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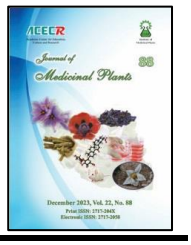

Research Article

Effect of *Terminalia chebula* Retz extract on blood coagulation parameters of mouse

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ABSTRACT

Background: Coagulation disorders involve a wide range of medical issues resulting from a deficiency or absence of certain coagulation factors in plasma. While existing therapies exist, herbal medicine presents an alternative and effective approach for treating bleeding disorders. *Terminalia chebula* Retz has been reported for its efficacy in ethnobotanical practices for bleeding stopping. **Objective:** The in vivo effectiveness of *T. chebula* Retz as an herbal medicine was examined using mice. **Methods:** Thirty-two mice were randomly divided into three treatment groups (n = 8) receiving different doses (2000, 1000, and 500 mg/kg/day), along with a negative control group. Blood samples were collected from the tail and heart on the 16th and 18th day of treatment, respectively, to determine coagulation parameters, including bleeding time (BT), clotting time (CT), prothrombin time (PT), activated partial thromboplastin time (aPTT), and platelet count (PLT). **Results:** The results demonstrated the effectiveness of *T. chebula* hydroalcoholic extract in promoting primary and secondary homeostasis. There was a significant decrease in bleeding time (70 %) and a substantial increase in platelet count (80 %). Moreover, the extract led to a notable reduction in clotting time (80 %) indicating its impact on primary homeostasis through platelet aggregation. Additionally, *T. chebula* extract shortened the coagulation time in both PT (70 %) and aPTT (20 %) tests, reflecting its influence on the extrinsic and intrinsic pathways of secondary hemostatic parameters, respectively. **Conclusion:** In conclusion, the optimal concentration (500 mg/kg/day) of hydroalcoholic extract of *T. chebula* appears to be effective in promoting blood coagulation through primary and secondary homeostasis.

1. Introduction

Up to 50 different substances have been discovered in blood and tissue that play an important role in blood clotting. These substances, called procoagulants and anticoagulants, maintain a delicate balance that determines the blood's ability to clot. Under normal conditions, anticoagulants predominate and prevent excessive clotting. However, when a blood vessel is damaged, clotting factors are

Abbreviations: T. chebula, *Terminalia chebula* Retz; CT, Clotting time; BT, Bleeding time; PLT, Platelet count; PT, Prothrombin time; aPTT, Activated partial thromboplastin time

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activated at the site of injury, leading to the formation of a blood clot [1]. Clotting is a natural process to prevents bleeding following a vascular injury. However, disruptions in this process can lead to multiple disorders [2]. Hemophilia, the most common and serious blood clotting disorder, results from a deficiency in clotting proteins that leads to impaired blood clotting. Hemophilia A and B, which result from deficiencies in coagulation factors VIII and IX, respectively, are the most severe forms of congenital bleeding disorders with X-linked heritability [3]. Although gene therapy appears promising in recent studies, current treatments primarily include liver transplantation or clotting factor replacement therapy. However, these therapies are expensive and have limited availability. Given the long-standing global use of medicinal plants and their historical significance, research into these sources could provide the opportunity to discover new hemostatic drugs [4].

Based on ethnobotanical studies, over a hundred medicinal plants known for their efficacy in stopping bleeding have been documented. These plants include *Madia sativa*, *Thymus vulgaris*, *Polygonum bistorta*, and many others [5]. The therapeutic effects of these medicinal plants on hemostasis can largely be attributed to their secondary metabolites, which stimulate various coagulation factors. These secondary metabolites facilitate the enhancement of factor XII activity, plasma fibrinogen levels, inhibition of fibrinolysis, and platelet aggregation [4]. The most important secondary metabolites with anticoagulant properties include tannins, glycosides, lignin, phenolic compounds, flavonoids and terpenoids [6, 7]. In addition, compounds such as gallic acid, vanillic acid and luteolin contribute to the hemostatic properties of these plants.In addition, saponins, known for their hemostatic properties, also play a role in coagulation [8].

Terminalia chebula Retz, a member of the Combretaceae family, has been identified as a potentially effective plant for promoting blood clotting. This plant has a rich variety of phytochemicals that contribute to its coagulating properties. Phenolic compounds, glycosides, luteolin and terpenoids were isolated from the fruits of *T. chebula*. In addition, *T. chebula* contains a significant amount of tannins, which make up about 32 % of its composition. The hydrolyzed form of tannin of *T. chebula* consists of hydrolyzable tannin components, including gallic acid, which is one of the main endogenous phenolic acids of the plant and has a coagulant effect. Although *T. chebula* has been extensively studied for its effects on wound healing through its tannin compounds [9-11], some studies have also highlighted its potential in stopping bleeding, particularly in cases of bleeding gums and dysentery [12, 13]. Tannins inhibit the formation of prostacyclins as factors that prevent the formation of platelet plugs [7]. Based on previous in vitro experiments confirming the blood clotting properties of *T. chebula* extract [14, 15], aims of this study is to examine the effect of the extract on coagulation *in vivo*, using mice as an experimental model.

2. Materials and methods

2.1. Extraction

T. chebula was obtained from a spice supplier in Sabzevar, Iran, its authenticity was confirmed by an experienced botanist, and the voucher specimen was stored in the herbarium of Hakim Sabzevari University (HSUH) under number HSUH0211. The dried fruits were ground into powder and then mixed with 70% ethanol at the rate of 100 g of powder per 250 ml of ethanol. The mixture was placed on a shaker and shaken at 100 rpm for 72 hours. The extract was then separated by filtration and concentrated on a

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rotary evaporator at 55 °C for 60 minutes.The resulting extract was then incubated in an oven at 50 °C for 48 hours to remove residual ethanol and excess water. The dry extract weighing 75 g was stored at 4 °C and used for further studies.

2.2. Experimental design and animal grouping

Thirty-two male NMRI mice (25-30 g, 6–8 weeks old) were purchased from the Animal Center, Royan Karaj, Iran. Mice were housed in standard laboratory conditions (21 ± 2 °C, 12/12-h light/dark cycle), with unrestricted access to a rodent diet and water. The animals were adapted for 2 weeks before to the experiment. Based on statistical analysis with G-POWER software, instruction of Hakim Sabzevari University's animal ethics committee, three groups (n = 8) were designed for dosage of 500, 1000, and 2000 mg/kg/day of *T. chebula* extract. Negative control group (n = 8) were administered orally with 0.3 mL distilled water. The present research was performed in accordance with the Guidelines in the Care and Use of Animals and was approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.022).

2.3. Coagulation tests

Mice were treated with various concentrations of *T. chebula* extract for 17 days. On day 16, clotting time (CT), bleeding time (BT) and platelet count tests and on day 18, prothrombin time (PT) and activated partial thromboplastin time (aPTT) tests were carried out.

2.4. Bleeding Time (BT)

To assess primary hemostasis, the bleeding time is used, i.e. the time that the superficial wound needs to clot. Bleeding time was measured on day 16 using IVY [16]. Bleeding time was determined by removing 2 mm from the tail tip and carefully ejecting buffered blood every 15 seconds (s) using the side roughness of the filter paper. Upon bleeding stoping, the number of blood spots on the filter paper was counted and the bleeding times were calculated by multiplying the total number of blood spots by 15.The normal range for a BT test is between 2 to 7 minutes.

2.5. Prothrombin time test (PT)

The PT test is a diagnostic tool designed to assess the function of the extrinsic coagulation pathway, with a typical reference range from 10 to 14 seconds. The mice were anesthetized employing a Ketamine-Xylazine (KX) on day 18 and blood samples were taken from the heart of the mice. Blood samples were mixed with 3.2 % sodium citrate (1 mL of citrate: 9 mL of blood) and plasma was separated at 2500 rpm centrifuge for 15 minutes. For the PT assay, 50 µl citrated plasma and 50 µl of warmed thromboplastin solution (Thermo Fisher) were mixed, incubated for 7 seconds at 37 °C and bleeding time (formation of the first white fibrin filaments) was recorded.

2.6. Activated partial thromboplastin time (aPTT)

The APTT, is a diagnostic assay that evaluates the efficiency of the intrinsic blood coagulation pathway. By measuring the coagulation time of plasma following the activation of coagulation factors in the absence of tissue thromboplastin, this test provides valuable insight into potential defects in the intrinsic pathway. The normal aPTT time is 30 to 45 seconds. For the aPPT assay, 50 µl of prewarmed aPTT reagent (Thermo Fisher) was mixed with 50 µl of citrated plasma and incubated for 3 min at 37℃. The

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clotting time was recorded after adding 50 µl of prewarmed CaCl2 solution (1 mM) to the mixture.

2.7. Clotting time (CT)

The CT test is a diagnostic tool for evaluating the effectiveness of the intrinsic pathway in initiating clot formation and the function of the common pathway in the blood clotting process. Lee and White method [17] was used to perform this experiment. On the 16th day, tail tip was punctured with a scalpel and a drop of blood from the supraorbital vein was collected on a glass slide. The clotting time was recorded between blood collection and fibrin formation [18]. The normal time for CT test is 2 to 6 minutes.

2.8. Platelet counts test (PLT)

Platelet counts test which determines the number of platelets in blood is an essential tool in assessing of primary hemostasis. For this manual test, on the 16th day, each tail tip was punctured and a drop of blood was collected and smeared on a glass slide. Dried blood smear was incubated with methanol for 3 minutes and stained with Gimsa dye for 15 min. After washing and drying in room temperature, platelets were counted from 10 scope and their mean was recorded [19].

2.9. Statistical analysis

Analysis of Variance (ANOVA) was employed to assess the means differences of multiple groups, followed by the Tukey's multiple comparison test. Statistical significance was considered for $P < 0.05$.

2.10. GC-MS analysis

To identify individual components, a hexane solution (50:50) of *T. chebula* extract was subjected to analysis on an Agilent GC-MS system (Agilent GC 6890A equipped with an Agilent 5973 mass detector) using ZB-5ms capillary column (30.0 m × 0.25 mm i.d.; 0.25 µm film thickness; from Zebron). The employed conditions was explained before [20]. The detection of compounds was based on a comparison of the measured retention indices and mass spectral patterns with those available in the literature. All the peaks with a match quality of ≥ 90 % were considered and their names were specified.

3. Results

3.1. Bleeding time (BT) test

The mean value of BT in control and treated groups with 500, 1000, and 2000 mg/kg/day, was 115 ± 4, 32 ± 2, 38 ± 1, and 37 ± 2 seconds, respectively (Fig. 1). These results indicate the *T. chebula* hydroalcoholic extract-treated groups dosages was significantly reduced BT up to 70 % than that of negative control ($P < 0.05$).

3.2. Clotting time (CT) test

Although the CT was reduced in all treated groups, there was a significant ($P < 0.05$) decrease in CT of the 500, and 2000 mg/kg/day compared with the control (Fig. 2). The mean value of CT in control and treated groups with 500, 1000, and 2000 mg/kg/day, were 122 ± 3, 26 ± 1, 106 ± 3, and 52 ± 2 seconds, respectively which indicate a 60 and 80 % decreas in CT.

3.3. Prothrombin time (PT) test

PT test results for mice treated with *T. chebula* hydroalcoholic extract showed a decrease in PT at all doses compared to the control group, but the mean time at doses of 500 and 1000 mg/kg/day was significantly different from control group ($P < 0.05$) (Fig. 3). PT time for control mice was 13±0.5 seconds, although it was

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4 ± 0.3, 8 ± 0.4, and 11 ± 0.5 seconds at the dose of 500, 1000, and 2000 mg/kg/day dosage, respectively, showing a reduction of 70 % and 60 % for 500 and 1000 doses.

3.4. Activated partial thromboplastin time (aPPT) test

Although a prolonged aPPT was observed at a dose of 1000 mg/kg/day (Fig. 4), the results indicated that the aPPT in the 500 and 2000 treatment groups was significantly lower than the control group ($P < 0.05$). The mean aPPT time for control mice was 16 ± 0.8 seconds, whereas it was 13 ± 0.5, 19 ± 0.4, and 13 ± 0.7 seconds in 500, 1000, and 2000 mg/kg/day dosage, respectively. Compared to the control group, a 20 % decrease in aPPT was observed at the 500 and 2000 mg/kg doses ($P < 0.05$).

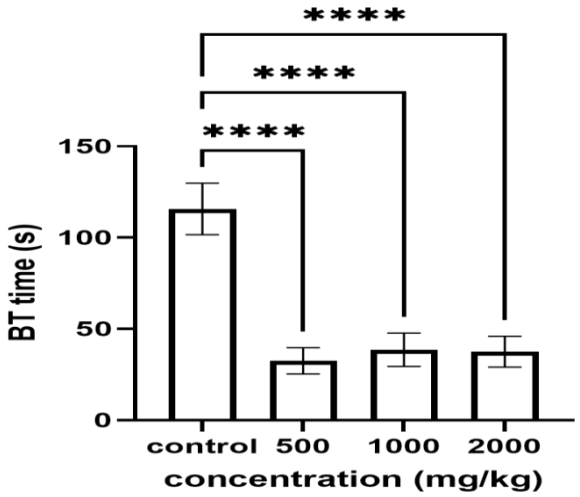

Fig. 1. The effect of various dosage of *T. chebula* hydroalcoholic extract on BT test in mice. The data are the means ± SD of three individual experiments and significance of the data was shown with star

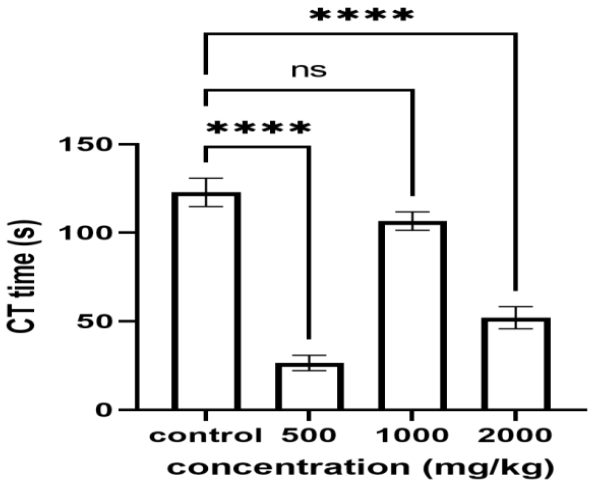

Fig. 2. The effect of various doses of *T. chebula* hydroalcoholic extract on CT test in mice. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

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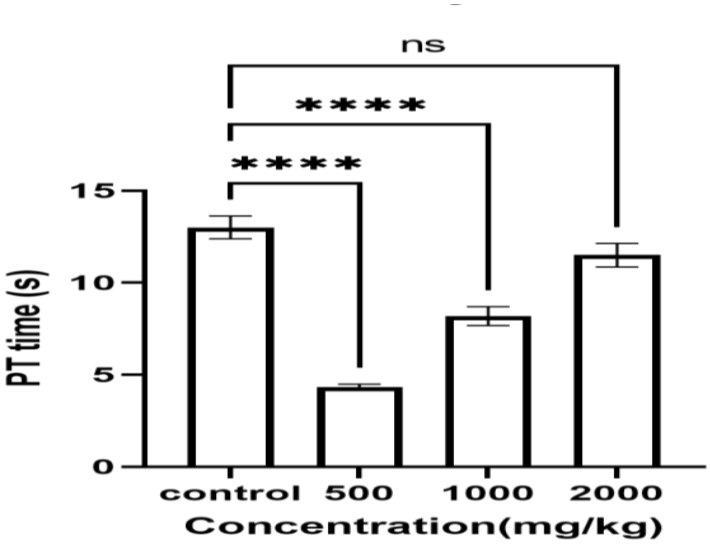

Fig. 3. The effect of various doses of *T. chebula* hydroalcoholic extract on PT test in mice. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

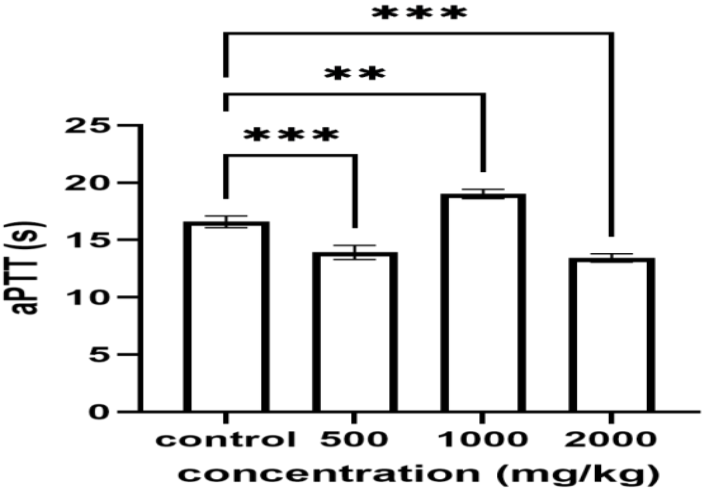

Fig. 4. The effect of various doses of *T. chebula* hydroalcoholic extract on aPPT test in mice. The data are the means ± SD of three individual experiments and significance of the data was shown with star.

3.5. Platelet count test (PLT)

Platelet count analysis revealed an increase in PLT in the *T. chebula* extract treated group, although the mean time in only doses of 500 mg/kg/day is significantly different from the control group ($P < 0.05$) with an increase of 80% (Fig. 5). The mean platelet count in control mice was 6, while the mean at doses of 500, 1000, and 2000 were 10, 8, and 9, respectively.

3.6. Analysis of extract compounds

Qualitative determination of the different biologically active compounds from *T. chebula* extract using GC/MS technique revealed the presence of two main compounds (Table 1).

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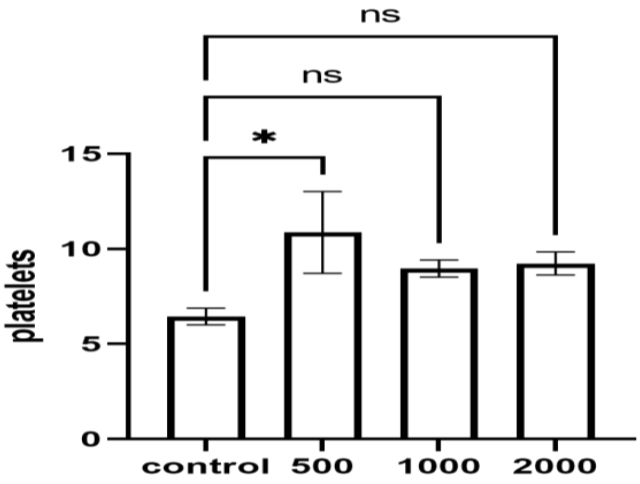

Fig. 5. The effect of various doses of *T. chebula* hydroalcoholic extract on platelet count in mice. The data are the means ± SD of three individual experiments and significance of the data was shown with star

Table 1. Gas Chromatography mass spectroscopy (GC-MS) analysis of the hydroalcoholic extract of *T. chebula*

No	RT (min)	Area %	Name	Quality
1	16.438	89.38	4,4-Dimethyl-2-(3-phenyl-2-thienyl) oxazoline	83
2	44.094	10.62	4-Cyclohexene-1,2-dicarboximide, N-butyl-, cis-	53

4. Discussion

In light of the bioactive compounds present in *T. chebula* and the promising results from previous *in vitro* studies on coagulation parameters [14, 15], an animal study was conducted to investigate the effects of its hydroalcoholic extract in mice. Results indicate that the *T. chebula* extract influences coagulation time effectively, with the optimal dosage observed at 500 mg/kg/day. The hydroalcoholic extract of *T. chebula* significantly reduced bleeding time in the BT test up to 70 %. The BT test which evaluates platelet count and function, is a common diagnostic tool for the detection of primary hemostasis disorders [21]. The decrease in BT demonstrates the extract's impact on enhancing platelet count, thereby influencing primary hemostasis. The findings regarding platelet count validate the BT results, as treatment with the hydroalcoholic extract leads to an increase in platelet numbers. Tannins, phenol and flavonoid compounds [22] in the *T. chebula* extract are possible factors in the coagulation time decrease. It is hypothesized that the compounds found in *T. chebula* can potentially decrease BT by inhibiting vasodilators like nitric oxide. Phenolic, flavonoid, and alkaloid compounds reduce the production of nitric oxide in macrophages and result in vasoconstriction, platelet number increasing and aggregation, and bleeding prevention [11]. Platelet accumulation was also confirmed by CT test. A decrease in clotting time in CT indicates the effect of *T. chebula* compounds on the primary hemostasis of blood coagulation. Tannins [22], phenolic, flavonoid [23] and alkaloid [24] compounds are effective in reducing CT by increasing platelet aggregation.

Our findings show the effect of hydroalcoholic extract of *T. chebula* on secondary hemostasis as it is effective on PT (extrinsic pathway), aPTT (intrinsic pathway) and CT (common pathway). Tannin [22], phenol and flavonoid [23] compounds in the extract are

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possible factors in the decline of coagulation time in these tests. Although the exact mechanism of these copmounds on the extrinsic pathway is not clear, it seems flavonoids and tannins can increase migration and accumulation of platelets to the damaged area and result in bleeding stop [25]. For example, the flavonoid quercetin stimulates the production of bone marrow stem cells and the expression of thrombopoietin mRNA as the main regulator of platelet production [26]. Tannins inhibit the formation of prostaglandins as factors that increase bleeding and cause coagulation [7].

Since the CT test is depending on platelet aggregation, decrease of clotting time in CT indicate effect of flavonoids and tannins. Moreover, tannins that have a phenolic ring in their structures and are among the polyphenolic compounds, when mixed with blood, immediately form a clot due to the coagulation of albumin [25]. Tannins as phenol compounds can be hydrolysed to gallic acid, catechol and ellagic acid [27]. Ellagic acid can activate the coagulation factor XII in the beginning of the intrinsic pathway [28] and result in decrease of coagulation time in aPTT. It appears that tannins, accounting for approximately 32 % of the compounds found in *T. chebula*, are considered effective in promoting blood coagulation.

Our previous results [29] indicated that desmopressin drug as a positive control result in a CT, PT, and aPTT of 13.9, 11, and 30.6 seconds respectively. Compared to desmopressin drug as a positive control, the extract of *T. chebula* increased and decreased CT and PT/aPTT, respectively, which indicates the efficiency of this extract in secondary homeostasis compared to desmopressin.

T. chebula extract is generally considered safe for consumption, and it has been used in traditional medicine for centuries. However, there are some potential side effects that have been reported with chronic consumption. *T. chebula* extract can act as a laxative and may cause diarrhea, abdominal discomfort, and other digestive problems if consumed in high doses or for extended periods. Hypoglycemia, allergic reactions (skin rash, itching, or swelling), liver toxicity and drug interactions are other side effects. Overall, while *T. chebula* extract has many potential health benefits, it is important to consume it in moderation and under the guidance of a healthcare provider to minimize the risk of side effects.

5. Conclusion

It is concluded that in addition to the efficiency of the optimal concentration (500 mg/kg/day) of *T. chebula* hydroalcoholic extract in blood coagulation through primary and secondary hemostasis, this extract can be more effective than desmopressin as a commercial drug in secondary hemostasis.

Author contributions

JV and RA conceived and designed the research. RA performed research and JV and TH analyzed data. JV and RA wrote the paper. TH and EK was an advisor in the animal experiment and plant extraction, respectively. All authors read and approved the manuscript.

Conflicts of interest

The authors declare that there is no conflict of interest.

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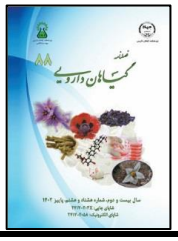

مقاله تحقیقاتی

اثر عصاره گیاه هلیله (*Terminalia chebula* Retz) بر پارمترهای انعقاد خون در موش

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اطلاعات مقاله

گل‌واژگان:

اختلالات انعقادی

طب سنتی

هموستاز

عصاره هیدروالکلی

گیاه هلیله

چکیده

مقدمه: کمبود یا عدم وجود برخی فاکتورهای انعقادی در پلاسما باعث اختلالات انعقادی می‌شود و یک رویکرد جایگزین و موثر برای درمان این اختلالات، طب گیاهی است. در مطالعات اتنوبوتانی، هلیله (*Terminalia chebula* Retz) به عنوان یک گیاه اثربخش برای توقف خونریزی گزارش شده است. **هدف:** اثربخشی گیاه هلیله به عنوان یک داروی گیاهی در موش مورد بررسی قرار گرفت. **روش بررسی:** ۳۲ موش به طور تصادفی به سه گروه تیمار (۸ سر) با دوزهای مختلف (۲۰۰۰، ۱۰۰۰ و ۵۰۰ میلی‌گرم بر کیلوگرم در روز) و یک گروه کنترل منفی (۸ سر) تقسیم شدند. نمونه‌های خونی از دم و قلب به ترتیب در روز ۱۶ و ۱۸ تیمار جمع‌آوری و پارامترهای انعقادی، از جمله زمان خونریزی (BT)، زمان لخته شدن (CT)، زمان پروترومبین (PT)، زمان ترومبوپلاستین جزئی فعال (aPTT) و شمارش پلاکت (PLT) بررسی شد. **نتایج:** کاهش قابل توجه در زمان خونریزی (۷۰ ٪)، افزایش قابل توجه در تعداد پلاکت‌ها (۸۰ ٪) و کاهش زمان لخته شدن (۸۰ ٪)، نشان دهنده تأثیر عصاره هیدروالکلی گیاه هلیله بر هموستاز اولیه از طریق تجمع پلاکتی است. همچنین، کاهش زمان انعقاد در هر دو آزمون PT (۷۰ ٪) و aPTT (۲۰ ٪) به ترتیب تأثیر عصاره گیاه را بر مسیرهای بیرونی و درونی پارامترهای هموستاتیک ثانویه منعکس می‌کند. **نتیجه‌گیری:** غلظت بهینه (۵۰۰ میلی‌گرم بر کیلوگرم در روز) عصاره هیدروالکلی *T. chebula* در ارتقای انعقاد خون از طریق هموستاز اولیه و ثانویه موثر است.

مخففها: T. chebula، گیاه هلیله؛ CT، زمان لخته شدن؛ BT، زمان خونریزی؛ PLT، شمارش پلاکت؛ PT، زمان پروترومبین؛ aPTT، زمان ترومبوپلاستین جزئی فعال

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