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Original Article

Effect of aqueous extract of *Tribulus terrestris* on the activity of major platelets factors and the treatment of immune thrombocytopenic purpura

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Abstract

Introduction

Idiopathic thrombocytopenic purpura (ITP), an autoimmune disease with decreased platelet counts needs corticosteroids, intravenous immunoglobulin (IVIg), and anti-Rho in the first line and splenectomy, rituximab, and thrombopoietin in the second line of therapy. The present study reveals the application of the aqueous extract *Tribulus terrestris* (*T. terrestris*) plant in ITP treatment.

Methods

A standard aqueous extract of *T. terrestris* was given orally to twenty patients who were newly diagnosed with ITP in the form of 500 mg capsules (three times per day for14 days). Blood samples were taken from patients before, during, and after two weeks of treatment to count plasma Platelet Factor 4 (PF4), serotonin factor, and Von Willebrand Factor (VWF).

Results

We observed a significant increase in the platelet count among all patients, while plasma platelets and assay factor 4 decreased and VWF increased during and after treatment. Serotonin was increased on days 1 and 14 and slightly decreased once the treatment was completed.

Conclusion

Our findings suggest that *T. terrestris* extract can be considered an efficient alternative medicine for the treatment of ITP.

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Introduction

Idiopathic thrombocytopenic purpura (ITP) is one of the most common forms of autoimmune disease. The main characteristic of this pathology is increased peripheral destruction of platelet[1, 2]. ITP is ascribed to the early destruction of platelet due to the presence of antiplatelet glycoprotein autoantibodies[3,4]. Two glycoproteins that are mainly attributed to ITP are GpIIb-IIIa and GpIb-Ix-V complex, which are localized on the platelet membrane[5, 6]. ITP abundance among adults is more common in women than men with a 2.5 to 1 abundance ratio[7]. The symptoms are

clinically featured as cutaneous purpura, gum bleeding, menorrhagia, petechiae, and purpura[3]. The first-line therapy is corticosteroids such as prednisone, IVIG, and anti-Rh (D). In the case of unresponsiveness to the first-line therapy, the second line of therapy includes splenectomy, rituximab, and thrombopoietin (TPO) receptor agonist[4,8]. However, these treatments accompany several side effects including immunosuppression and increased risk of bacterial infections (e.g., Pneumococcus and meningococcal bacteria).

T. terrestris belonging to the family Zygophyllacea is an annual plant native to the Mediterranean region. It has long stems, small yellow flowers, and large spined fruits. Pharmacological studies have shown that saponins of this plant are the main active component and it has been used against various diseases, such as sexual dysfunction, high blood pressure, and cardiovascular disease[9-13].

Long-term use of chemically synthesized drugs could be burdensome and costly. As a result, herbal medicine has received increased attention as a safe and effective alternative to chemically synthesized medicine due to potentially fewer side effects. In this clinical study, we investigated the natural extract of aqueous extract as an herbal drug for the treatment of ITP disease. In particular, we studied the effect of aqueous extract of *T. terrestris* on the platelet count of patients with ITP disease and determined the effects of this extract on the secretion/activity of major platelets related factors.

The rest of this article is organized as follows. First, we present the materials that are utilized for the preparation of the drug as well as a brief description of patients who participated in the study. We then discuss the methods that are employed for measuring the PF4, serotonin, and VWF assays. A summary of our results including the effects of *T. terrestris* extract on patients' platelet counts and relevant factors along with the conclusion are discussed at the end.

Methods

Ethical statement and trial registration

The protocol study was approved by the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.REC.1394.13) and written informed consent was received from all participants. This design study is registered in the Iranian Registry of Clinical Trials under IRCT 2015062322884N1 identification number.

Extract preparation

T. terrestris, a naturally grown plant, is harvested in Kermanshah Province, located in the west of Iran. We have authenticated the plant at Agricultural Laboratory, Kermanshah University. To prepare the aqueous extract, one weight of air-dried fine-powdered thistle, seeds, and leaves of *T. terrestris* were mixed with 20 weights of distilled water and stirred at room temperature for 24 hours. The extract was dried under 40°C. The resulting powder was packed in 500 mg capsules. The capsules were packed in bottles and kept in an environmentally controlled room (25±5°C and relative humidity of 65±5%) until usage.

Patients

Twenty patients with ITP (13 women and 7 men) within the 20- to 40-year-old range participated in this study. All patients were recently diagnosed with thrombocytopenia with platelet counts less than 100×103/µl. Major common symptoms of patients were nose and gum bleeding, petechiae,

and purpura. None of the patients went under any kind of treatment including chemically synthesized drugs. ITP patients were recruited from the Hematology and Oncology Clinic, Taleghani Hospital at in Kermanshah, Iran.

Drug doses of 10 mg/kg/day were established based on internal animal studies[14]. The capsules were orally administrated at a frequency of three times per day for 14 consecutive days.

Blood samples

Blood samples were taken before the start of treatment, and on the 1st, 14th, and 28th days after the start of treatment. Platelet counts were estimated using a cell counter (KX-21 Hematology Analyzer, Sysmex Corporation). The samples were collected into tubes (Kang Jian Medical Technology) containing 3.2% (w/v) Sodium Citrate (nine parts blood and one part citrate) and subsequently centrifuged (2000 g, 15 minutes) to remove cellular components and to achieve fresh citrated plasma samples. All plasma samples were aliquoted into 500-µl volumes and stored at -70 ℃ refrigerating conditions until needed.

PF4 determination

The concentration of human PF4 was measured by a DY795 ELISA kit, Thermo Fisher Scientific. Microplate wells were coated overnight with 100 µl capture antibody (mouse anti-human PF4; R&D system) a diluted the working concentration of 2 µg/ml using phosphate-buffered saline (PBS) at room temperature. 100 µl of patient's plasma were diluted 1:240 in PBS containing 1% BSA and were added to each well and incubated for 2 hours at room temperature. After adding the detection antibody (1:180 in PBS/1% BSA), the plate was incubated for 2 hours at room temperature. Streptavidin was conjugated to horseradish-peroxidase that diluted 1:200 in PBS/1%BSA and was added (100 µl) for 20 min at room temperature. After five times washing, 100 µl of Substrate solution was added (TMB; R&D system; DY999). The plate was incubated in a dark area at room temperature for 20 min and finally 75 µl of 0.5 M sulfuric acid was added and the absorbance was measured at 450 nm.

Serotonin determination

ELISA assay for serotonin content was performed using a serotonin ELISA kit (EIA-5061; DRG instruments GmbH, Germany). Acylation reagent and acylation buffer were added to 25 µl of each plasma sample. 25 µl of prepared samples were added to duplicated wells of the microplate. Serotonin antiserum then was added to the wells followed by incubation for 30 min at room temperature. 100 µl of the conjugate was added (Goat anti-rabbit IgG conjugated with peroxidase) and the plate was incubated at room temperature for 15 min. The wells were washed three times and substrate solution was added (TMB). The plate was incubated at room temperature for another 15 min and 50 µl stop solution (0.5 N sulfuric acid) was added, and the absorbance was measured at 450 nm.

VWF assay

The VWF concentration was determined by the VWF ELISA kit (Diagnistica Stago Asniererssur seine, France) similarly as described in section 2.4.2. Briefly, the capture antibody (Rabbit anti-VWF monoclonal antibody) was coated and samples and standards (1:1000 dilutions) were added to the wells. The wells were washed and a detection antibody (Goat anti-VWF antibody)

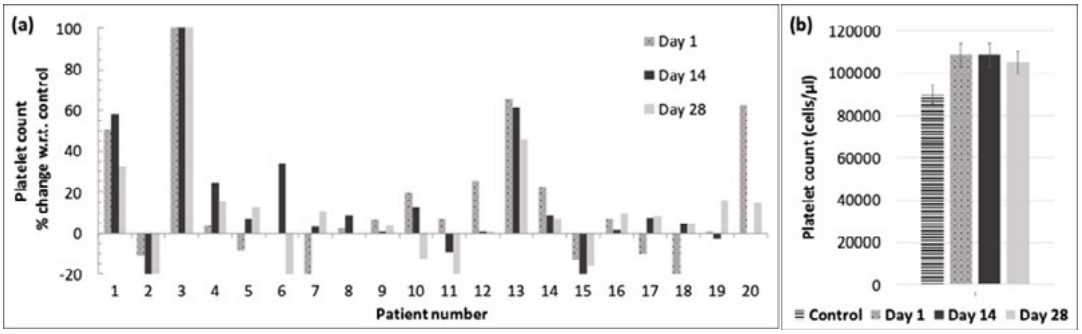

Figure 1 (a). Platelet count percentage change after administration of *T. terrestris* extract for all participants with respect to control, and (b) average change of platelet concentration among all patients. Note that the control is defined as the platelet concentration of each individual patient before beginning the treatment

was added. Streptavidin peroxidase was added and after washing, substrate (TMB) was added (for7 min). Finally, the stop solution (sulfuric acid) was added and the absorbance was measured at 450 nm.

Statistical analysis

Statistical analysis was performed using SPSS version 22. Data were expressed as means ± SD and analyzed by the Friedman test for non-parametric ($P<0.05$)

Results

The aqueous extract of *T. terrestris* increased the count of platelets in patients' blood. figure 1 a, shows the effect of *T. terrestris* aqueous extract on the platelet count during and after treatment.

The increased percentage of platelet counts on the 1st and 14th days of treatment and 14 days after treatment was completed (day 28) were 21%, 21%, and 17%, respectively as shown in figure 1 b. It is important to mention that some of the participants, i.e., patients # 4, 7, 15, 17, and 18, were suffering from Helicobacter pylori infection and the initial treatment on day 1 did not positively affect their platelet count. However, after conducting simultaneous H. pylori therapy with Clarithromycin, the platelet count increased in most of them. Note that ITP patients are usually characterized by their platelet count is less than 100×103/µl[15]. After two weeks of *T. terrestris* treatment, the average platelet counts of the patients surpassed the level, which is another indication of *T. terrestris* effectiveness.

Figure 2, shows the effect of *T. terrestris* aqueous extract on the PF4 level during and after treatment. As can be seen from this figure, the PF4 level significantly decreased in patients

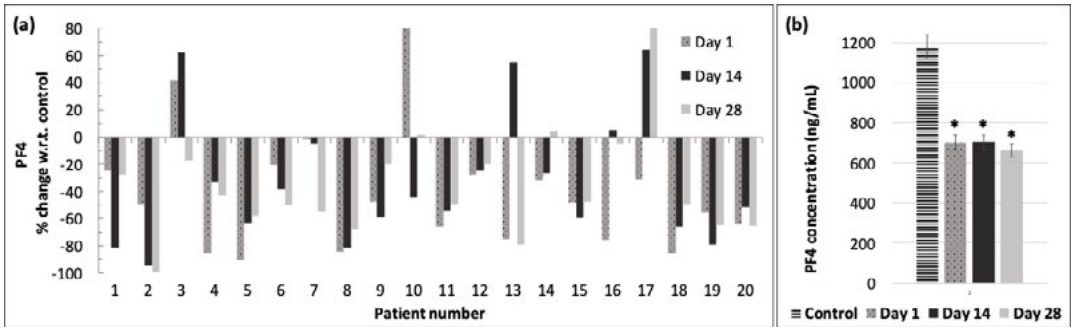

Figure 2 (a). PF4 percentage change after administration of *T. terrestris* extract for all participants with respect to control, and (b) average change of PF4 concentration among all patients. Note that the control is defined as the PF4 concentration of each individual patient before beginning the treatment

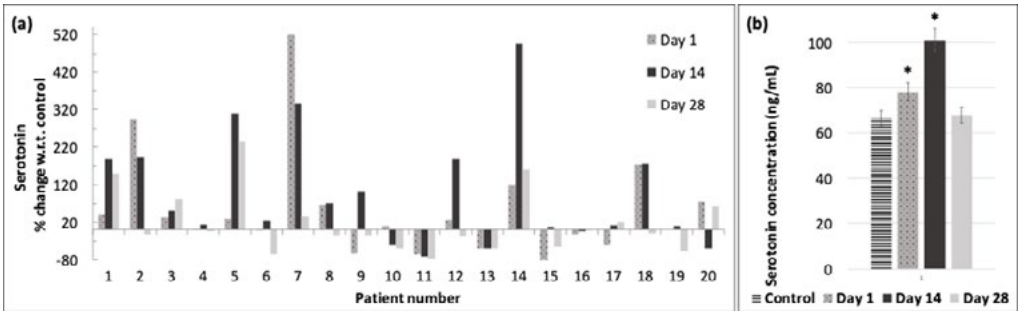

Figure 3 (a). Serotonin percentage change after administration of *T. terrestris* extract for all participants with respect to control, and (b) average change of serotonin concentration among all patients. Note that the control is defined as the serotonin concentration of each individual patient before beginning the treatment

from ~1200 to ~700 pg/ml during the first day of treatment and remained constant afterward.

Figure 3, shows the effect of *T. terrestris* aqueous extract on the plasma serotonin level during and after treatment. As shown in this figure, serotonin levels increased on days 1st and 14th (78.2 and 101.0 ng/ml respectively, P<0.001). However, two weeks after the treatment completion (day 28), the serotonin level decreased and reached its value before treatment.

Figure 4, shows the effect of *T. terrestris* aqueous extract on the plasma VWF level during and after treatment. Plasma VWF concentration significantly increased during treatment (8869.5, 12138.5 ng/ml on days 1 and 14, respectively). The VWF level remained high even after treatment completion (11602.3ng/ml on day 28, P<0.001).

The present study explored the effectiveness of *T. terrestris* extracts in the treatment of ITP patients. Our results suggest that *T. terrestris* extract positively affects the count of platelet and their activity. *T. terrestris* may affect the spleen or bone marrow by decreasing platelet disruptions or increasing its production. Our recent animal study[14] shows that *T. terrestris* extract decreases the weight and size of the spleen (as the main site of platelet destruction) in cyclophosphamide immune-suppressed rats, which reduces platelet depletion and results in an increased platelet count in the blood. For the case of a human study, a significant increase in the platelet count was observed after only two weeks of treatment with *T. terrestris* extract.

The observed decrease in PF4 level suggests that *T. terrestris* extract may reduce platelet activity, resulting in a reduced release of their granule components. Additionally, the observed increase in VWF level can facilitate blood

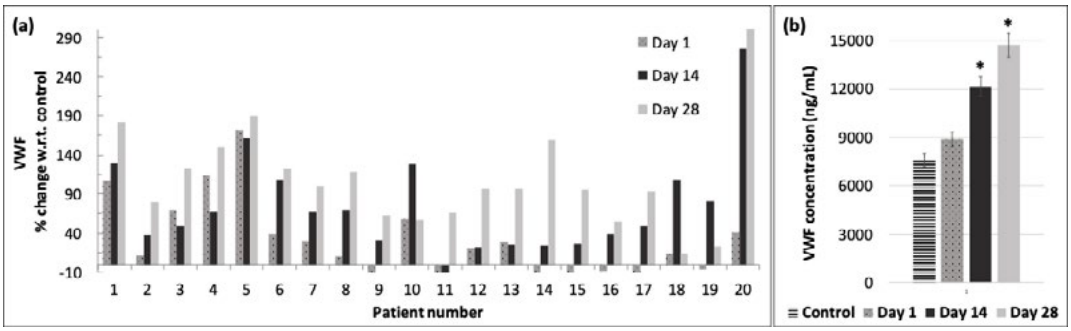

Figure. 4 (a). VWF percentage change after administration of *T. terrestris* extract for all participants with respect to control, and (b) average change of VWF concentration among all patients. Note that the control is defined as the VWF concentration of each individual patient before beginning the treatment.

coagulation and improve bleeding symptoms such as petechiae and purpura. Other herbal medicines such as Xiayuxue Decoction, chicory extract ginsenosides, beta-sitosterol, calycosin, Hippo phaethontids L., and Angelica S extracts have also shown therapeutic effects on arterial thrombosis and cardiovascular diseases, where the extracts pronounced the inhibition of VWF expression in endothelial cells [16, 17]. Platelet receptors can uptake serotonin from blood and store them in granules [18]. An increased level of VWF factor increases its interaction with FcγRIIA on platelets and may lead to the activation of signaling pathway and serotonin release from delta granules [19, 20]. Other studies suggested that herbs such as ginseng increase the serotonin level in blood by inhibiting serotonin uptake [21]. Our study also suggests that *T. terrestris* extract positively affects the serotonin level in the blood by inhibiting serotonin transporters on the platelet surface and platelet decreasing the serotonin adsorption by the platelets, which increases the serotonin level of the blood. Thus, the enhanced serotonin level observed in the current study may be related to FcγRIIA interactions or/and inhibitory effects.

Conclusion

Our study indicates the potential of *T. terrestris* aqueous extract as effective herbal medicine for the treatment of patients with ITP disorder. Major common symptoms of patients including nose and gum bleeding and purpura disappeared after two weeks of treatment. However, further studies are required to be better *T. terrestris* understand *T. terrestris* mechanism of action in ITP treatment. Applications of *T. terrestris* extract for other platelet disorders may be explored in the future.

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Conflicts of interest

The authors declare that there is no conflict of interest.

Ethics approval

All the protocols were approved by the Ethical Committee (Ethics code: IR.KUMS.REC.1394.13).

Consent to participate

'Not applicable' for that section.

Consent for publication

The authors declare that they consent to manuscript publication.

Code availability

'Not applicable' for that section.

Authors contributions

Conceptualization, Ali Mostafaie; Methodology, Ali Mostafaie, Zahra Koolivand, Soodabeh Aghapour, Kamran Mansouri, Mehrdad Payandeh, Fariba Sohrabi; Investigation, Ali Mostafaie, Mehrdad Payandeh; Writing- Original Draft, Zahra Koolivand, Soodabeh Aghapour; Writing- Review & Editing, Ali Mostafaie, Zahra

Koolivand; Funding Acquisition, Ali Mostafaie.

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