

Original Article

The Coagulant Effect of the *Medicago sativa* L. Hydroalcoholic Extract: An in vivo Study on Mice

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Abstract

Background and Aim: Herbal medicines are used in the treatment of many diseases, including blood-related disorders. *Medicago sativa*, which is used in traditional medicine to stop bleeding, contains several secondary compounds, is assumed to be effective in primary and secondary blood coagulation. The aim of this study was to evaluate the effect of *M. sativa* extract on blood coagulation system in vivo.

Materials and Methods: Twenty-four male mice were randomly divided into 3 groups (n = 8) as follows: negative control (receiving 0.3 ml of distilled water), positive control (receiving 0.3 ml of tranexamic acid, 1200 mg/kg/day) and the treatment group (receiving 0.3 ml of the hydroalcoholic extract of *M. sativa*, 300 mg/kg/day). On the 13th day of treatment, collection of blood samples from the tail tip for bleeding time (BT), clotting time (CT), and number of platelets was carried out. On the 14th day, the blood samples were collected from the heart and prothrombin time (PT), activating the partial thromboplastin time (aPTT) as indicators of coagulation rate that were determined. The GC-MS was also used to determine extract compounds.

Results: The results indicated that *M. sativa* extract had a significant prolonged effect on the aPTT and PT. Furthermore, this extract increased platelet count, though it significantly reduced coagulation time in BT and CT. It was concluded that *M. sativa* extract could have beneficial effects on the initial blood homeostasis.

Conclusion: Given the highly beneficial effect on coagulation and then tranexamic acid, this extract can be recommended as a potential homeostatic remedy after further clinical evaluations.

Keywords: *Medicago sativa*., Phytochemical analysis, Intrinsic and extrinsic pathways, Blood coagulation

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Introduction

coagulation factors that leads to the activation of fibrin from fibrinogen, formation of fibrin network, and blood coagulation. This process causes platelets accumulation and a stable hemostatic plug that prevents blood loss. Reduction in any factors involved in extrinsic, intrinsic or common clotting pathways, or

the presence of inhibitors of these factors can increase blood clotting time in a variety of bleeding disorders (1).

Current therapies for bleeding disorders, including replacement therapy with plasma derivatives or recombinant factors, gene therapy, and liver transplantation have low efficacy and significant adverse complications. Viral infections transmission

such as HIV and HCV, production of antibodies against injected factors, high-cost production, allergic reactions, and thrombosis following drug consumption are some of these side effects (2).

The use of herbal medicines can be considered as one of the alternative remedies. Despite great significant achievements in modern medicine, herbal medicines are still used as effective remedies in the treatment of some diseases. Today, more than 80% of the world's population still use herbal medicines to treat their diseases. Although about a quarter of the world's medicines are either obtained directly from plants or use herbal compounds in their production (3), most of them have not been previously studied in clinical trials, and none of them have been evaluated enough to be conclusively recommended against blood coagulation (4). Thus, further clinical trials should be conducted to determine the efficiency of medicinal plants and their components that may have coagulant properties.

Medicago sativa (Linn) has been used as a traditional remedy in the treatment of bleeding disorders. The first forage in the world is *M. sativa*, which has been used for more than 8000 years. Caucasus, Minor Asia, Turkmenistan and Iran are known as the main sources of *M. sativa* (5). *M. sativa* is widely grown in central and southern Iran. In traditional medicine, *M. sativa* is used as a blood supplement and blood coagulant. Moreover, it is used as an antidote for bleeding due to warfarin abuse. *M. sativa* with its phytoestrogenic compounds can probably be an effective plant in the blood coagulation (6). It was shown that phytoestrogen compounds in *M. sativa* increase plasma estrogen and subsequently increase fibrinogen. The hydroalcoholic extract of *M. sativa* increased the number of platelets, white and red blood cells in male rats with cyclophosphamide-induced thrombocytopenia (7). Moreover, in the pharmacology studies of Kurdish regions, *M. sativa* is used to prevent skin hemorrhages and accelerate the blood coagulation process (6). Due to some coagulant compounds in this plant, traditional use in bleeding reduction and previous results in the effect of its extract on increasing plasma fibrinogen, the clinical effect of the hydroalcoholic extract of *M. sativa* on mice was investigated.

Materials and Methods

Extraction

Fresh leaves of *M. sativa* were prepared from farms around Sabzevar, Iran. After identification, the fresh leaves of *M. sativa* were washed under running water, shade dried, powdered into small pieces, and mixed with 70% ethanol to be placed on a shaker for 48 hours at 100 rpm. The extract was filtered and concentrated at 55°C by rotary evaporation. It 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 investigations.

Animals

Twenty-four male NMRI mice (25-30 g, 6–8 weeks old) were purchased from the Animal Center, Royan Karaj, IRAN. The mice were kept under normal laboratory conditions (21 ± 2 °C, 12/12-h light/dark cycle). They had free access to standard rodent chow and water. The animals were adapted for two weeks prior to the experiment. As it was revealed by the statistical analysis performed using G-POWER software based on the instructions issued by Hakim Sabzevari University's Animal Ethics Committee, the mice were divided into 3 groups of 8 animals: the first group received distilled water and served as the negative control group; the second group was administered orally with *M. sativa* leaves extract with the dosage of 300 mg/kg/day. The third group was treated with 1200 mg/kg/day the tranexamic acid and served as the positive control group. The present research was conducted based on the guidelines issued by the Care and Use of Animals and was approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.002).

Prothrombin Time (PT)

Each group was administered by gavage during 14 days. Subsequently, the mice were anesthetized using a Ketamine-Xylazine (KX). 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. Bleeding time (formation of the first white fibrin filaments) was recorded. Since thromboplastin reagents produced by different

companies have different international sensitivity index (ISI), the international normalized ratio (INR) was used to compare the results of the 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 using the international reference thromboplastin reagent developed by WHO.

Activated Partial Thromboplastin Time (APTT)

For the aPPT assay, 100 μ l of prewarmed aPTT reagent (Thermo Fisher) was mixed with 100 μ l of citrated plasma and incubated for 3 min at 37°C. The clotting time was recorded after adding 100 μ l of a prewarmed CaCl₂ solution (1 mM) to the mixture.

Bleeding Time (BT)

Bleeding time was measured based on Dejana method with some modifications (8) on the 13th day. Bleeding time was assessed by amputating 2 mm of the tail tip, and issuing blood was carefully blotted every 15 second using the rough side of a filter paper. When blood was not observed on the filter paper any more, we counted the number of bloodstains on the filter paper, and calculated the bleeding time (Sec) by multiplying the total number of blood stains by 15.

Clotting Time (CT)

Calculation of the clotting time was carried out based on the method developed by Li and White (22). On the 13th day, the tail tip was punctured using a scalpel and a drop of blood from the supraorbital vein that was collected on a glass slide. The clotting time was recorded between blood collection and fibrin formation.

Platelet Test

Platelet count 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. Dried blood smear was incubated with methanol for 3 minutes and stained with Gimsa dye for 15 min. After washing and drying at room temperature, the platelets were counted from 10 scopes and their mean was recorded (9).

Measurement of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The TPC of the plant extract was measured using a spectrophotometric method, although certain alterations were made (10). We prepared the reaction mixture by blending 0.5 ml of the methanolic solution

of the extract (1 mg/ml), 0.5 ml of 10% Folin-Ciocalteu's reagent in water, and 2 ml of NaHCO₃ (10%). Blank was also concomitantly prepared. The samples were incubated in a dark space for 2h 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. The same procedure was repeated for the standard solution of gallic acid and the calibration line was drawn. Based on the measured absorbance, the TPC was calculated using a calibration curve. The TPC was expressed in terms of gallic acid equivalent (mg of GAE/g of dry extract) (10).

The TFC of *M. sativa* extract was also determined using the aluminum chloride method (10) with some modifications. The sample contained 1 ml of methanol solution of the extract in the concentration of 1 mg/ml and 1 ml of 2% AlCl₃ solution dissolved in methanol. The samples were incubated for 30 min at room temperature. The absorbance was determined using spectrophotometer at 415 nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration curve was drawn. Based on the measured absorbance, the TFC was calculated using rutin calibration curve. The TFC of *M. sativa* extract was expressed in terms of rutin equivalent (mg of RuE/g of dry extract) (11).

GC-MS Analysis

To determine individual constituents, a hexane solution of *M. sativa* 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 \times 0.25 mm i.d.; 0.25 μ m film thickness; from Zebron). The temperature of the employed oven was as follows. Accordingly, its initial temperature was adjusted at 50°C for 5 minutes, and was subsequently raised to 150°C by a ramp of 5°C/minute. The oven was set at this temperature for 10 minutes. Eventually, the open temperature was again raised to the final temperature of 260°C using a ramp of 5°C/minute. It was kept 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 at splitless mode. The either adjusted operational parameters for the mass detector

there as follows. The ionization voltage: 70 eV, ion source temperature: 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 conducted for all the peaks using the NIST Mass Spectral Library software. The homologous saturated hydrocarbon standards (C₈ to C₂₀ and C₂₁ to C₄₀) were investigated using the same column and conditions to calculate the retention indices (RI) of the compounds (12). The detection of compounds was based on a comparison of the measured retention indices and mass spectral patterns with those available in the literature. The entire peaks with a match quality of $\geq 90\%$ were taken into account and their names were specified.

Statistical Analysis

Analysis of Variance (ANOVA) was used to analyze the difference between the means of more than two groups followed by the Tukey multiple, Games Howel comparison test and transformation test. Statistical significance was accepted for P values < 0.05 .

Results and Discussion

PT and APTT Test

Prolonged PT was observed in a dose-dependent manner from 13 to 15.4 sec (Fig 1a). Moreover, the treated groups showed an INR numerical value more than of 1 in comparison with the control. Moreover, remarkable ($P < 0.05$) prolongation in aPTT test with the *M. sativa* extract compared with the control was observed. The mean time in the control mice was 16.6, while it was 27.6 sec in *M. sativa* extract group. Significance analysis of the data showed that this time in the treated group with *M. sativa* extract had significantly increased compared to the control ($P < 0.05$) (Figure 1a).

CT test

The results indicated that the mean value of CT in positive and negative control groups and the treated group was 53.5, 108.7 and 15 sec, respectively. The effect of the ethanolic extract of *M. sativa* as presented in Fig 1b, that was accompanied by a significant decrease in CT ($P < 0.05$).

BT test

There was a significant ($P < 0.05$) decrease in BT of the treated group with *M. sativa* extract compared with the

controls. The mean value of BT in negative and positive control groups and treated group was 115.7, 32.14 and 10.71 sec, respectively (Figure 1c).

Platelet count test

The results of platelet count showed that the mean platelet count in the positive and negative control mice was 35.4 and 6.5×10^4 cell / μ l, respectively, while this average in the treated mice with *M. sativa* extract was 74.4×10^4 cell / μ l which had increased significantly ($P < 0.05$) (Fig 1d).

Analysis of Extract Compounds

Qualitative determination of the different biologically active compounds from *M. sativa* extract using GC/MS technique revealed the presences of 9 different compounds, in which Mome inositol, phytol and scandanone with 49, 22 and 15%, respectively were the main ingredients of *M. sativa* (Table 1). TPC of the extract was 12.6 mg GAE/g of the sample in dry weight (mg/g) and TFC of the extract was also 1.025 mg RuE/g of the sample in dry weight (mg/g).

One of the important uses of medicinal plants is their use in the treatment of coagulation disorders as one of the specific types of diseases. One of these herbs is *M. sativa*, which has been used as a coagulant in traditional medicine, which is probably due to the presence of phytoestrogenic compounds (6). To investigate the *in vivo* effects of *M. sativa* extract on blood coagulation, the effect of its hydroalcoholic extract in mice was studied.

A significant increase in coagulation time was observed in the PT test in the *M. sativa* treated group compared to the control that can be due to a decrease in any of the coagulation factors or increasing of its inhibitors in the external pathway. The tissue factor (TF), as one of the external pathway initiators involved in the formation of active FVII from FVII, is indirectly affected by the consumption of *M. sativa*. Some studies showed that *M. sativa* consumption could significantly increase HDL cholesterol and lead to increased PT time (13). In fact, exchange of apolipoprotein A-I between HDLs and TF containing vesicles might limit the access of TF to calcium as a cofactor or cause a structural change in TF that lead to the inhibition of interaction with FVII, and prevention of the external pathway (14). Moreover, HDLs can reduce the concentration of coagulation factors, possibly by increasing proteins C and S as inhibitors of FVII (15).

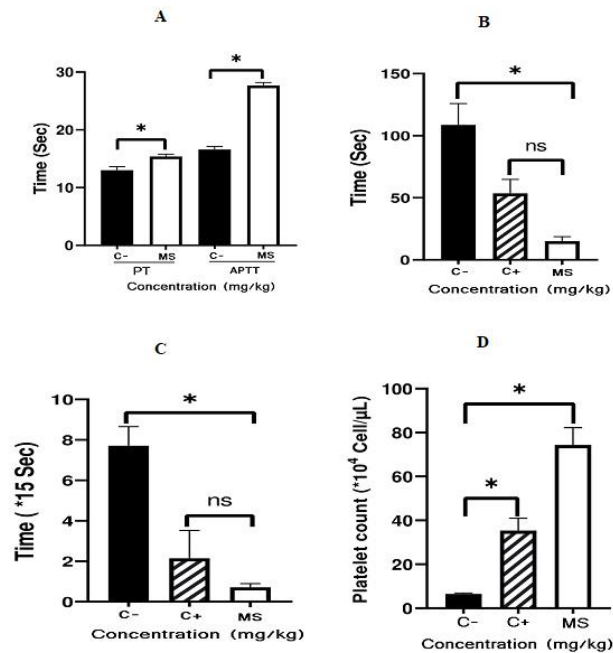


Figure 1. Coagulation assays. (A) PT and APTT clotting time significantly increased in the *M. sativa* extract treated group (\square) in comparison with the control (\blacksquare). Clotting time (B) and bleeding time (C) significantly decreased in the *M. sativa* extract treated group in comparison with both negative and positive controls. (D) *M. sativa* extract increased platelet number in the treated mice.

Quercetin in *M. sativa* can be another possible factor in increasing external coagulation time. The antithrombin (ATIII) amount is directly associated with the concentration of quercetin (16) and an increase in this enzyme can inhibit TF and FVII in the external pathway of blood coagulation (16). It was also shown that vitamin E or C causes an increase in quercetin survival by preventing its oxidative degradation as well as its absorption by the body (17). Thus, synergistic effects of quercetin with vitamin E and C increase its inhibitory action on TF, which increases the coagulation time in the external pathway and PT test (18). Pentyl acetate in *M. sativa* extract is another possible cause of increased PT because it can inhibit TNF- α by affecting macrophages (the main site of TNF- α), preventing the development of blood vessels, and subsequently increasing PT (19). The aPTT test that reflects intrinsic pathway function significantly increased in the treated group compared to the control group ($P < 0.05$). These results are in line with in vitro results that showed that *M. sativa* extracts increase aPTT (20). It is supposed that the synergistic effects of flavonoids and phytoestrogens with ascorbic acids are among the possible reasons for the inhibitory

effects of clotting time in *M. sativa*. Luteolin and compesterols are phytoestrogen, and Rutine and quercetin are flavonoid compounds in *M. sativa*. Phytoestrogens such as Luteolin inhibit the 3 α reductase enzyme leading to the inhibition of the intracellular conversion of testosterone to dihydrotestosterone (DHT) (21). Decreased dihydrotestosterone in the blood is one of the possible causes of increased aPTT (20). It was shown that Luteolin as one of the phytoestrogen compounds in *M. sativa* can increase coagulation time in aPTT in *in vitro* and *in vivo* studies (22). Compesterols increase aPTT via the reduction of LDL cholesterol levels (23). Blann *et al.* reported that oxidized LDL (Ox LDL) promotes the release of von Willebrand factor from human endothelial cells that with binding to FVIII increases the half-life of FVIII (24). With the consumption of *M. sativa*, LDL cholesterol and subsequently von Willebrand factor levels decrease (13) resulting in the reduction of the survival of the FVIII and increasing of aPTT (25). It was also shown that the inhibition of FVIII and FIX activity by Rutine (26) and FIX, FXI and FXII by quercetin increase the aPTT (16). Furthermore, some flavonoids and phenolic acids

Table 1: Some GC – MS identified the phytochemical components of the hydroalcoholic extract of *M. sativa* leaves.

NO	Name	Area %	Type of compound
1	Mome Inositol /3-O-Methyl-d-glucose	49.14	Vitamin B
2	Phytol	22.37	Alcoholic di terpenoid
3	Scandenone	15.24	Flavonoid
4	4.α.,14.α.,24-trimethyl-11.β.-hydroxycholestan-3.β.-yl acetate	5.38	Steroids
5	3,5-diiodoanthranilic acid	2.63	D-carboxylic acid
6	2-Myristinoyl-glycinamide	2.06	Derivative of amide
7	4-Benzylamino-1,3-diphenyl-5,6,7,8-tetrahydro-quinolin-2(1H)-one	1.64	Quinolone
8	Methyl 9,12,15-octadecatrienoate	0.78	Fatty acid
9	Pentyl acetate	0.77	Ester

increase ATIII synthesis. ATIII is an activated form of protein C that increase aPTT by proteolytic cleavage of FVIIIa and FVa. Protein S also accompanies protein C in inactivating factors. Coumarin compounds in *M. sativa* significantly increase aPTT by inhibiting vitamin K-dependent factors such as FII, FIX, and FX (27). Scopolin as a methoxy coumarin in *M. sativa* can also increasing the time of blood coagulation in the internal pathway (28).

Polysaccharide compounds in *M. sativa* are another possible factor 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 activity (29). Polysaccharides can also inhibit the thrombin and internal coagulation pathway by the cofactor heparin II (HCII) (30). It was shown that extracted polysaccharide compounds from *Undaria pinnatifida* (31), *Codium Fragile* (32), *Porana volubilis* (30), *Camellia sinensis* (33), Rosaceae and Asteracea family plants (34) prolong aPTT.

Platelet levels in the *M. sativa* treatment group significantly increased compared to the negative and positive control groups ($P < 0.05$). Thrombopoietin is the main regulator of platelet production which is synthesized in the liver (35). This hormone binds to its receptor on the surface of platelets and megakaryocytes, increasing the level of TPO. The resulting TPO stimulates platelet production and increases platelet production (35). Quercetin in *M. sativa* extract is one of the possible causes of the

increased platelet amount through effecting the thrombopoietin (35). In fact, quercetin increases the thrombopoietin mRNA expression in bone marrow stromal cells (36).

It was also shown that polysaccharides in *M. sativa* increase platelets by affecting the RUNX-1 and SCF genes. RUNX-1 is a transcription factor that induces megakaryocyte maturation resulting in increased platelet production. The SCF gene is a stem cell and blood cytokine factor that causes megakaryocytes to mature (37). Arachidonic acids in *M. sativa* extract might stimulate the synthesis of megakaryocytes and platelet production through Notch / AKT signaling pathways (38).

The CT test reflects the function of common and intrinsic pathways and platelet aggregation. The coagulation time in the CT test in the *M. sativa* treatment group was significantly reduced compared with the negative and positive control groups ($P < 0.05$). It is supposed that beta-carotene in *M. sativa* increases iron and the number of red blood cells. Subsequently, this leads to high hematocrit and activation of platelet aggregation (39). Red blood cells are effective in inducing platelet aggregation by releasing a significant portion of their ADP (40). Released ADP from red blood cells contributes about 60% to the reduction of individual platelets and 28% to platelet adhesion (40). It has been reported that alkaloids can reduce blood clotting time by inducing epinephrine (adrenaline) secretion (41) resulting in increased FV amount (42). Linoleic acid in *M. sativa* extract is another possible

factor in reducing CT. In fact, linoleic acid by desaturase enzyme produces arachidonic acid that is converted to prostaglandin H₂ by the cyclooxygenase enzyme. Conversion of prostaglandin to thromboxane α_2 by thromboxane synthetase in platelets causes platelet aggregation and vasoconstriction (43). Thromboxane α_2 synthesis can also be induced by tannins in *M. sativa* extract. Inhibition of thromboxane α_2 by quercetin and resveratrol reduces platelet aggregation and CT increase (44). Gallic acid in *M. sativa* extract in interaction with resveratrol and quercetin can inhibit their inhibitory effects on thromboxane α_2 , and cause platelet aggregation and CT increase (44). 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. The bleeding time was reduced in the treatment group with *M. sativa* compared with the negative and positive control groups. It is supposed that the B vitamins in *M. sativa* extract can reduce BT by increasing serotonin (45). Serotonin (5-hydroxytryptamine) causes vasoconstriction by binding to its receptors in blood vessels and inhibiting vasodilators such as nitric oxide or calcitonin dependent peptides.

Conclusion

The present experiment showed that *M. sativa* hydroalcoholic extract is a broad-spectrum medicinal product that has different effects on primary and secondary homeostasis. According to our results, treatment with *M. sativa* extract seemed to be effective in the primary homeostasis process, although it had no positive effects on the intrinsic (aPPT) and extrinsic (PT) pathways of secondary hemostasis. Moreover, since this extract had superior effects on tranexamic acid as a positive control, it can be concluded that the compounds in this extract (by examining more clinical trials and confirming the coagulation effects) probably can be used as an alternative to tranexamic acid. The separate extraction of secondary metabolites affecting each coagulation pathway from *M. sativa* extract can be helpful in targeting different pathways of coagulation. However, further studies are needed to discover the exact mechanism of action of the active component.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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