UMSHA Press

AJPR
Avicenna Journal of Pharmaceutical Research
Avicenna J Pharm Res, 2023; 3(2):74-81. doi:10.34172/ajpr.1058
http://ajpr.umsha.ac.ir

Original Article

In Vivo Coagulation Effects of *Thymus vulgaris* Leaves Extract in Mice

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Article history:
Received: August 29, 2022
Revised: December 9, 2022
Accepted: December 20, 2022
ePublished: September 1, 2023

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Abstract

Background: In addition to current therapies, herbal medicine is still used as an effective method for the treatment of some diseases such as bleeding disorders. *Thymus vulgaris* has been employed as traditional remedy in the treatment of bleeding disorders, although its clinical effects have not been investigated yet. Accordingly, this study evaluated the effects of the *T. vulgaris* extract on coagulation parameters, including bleeding time (BT), clotting time (CT), prothrombin time (PT), activated partial thromboplastin time (aPTT), and the number of platelets (PLT).

Methods: Forty male mice were randomly divided into 5 groups (n=8). The treated groups were administered with 100, 200, or 300 mg/kg/daily of the hydroalcoholic extract, as well as negative and positive control groups. Blood samples were taken from animals on the 13th and 14th day after treatment, and then coagulation indices were determined finally.

Results: The results demonstrated a significant reduction in the BT and CT tests while a significant increase in PT, aPTT, and platelet numbers. Phenolic and flavonoid compounds in the hydro-alcoholic extract of *T. vulgaris* are the most affecting compounds confirmed by gas chromatography-mass spectrometry and phytochemical experiments.

Conclusion: In general, the results indicated the coagulation effect of the *T. vulgaris* extract through primary homeostasis and a common pathway of secondary hemostasis.

Keywords: *Thymus vulgaris*, Primary hemostasis, Blood coagulation

Please cite this article as follows: Mashkani Z, Vatandoost J, Hajjar T, Mahdavi B. *In vivo* coagulation effects of *Thymus vulgaris* leaves extract in mice. Avicenna J Pharm Res. 2022; 3(2):74-81. doi:10.34172/ajpr.1058

Introduction

Homeostasis including procoagulant, anticoagulant, and fibrinolysis system (1) may damage during coagulation disorders (2). Despite great advances in current therapies for bleeding disorders, including replacement therapy, gene therapy, and liver transplantation, they have low efficacy and significant adverse complications. Medicinal plants have played an important role in the treatment of diseases, and the use of their effective compounds is a new method for the treatment of bleeding disorders (3,4).

Thymus vulgaris of the Lamiaceae family grows in southern Europe, including Spain, Italy, Greece, Portugal, and France (5). Medicinal uses of this plant are related to various compounds (6). The chemical composition of *T. vulgaris* includes polyphenols and flavonoids (5), carvacrol, para-cymene, borneol, and gamma-terpinene (7), naringin and luteolin (8), tannins, as well as resins, terpenes, saponins, thymol, beta caryophyllene, and paracetamol (9). It has been demonstrated that tannins and flavonoids have a positive effect on the blood clotting process (5,10,11). It was also reported that alkaloids extracted from *T. vulgaris* had a positive effect on the reduction of bleeding time (BT) (12). The coagulation effects of the *T. satureioides* extract, a family of *T. vulgaris*, have been proven as well. The effect of the *T. vulgaris* extract on thrombosis and hemostasis was also demonstrated *in vitro* (13). Therefore, the *in vivo* assay of *T. vulgaris* as one of the effective coagulation plants will be useful.

Materials and Methods

Extraction

The fresh leaves of *T. vulgaris* were prepared from farms around Sabzevar, Iran. After identification, these leaves were washed under running water, shade dried, powdered into small pieces, mixed with 70% ethanol, and finally placed on a shaker for 48 hours at 100 rpm. The extract was filtered and concentrated at 55°C by rotary evaporation. The extract was then placed in a drying oven at 40°C to drive off the ethanol and water excess. The dried extract was kept at 4°C and used for further study (14).

Animals and Treatment Groups

Forty male NMRI mice (25-30 g, 6-8 weeks old) were purchased from the Animal Center, Royan Karaj, Iran. They were housed under normal laboratory conditions (21 ± 2°C, 12/12-hour light/dark cycle) with free access to standard rodent chow and water. The animals were adapted for 2 weeks prior to the experiment. Based on the statistical analysis with G*Power software, the instruction of Hakim Sabzevari University's animal ethics committee as well as similar articles (15-17), three groups (n = 8) were designed for dosage of 100, 200, and 300 mg/kg/d. Negative and positive control groups (n = 8) were orally administered with 0.3 cc distilled water and 1200 mg/kg/d tranexamic acid, respectively.

Prothrombin Time

Each group was administered by gavage for 14 days and after this period, the mice were anesthetized using a ketamine-xylazine. Blood samples taken from the heart of the mice were collected into 3.2% sodium citrate (1 mL of citrate: 9 mL of blood) and centrifuged at 2500 rpm for 15 minutes. For the PT assay, 100 µL citrated plasma and 100 µL of warmed thromboplastin solution (Thermo Fisher) were mixed and incubated for 7 seconds at 37°C, and BT (the formation of the first white fibrin filaments) was recorded accordingly. Considering that thromboplastin reagents produced by different companies have different international sensitivity indexes, the international normalized ratio (INR) was used to compare the results of the prothrombin time (PT) test in different laboratories and eliminate the interference between the sensitivities of different reagents. The INR represents the ratio of experimented PT divided by a control PT obtained by using the international reference thromboplastin reagent developed by the World Health Organization (18,19).

Activated Partial Thromboplastin Time

For the activated partial thromboplastin time (aPPT) assay, 100 µL of the prewarmed aPTT reagent (Thermo Fisher) was mixed with 100 µL of citrated plasma and incubated for 3 minutes at 37°C. The clotting time (CT) was recorded after adding 100 µL of a prewarmed $CaCl_2$ solution (1 mM) to the mixture.

Bleeding Time

BT was measured based on the Dejana method with some modifications (20) on the 13th day. BT was assessed by amputating 2 mm of the tail tip, and the issuing blood was carefully blotted every 15 seconds using the rough side of a filter paper. When no further blood appeared on the filter paper, the number of bloodstains on the filter paper was counted, and BT (seconds) was calculated by multiplying the total number of blood stains by 15.

Clotting Time

The CT was evaluated based on the method suggested by Li and White (21). On the 13th day, the tail tip was punctured with a scalpel, and a drop of blood from the supraorbital vein was collected on a glass slide. The CT was recorded between blood collection and fibrin formation (21).

Platelet Count

Platelet count (PC) was performed manually. On the 13th day, each tail tip was punctured, and a drop of blood was collected and smeared on a glass slide. The dried blood smear was incubated with methanol for 3 minutes and stained with Giemsa dye for 15 minutes. After washing and drying at room temperature, platelets were counted from 10 scopes and their mean was recorded as well (22).

Measurement of Total Phenolic and Flavonoid Compounds

The total phenolic content (TPC) of the plant extract was measured using a spectrophotometric method with some modifications (23). The reaction mixture was prepared by mixing 0.5 mL of methanolic solution of the extract (1 mg/mL), 0.5 mL of 10% Folin-Ciocalteu's reagent in water, and 2 mL of $NaHCO_3$ (10%). Blank was also concomitantly prepared, and the samples were incubated in a dark space for 2 hours at room temperature. The absorbance was determined using a spectrophotometer at 765 nm. The samples were prepared in triplicate for each analysis, and the mean value of absorbance was obtained accordingly. The same procedure was repeated for the standard solution of gallic acid, and the calibration line was drawn from it. Based on the measured absorbance, the TPC was calculated using a calibration curve and expressed in terms of gallic acid equivalent (mg of GAE/g of the dry extract) (23).

The TFC of the *T. vulgaris* extract was also determined using the aluminum chloride method (23) with some modifications. The sample contained 1 mL of the methanol solution of the extract at the concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for 30 minutes at room temperature. The absorbance was computed using a spectrophotometer at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained accordingly. The same procedure was repeated for the standard solution of rutin, and the calibration curve was drawn accordingly. Based on the measured absorbance, the TFC was calculated using the rutin calibration curve. The TFC of the *T. vulgaris* extract was expressed in terms of rutin equivalent (mg of RUE/g of the dry extract) (24).

Gas Chromatography-Mass Spectrometry Analysis

To identify individual components, a hexane solution of the *T. vulgaris* extract was subjected to analysis on an Agilent gas chromatography-mass spectrometry (GC-MS) system (Agilent GC 6890A equipped with an Agilent 5973 mass detector) using the ZB-5ms capillary column (30.0 m × 0.25 mm i.d., 0.25 µm film thickness, from Zebron). The employed oven temperature programming

is explained as follows:

Accordingly, its initial temperature was adjusted at 50°C for 5 minutes and then raised to 150°C by a ramp of 5°C/min. The oven was set at this temperature for 10 minutes. Finally, the open temperature was again raised to the final temperature of 260°C using a ramp of 5°C/min and held at this pressure for 20 minutes. The injector temperature was 260 °C. Helium was used as a carrier gas with a flow rate of 1.0 mL/minute. The samples were injected in the splitless mode. The adjusted operational parameters for the mass detector were the ionization voltage of 70 eV and ion source temperature of 200°C over a mass range of 500-500 amu. The peak area was determined using MSD ChemStation from Agilent Technology. A library search was performed for all the peaks using the NIST Mass Spectral Library software. The homologous saturated hydrocarbon standards (C_8-C_{20} and C_{21}-C_{40}) were analyzed using the same column and conditions to calculate the retention indexes (RI) of compounds (25,26). The detection of compounds was based on a comparison of the measured RI 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 accordingly.

Statistical Analysis

The analysis of variance was used to analyze the difference between the means of more than two groups followed by Tukey's multiple, Games-Howell comparison test, and transformation test. The statistical significance level was accepted for $P < 0.05$.

Results

Prothrombin Time and Partial Thromboplastin Time

The increased coagulation time was observed in the PT test of treated groups with the *T. vulgaris* extract in a dose-dependent manner up to 16.3 seconds for a dosage of 300 mg/kg/d. The significance analysis of data demonstrated that this time in the treated groups has significantly increased by 1.2 folds compared to the control ($P < 0.05$, Figure 1a). The same results were obtained for the aPTT test and in a dose-dependent manner, the coagulation time was significantly increased from 16.6 up to 30.3 seconds (1.8 folds) for a dosage of 300 mg/kg/d ($P < 0.05$, Figure 1b).

Bleeding Time and Clotting Time

Although prolonged CT was observed in a dose-dependent manner, the findings indicated that the CT in all groups, the same as the positive control, was less than the negative control (Figure 1c). The effect of the ethanolic extract of *T. vulgaris* (Figure 1d) was accompanied by a non-significant increase in BT.

Platelet Count Test

The results of PC showed that the mean platelet number in negative control mice is 6.5×10^4 cell/µL, while this average in treated mice is 75, 67, and 35×10^4 cell/µL for TV100, TV200, and TV300, respectively which has increased significantly ($P < 0.05$, Figure 1e). Although a reduction was seen in treated groups in a dose-dependent manner, there was a significant increase of up to 11.5 fold compared with the control.

Analysis of Extract Compounds

The qualitative determination of different biologically active compounds from the *T. vulgaris* extract using GC-MS revealed 8 different compounds, in which phytol (30%), 2,6-dimethoxyphenol (23%), and thymol (19%), were the main compounds of *T. vulgaris* (Table 1). Moreover, the total phenolic content and the total flavonoid content of the extract were 1134 mg gallic acid/g and 0.08 mg Rutin/g of the sample in dry weight (mg/g), respectively.

Discussion

In addition to current therapies for bleeding disorders such as replacement therapy, gene therapy, and liver

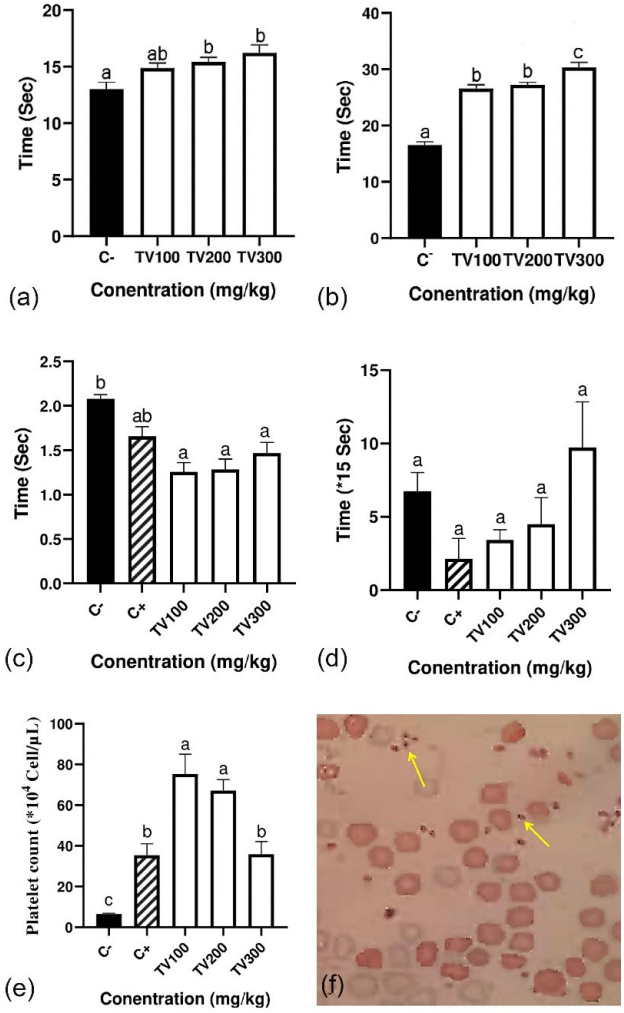
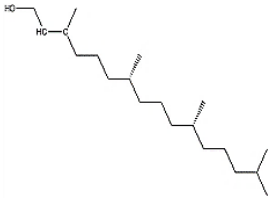
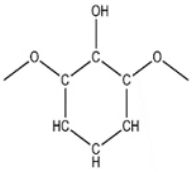
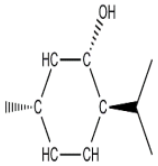
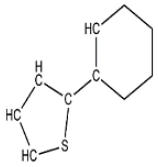
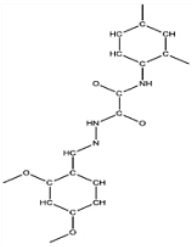
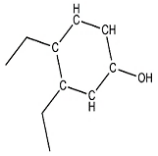
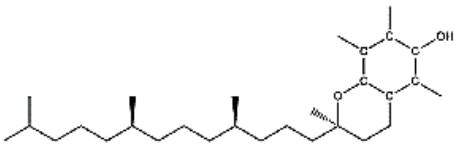
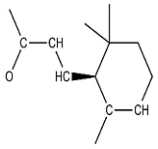


Figure 1. *In Vivo* Coagulant Effect of the *Thymus vulgaris* Hydro-alcoholic Extract on Blood Coagulation Parameters: (a) PT, (b) aPTT, (c) CT, (d) BT, (e) Platelet Number, and (f) Microscopy Image of Platelet. *Note*. PT: Prothrombin time; aPTT: Activated partial thromboplastin time; CT: Clotting time; BT: Bleeding time; SD: Standard deviation. The data are the means ± SD of three individual experiments, and the significance of the data was shown with different letters

Table 1. Some GC-MS Identified Phytochemical Components of the Hydroalcoholic of *Thymus vulgaris* Leaf Extract

Compound Name	Area %	Compound Type	Structure
Phytol	30.18	Diterpene alcohol/diterpenoid	
2,6-Dimethoxyphenol	23.57	Phenol	
Thymol	18.93	Terpenoids	
1-dienylcyclohexane	7.4	Sulfur heterocycle	
2',4'-Dimethyloxanilic acid N'-veratrylidenehydrazide	6.75	Alpha amino acid	
3,4-Diethylphenol	4.84	Phenol	
D,alpha.Tocopherol	4.44	Phenols	
α-Ionone	3.89	Methyl ketones/ noreisoprenoids	

Note. GC-MS, gas chromatography-mass spectrometry.

transplantation, herbal medicines can be among the alternative methods. Although *T. vulgaris* has been used as a traditional remedy in the treatment of bleeding disorders, its clinical effects have not been investigated yet. Considering probabilistic coagulation compounds in *T. vulgaris*, an animal study was performed to evaluate the effect of its hydroalcoholic extract on mice. In line with *in vitro* experiments (27), our findings represented increasing the dose-dependent effect of the hydroalcoholic extract of *T. vulgaris* on the extrinsic coagulation pathway as prolonged coagulation time was observed in the PT test. Phenolic and flavonoid compounds, which were confirmed in quality tests, are possible factors affecting the extrinsic coagulation pathway (28). Thymol as an identified flavonoid compound in *T. vulgaris* (Table 1) can increase blood CT by reducing triglycerides (29) and subsequently reducing FVII activity (30). This mechanism was also demonstrated for the other flavonoid compounds

such as Carvacrols (29,31) and Saponins (32, .(33Moreover, a decrease of triglycerides in the plasma serum has been reported in rats treated with *T. vulgaris* (34) or the *Satureja hortensis* oil extract (35). Another identified compound in the *T. vulgaris* extract, α-Ionone as a vitamin A precursor (36,37) can be another possible factor in the increase of PT since an increase in PT has been observed in chickens with vitamin A diet (38). Rosmarinic acid and hesperidin identified by other researchers (30,39,40) in the *T. vulgaris* extract indicated that they are the possible causes of increased PT (41).

The aPTT test reflecting intrinsic pathway function had a significant increase in the treated group compared to the control group ($P < 0.05$). These results are in line with *in vitro* results, showing that *T. vulgaris* extracts increase aPTT (27). It is supposed that some flavonoids and phenolic acids cause an increase in antithrombin III (ATIII) synthesis. ATIII is an activated form of protein C, leading to an increase in aPTT by the proteolytic cleavage of FVIIIa and FVa 82, 42, and 32. Polysaccharide compounds in *T. vulgaris* are the other possible factors in the increase of aPTT. The negatively charged polysaccharide and polyphenol-polysaccharide compounds can increase aPTT by increasing ATIII activity and inhibiting FVIII, FIX, and FXI activities (42). Polysaccharides can also inhibit the thrombin and internal coagulation pathway by the cofactor heparin II (43). It was shown that polysaccharide compounds extracted from *Undaria pinnatifida* (44), *Codium Fragile* (45), *Porana volubilis* (43), *Camellia sinensis* (46), Rosaceae, and Asteraceae family plants (47) prolong aPTT.

Platelet levels in *T. vulgaris* treatment groups increased significantly compared to the control group ($P < 0.05$). Thrombopoietin (TPO) is the main regulator of platelet production which is synthesized in the liver (48). This hormone binds to its receptor on the surface of platelets and megakaryocytes, increasing the TPO level and stimulating platelet production (48). Quercetin, which was previously identified by other researchers in the *T. vulgaris* extract, is the possible cause of increased platelet number through TPO effecting (48). In fact, quercetin increases TPO mRNA expression in bone marrow stromal cells (49).

It was also shown that polysaccharides in *T. vulgaris* increase platelets by affecting the runt-related transcription factor 1 *(RUNX-1)* and stem cell factor *(SCF)* genes. *RUNX-1* is a transcription factor that induces megakaryocyte maturation, resulting in increased platelet production (50). The *SCF* gene is a stem cell and blood cytokine factor that causes megakaryocytes to mature (51).

The CT test reflects the function of a common and intrinsic pathway and platelet aggregation (52). The coagulation time in the CT test in the *T. vulgaris* treatment group was significantly reduced compared with the control ($P < 0.05$). It is supposed that beta-carotene in *T. vulgaris* increases iron and subsequently the number of red blood cells (RBCs), causing high hematocrit and activating platelet aggregation (53). RBCs are effective in inducing platelet aggregation by releasing a significant portion of their adenosine diphosphate (54). Released adenosine diphosphate from RBCs has a 60% and 28% contribution to the reduction of individual platelets and adhesion of platelets, respectively (54). It has been reported that alkaloids can reduce blood CT by inducing epinephrine (adrenaline) secretion (55), increasing the Factor V (FV) amount (56)

Linoleic acid in the *T. vulgaris* extract is another possible factor in reducing CT. In fact, linoleic acid by the desaturase enzyme produces arachidonic acid (57) that is converted to prostaglandin H2 by the cyclooxygenase enzyme. The conversion of prostaglandin to thromboxane α2 by thromboxane synthetase in platelets causes platelet aggregation and vasoconstriction (58). Thromboxane α2 synthesis can also be induced by tannins in the *T. vulgaris* extract. The inhibition of thromboxane α2 by quercetin and resveratrol reduces platelet aggregation (58). The gallic acid in the *T. vulgaris* extract in interaction with resveratrol and quercetin can inhibit their inhibitory effects on thromboxane α2, increasing platelet aggregation and CT (59). Polyphenols can stabilize low-density lipoprotein (LDL) through interaction with apoprotein B (60). LDL stabilization increases the possibility of OxLDL formation which can activate platelets through surface receptors and increase platelet aggregation (61).

The BT test, which is related to the number of platelets and vasoconstriction, is one of the most common tests for the identification of primary homeostasis disorders (62). This test indicates the formation of plaque hemostasis, which depends on sufficient platelet number and adhesion and reduced blood fluidity (63). The BT was reduced in the treatment group (except for TV300) with *T. vulgaris* compared to the control. It is supposed that the inhibition of vasodilators such as nitric oxide by *T. vulgaris* compounds can reduce BT. *T. vulgaris* compounds such as caffeic acid (64), retinoic acid (65), vitamin A (66), linalool (67), and carvacrol (68,69,70) reduce the production of nitric oxide in macrophages and result in vasoconstriction, platelet aggregation, and bleeding prevention.

Conclusion

According to CT, BT, and platelet tests, treatment with the *T. vulgaris* extract seems to be effective through the primary and common pathway of secondary hemostasis. In contrast, the *T. vulgaris* extract had prolonged effects on the intrinsic (aPPT) and extrinsic (PT) pathways of secondary hemostasis. Although the effect of the *T. vulgaris* extract on hemostasis tests is dose-dependent, TV100 is the best dosage for affecting the primary and common pathway of secondary hemostasis (CT, BT, and platelet number) without a significant impact on the intrinsic (aPPT) and extrinsic (PT) pathway of secondary hemostasis.

Acknowledgements
The authors are grateful for the financial support provided by Hakim Sabzevari University.

Authors' Contribution
Conceptualization: Zahra Mashkani, Jafar Vatandoost.
Data curation: Zahra Mashkani.
Formal Analysis: Zahra Mashkani.
Investigation: Zahra Mashkani.
Methodology: Zahra Mashkani, Jafar Vatandoost, Toktam Hajjar, Behnam Mahdavi.
Project administration: Jafar Vatandoost.
Resources: Zahra Mashkani.
Supervision: Jafar Vatandoost.
Validation: Jafar Vatandoost, Toktam Hajjar, Behnam Mahdavi.
Visualization: Zahra Mashkani.
Writing–original draft: Zahra Mashkani.
Writing–review & editing: Jafar Vatandoost.

Competing Interests
The authors declare that there is no conflict of interests.

Ethical Approval
The present research was performed in accordance with the Guidelines for the Care and Use of Animals and was approved by the Animal Ethics Committee of Hakim Sabzevari University (IR.HSU.REC.1399.002).

Funding
This research received no grant from funding agencies in the public, commercial, or non-profit sectors.

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