treated with the extract (at the doses of 100 and 200), the time of clotting was significantly reduced compared to that in the thrombocytopenic group (Figure 2A). The results of the PF4 measurement in the study groups showed a decrease of this factor in all the experimental groups compared to the

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control group, which was significant in the groups receiving 100 and 200 doses of the extract (P = 0.004 and P < 0.000, respectively) (Figure 2B).

Body and spleen weights

The final weight of the rats in the CP group was significantly lower than that in the control group (P = 0.017), but it had no change in the thrombocytopenic group receiving 100 doses of the extract (P = 0.022). In the thrombocytopenic rats treated with 200 mg/kg of the extracts, the body weight was increased with no significant difference from the control group (Fig. 3A). In the CP group as well as the thrombocytopenic groups receiving the extract at the doses of 100 and 200, the spleen weight significantly increased compared to the control group (P = 0.008, P = 0.035 and P = 0.001, respectively) (Fig. 3B).

Megakaryocyte count

The statistical analysis of the number of megakaryocytes in different groups showed a significant decrease in the CP group compared to the control group (P < 0.001). In the extract-treated thrombocytopenic groups, the number of megakaryocytes was significantly higher than that in the CP group (P < 0.001) (Fig. 4A). Furthermore, based on the bone marrow sections of the rats receiving CP, the hypocellularity and increased adipose cells were compared to those in the extract-treated groups (Fig. 4B).

Splenic tissue changes

By the examining of the tissue sections prepared from the spleens of different groups, a reduction of the white pulp was observed in the CP groups versus the control group, but it was increased in the treated groups (Fig. 5). Furthermore, as the tissue sections of the extract-treated groups were highly magnified, megakaryocytes emerged to have increased as compared to the control group, suggesting hematopoiesis in the spleen and after bone marrow suppression.

Table I: Compaction of CBC in different groups.

Groups/ Cell number	Control	CP	CP+100 mg TPE	CP+200 mg TPE	P
Platelets/µl	963500±214.53	87000±75.67	869000±179.66	925000±209.94	<0.001
WBC/µl	8790±469	910±124	6000±241	6470±246	0.02
RBC/µl	$7.1\times10^6\pm 1.12\times10^6$	$5.7\times10^6\pm 0.56\times10^6$	$6.8\times10^6\pm 1.04\times10^6$	$6.3\times10^6\pm 1.97\times10^6$	0.267

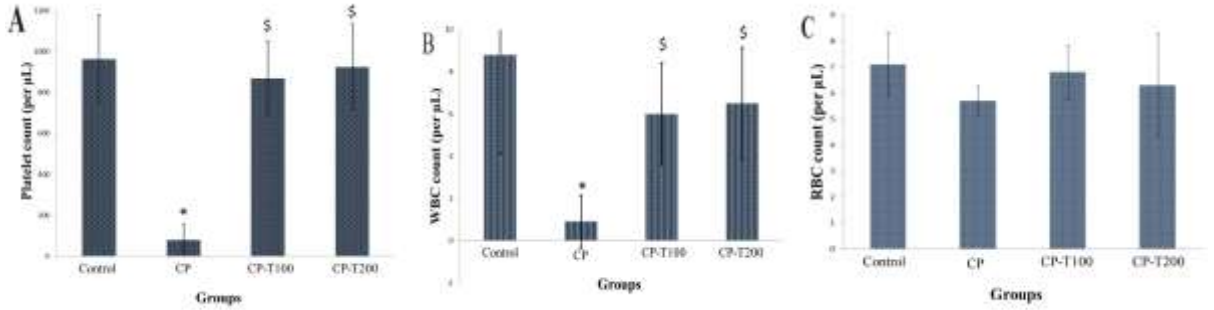

Figure 1. Changes in the blood cells: A) platelet, B) WBC and C) RBC in study groups: control, CP, CP-treated with 100 and 200 mg/kg TPE. Data were presented as mean ±standard deviation). *: Significant decrease over the control group $: Significant increase over the CP group.

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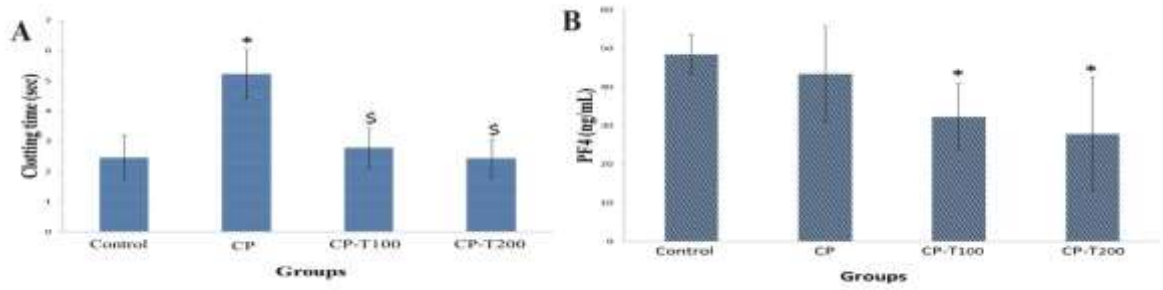

Figure 2. Changes in the clotting time (A) and PF4 (B) in study groups: control, CP, CP-treated with 100 and 200 mg/kg TPE. Data were presented as mean ±standard deviation). *: Significant difference with control group $: Significant difference with CP group.

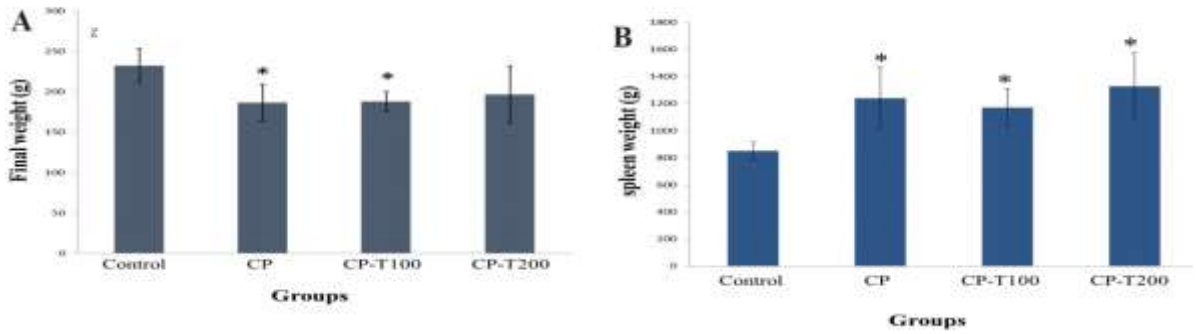

Figure 3. Average final body weight of rats (g) (A) and final spleen weight (B) in study groups: control, CP, CP-treated with 100 and 200 mg/kg TPE. Data were presented as mean ±SD *: Significant difference compared to control group.

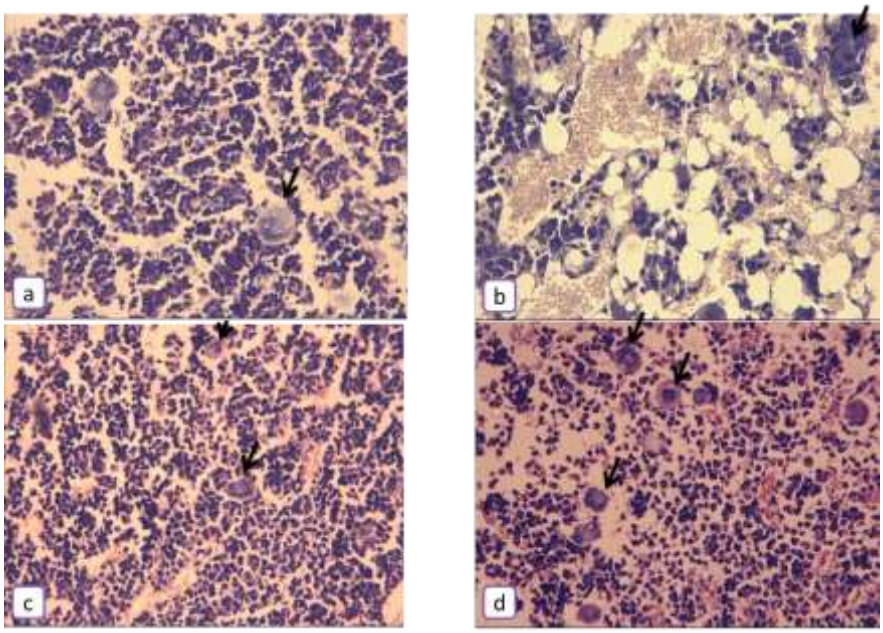

Figure 4. A) Changes of megakaryocyte in study groups: control, CP, CP-treated with 100 and 200 mg/kg TPE. *: Significant difference with control group, $: Significant difference with CP group. Bone marrow sections from different groups: a) control, b) CP, c) CP/100 and d) CP/200 mg/kg of extract (X400), the arrows indicate megakaryocyte.

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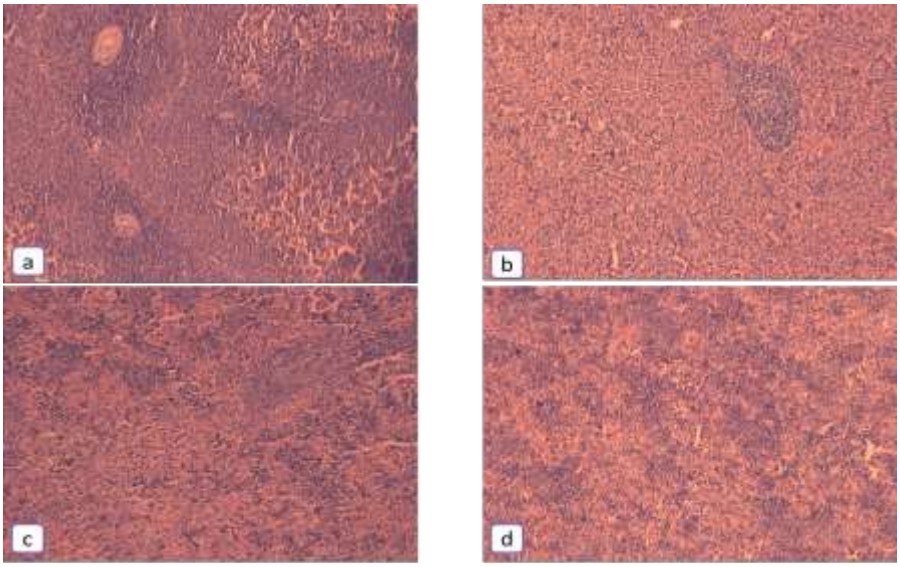

Figure 5. Spleen cross section from different groups: a) control, b) CP, c) CP/100 and d) CP/200 mg/kg extracts. H-E staining (X100)

Discussion

In this experimental animal study, the therapeutic effect of TPE on blood changes was evaluated in a rat model of CP-induced chemotherapy. CP decreased the platelets and WBCs, leading to severe thrombocytopenia and leucopenia through bone marrow suppression and increased clotting time. In contrast, TPE increased the platelet and WBC counts, significantly reducing the clotting time. Red clover has some therapeutic properties, but its effect on platelets and WBCs is poorly understood. Due to the strong antioxidant properties of this herb and the existence of isoflavones and saponins in it, considerable attention has been paid to it.

Tahir et al. (2014) reported that compounds such as alkaloids, flavonoids, and tannins improve megakaryocyte function and platelet production during chemotherapy (24). These compounds are also found in TPE. The present study is in accordance with that study in which an increase was observed in the number of the platelets and bone marrow megakaryocytes of the rats receiving TPE. As a study found, saponins from the stem and leaves of Chinese ginseng could significantly prevent CP-induced DNA damage in bone marrow cells (25); therefore, the improved bone marrow cellularity was attributed to *T. pratense* saponins.

Kolodziejczyk et al. (2013) suggested that two species of clover (scabrum and pallidum) can inhibit platelet activity due to compounds such as phenolic and clovamide (26). Another study found this factor has anticoagulant and coagulation properties (27). In our study, PF4 decreased in the extract-treated groups, as one of the hallmarks of platelet function depletion, but it did not affect the time of clotting and possibly the factors controlling it.

In the present study, the platelet function measured by PF4 analysis was found to have decreased in the CP group, where the extract could not increase it. This indicates a low platelet function in the bloodstream. In a related study, the tomato extract prevented platelet aggregation and adhesion, which can be attributed to decreased PF4 production (28). This study is consistent with our research where PF4 decreased in the TPE-treated groups. Several studies have also investigated the

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relationship between PF4 factor and clotting time, and PF4 has been shown to have different effects on clotting time (27, 29).

CP-induced bone marrow suppression results in varying degrees of thrombocytopenia and leucopenia. This leads to problems such as hospitalization, endangered quality of life, and increased healthcare costs. Thrombocytopenia increases the risk of bleeding, requiring platelet administration and repeated chemotherapy. Of course, the doses of the drugs used decrease, leading to a reduction in the efficacy of the treatments (22). Therefore, reducing the dose of agents and repeating the treatment are important issues during chemotherapy (23).

Apoptosis in megakaryocytes is the main cause of decreased platelet production during chemotherapy (30). In the present study, the examination of the bone marrow tissue showed decreased megakaryocyte cells and increased adipose tissue in the thrombocytopenic group, whereas TPE reversed these changes. Bano et al. (2013) induced thrombocytopenia in rats with oxaliplatin, in which the spleen enlarged due to platelet aggregation (31). In our study, the spleen size increased too. In addition to the spleen enlargement after the suppression of the bone marrow by CP, megakaryocytes were observed in the spleen sections, indicating hematopoietic activity in the spleen. Another study has found that bone marrow suppression by IL-12 in mice results in spleen weight gain as well as erythroid, myeloid and megakaryocyte hematopoietic cells in spleen tissue sections (32).

In the present study, RBCs in the rats injected with CP did not decrease significantly. After treatment with red clover extracts, the number of these cells returned to normal but did not increase significantly. However, a study reported that RBCs and platelets significantly decreased with CP and significantly increased after the consumption of Carica papaya leaf juice (21). Therefore, the reported changes of platelets are consistent with the results of this study, but the changes of RBCs are not, possibly due to the different components of the drug or different CP protocols.

Lata et al. (2014) administered the extract of Phyllanthus fraternus to CP-treated mice to assess hepatotoxicity and blood factors. The results showed decreased WBCs, and RBCs recovered significantly following the extract administration (33). The findings of that study are consistent with our research on WBCs, but the two studies do not differ on RBC changes. This may be due to the dose of the CP used in that study (200 mg/kg for 5 weeks), while we used very low doses of 100, 50 and 50 on three consecutive days.

The dose of CP used to induce thrombocytopenia in rats is controversial. Some studies used three consecutive doses of 100 mg/kg for three days intraperitoneally or subcutaneously (11, 21). In our previous study, doses of 100, 50, and 50 were intraperitoneally injected on three consecutive days. On the sixth day, the platelet count reached less than 100,000/µl. This method of chemotherapy–induced thrombocytopenia is recommended for rats (21).

Reduced body fat and muscle mass is one of the limiting side effects of chemotherapy. Garcia et al. (34) showed that, in mice receiving cisplatin, the expression of the fatty acid synthase gene, a key enzyme in lipogenesis, was suppressed. CP intake reduces the lipid precursors in mice and causes incorrect fat storage in the liver. In the present study, CP-treated rats lost weight and increased adipocytes in the bone marrow; the extract improved the bone marrow cellularity, and higher doses prevented weight loss.

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Considering the increasing spread of cancer, a problematic side effect of the drugs used for chemotherapy is thrombocytopenia. The treatments that have been reported to improve the conditions each have limitations. Because of the complications of cancer treatment during chemotherapy and the disruption of the treatment of cancer patients, an appropriate treatment is needed for thrombocytopenia. Furthermore, bone marrow suppression is a common side effect of chemotherapy, associated with decreased blood cell production and damage to normal cells. The clinical efficacy of chemotherapy, which often limits the severity of the cancer treatment doses, is due to the hematological problems leading to thrombocytopenia, leukemia and anemia (9, 22).

Conclusion

Red clover (TPE) increases the number of platelet and WBCs and improves the side effects of CP-induced thrombocytopenia, leukopenia and body weight loss. It can be used as a therapeutic adjunct to reduce chemotherapy complications.

Ethical Considerations

The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (KUMS.REC.1395.140).

Acknowledgments

All authors studied the authorship agreement of the journal and the manuscript was reviewed and approved by them.

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Author's Contribution

MR.Kh. and H.K. carried out the experiment; data collected and wrote manuscript. M.Kh. designed the study, performed statistical analysis and corrected the manuscript. Each author has carefully reviewed and given their final approval to the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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