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Short Communication Article

Blood coagulation effect of combined extract of *Thymus vulgaris* L. and *Medicago sativa* L.

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ABSTRACT

Thymus vulgaris and *Mediacgo sativa*, are used as a traditional remedy in the treatment of bleeding disorders. Considering their probabilistic coagulation compounds, in an animal study, forty male mice were randomly divided into 5 groups (n = 8) as well as negative and positive control. Coagulation indices include bleeding time (BT), clotting time (CT), and the number of platelets (PLT) were examined on the 13th day of treatment. A significant reduction in the BT and CT tests, as well as a significant increase in PLT in the treated groups was observed. It is concluded that although the *T. vulgaris* and *M. sativa* extracts have a coagulation effect through primary homeostasis and a common pathway of secondary hemostasis, combined extracts are more effective than individual extracts. Moreover, phenolic and flavonoid compounds are the most affecting compounds that affect platelet number and aggregation.

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1. Introduction

Blood coagulation is a process to prevent blood loss during bleeding (Elyasi et al., 2017). In a healthy person, when the blood vessels are damaged, the blood quickly turns into a gel form and a clot forms to prevent bleeding (Norris, 2003). During bleeding, with the activation of factor XII or VII, the blood coagulation system starts, and subsequently thrombin protein is activated and fibrin monomers are formed (Elyasi et al., 2017). So, vascular wall damage and deficiency of coagulation factors and platelets cause various types of bleeding with different severities (Palta et al., 2014). A variety of treatment methods such as gene therapy and replacement with plasma or recombinant factors are used to treat bleeding disorders, which have side effects such as inhibitory antibody production, short half-life, high production cost, and the transmission of viruses such as HIV (Lethagen, 2003; Franchini, 2013; Valentino, 2014; Windyga et al., 2014; Aghili and Zarkesh-Esfahani, 2016; Monroe et al., 2016; Enayati et al., 2017; Akbarzadeh et al., 2019; Fazeli-nasab et al., 2019; Vatandoost et al., 2022b). Herbal medicines are used by different populations (Abdallah et al., 2022) all over the world to treat various disorders. Despite the availability of modern drugs in the treatment of diseases; people's desire for herbal medicines is increasing (Emeka, 2021). These treatment methods are often considered effective and safe medicine due to their natural origin and natural compounds. Natural compounds such as tannins, saponins, glycosides and other phenols are effective in bleeding control (Rehman et al., 2019). *Thyme vulgaris* contains a variety of these secondary metabolites such as polyphenol groups, flavonoids and alkaloids, which affect bleeding in the blood coagulation process (Triratana et al., 1991; Mirza and Baher, 2003; Hosseini et al., 2010; Pawlaczyk et al., 2010; Raaof et al., 2013b; Hosseinzadeh et al., 2015; Klotoe et al., 2017; Rehman

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et al., 2019). It has been demonstrated that tannins and flavonoids have a positive effect on the blood clotting process (Chen et al., 2005; Fauzi et al., 2018). *Medicago sativa* also contains compounds that are effective in blood clotting, including phytoestrogens, Vitamin K, and Vitamin C (Klotoe et al., 2017; Vatandoost et al., 2022a). It was shown that phytoestrogen compounds in *M. sativa* increase plasma estrogen and subsequently increase fibrinogen. Moreover, in the pharmacology studies, *M. sativa* is used to prevent skin hemorrhages and accelerate the blood coagulation process (Karimi et al., 2013; Mancini et al., 2015; Bilen et al., 2016; Zagórska-Dziok et al., 2020). Although the effect of the *M. sativa* and *T. vulgaris* extracts on thrombosis and hemostasis was demonstrated *in vitro* (Klotoe et al., 2017; Vatandoost et al., 2022a), the *in vivo* assay of individual as well as combined extracts will be useful.

2. Experimental

2.1. Sample preparation and extraction

Fresh leaves of *M. sativa* (Fig. 1a) and *T. vulgaris* (Fig 1b) were prepared from farms around Sabzevar, Iran, authenticated by an expert botanist and the voucher specimen was kept in the herbarium of Hakim Sabzevari University (HSUH) with HSUH0209 and HSUH0210 number, respectivly. After identification, the fresh leaves were washed under running water, shade dried, powdered into small pieces, mixed with 70% ethanol (180 g with 470 mL), and placed on a shaker for 48 hours at 1000 rpm. The extracts were filtered and concentrated at 55 °C by rotary evaporation. Extracts were then placed in a drying oven at 40 °C to drive off the ethanol and water excess. The dried extracts were kept at 4 °C and used for further study.

2.2. Experimental design and animal grouping

Forty male NMRI mice (25-30 g, 6-8 weeks old) were purchased from the Animal Center, Royan Karaj, IRAN. The mice were housed under normal laboratory conditions (21 ± 2 °C, 12/12-h light/dark cycle) with free access to standard rodent water and chow. The animals were adapted for 2 weeks before the experiment. Based on statistical analysis with G-POWER software, instruction of Hakim Sabzevari University's animal ethics committee and previously identified effective dose, three groups (n = 8) were designed for dosages of 100, 300, and 100+300 mg/kg/day of *M. sativa*, *T. vulgaris,* and combined extract respectively. Negative and positive control groups (n = 8) were orally administered with 0.3 mL distilled water and 1200 mg/kg/day tranexamic acid, respectively. The present research was performed in accordance with the Guidelines in the Care and Use of Animals and was approved by Hakim Sabzevari University's Animal Ethics Committee (IR. HSU.REC.1399.02).

2.3. Platelet counts test

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 (Brahimi et al., 2009).

2.4. Bleeding time (BT)

Bleeding time (BT) was measured based on the Dejana method with some modifications (Vatandoost et al., 2022c) 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.

2.5. Clotting time (CT)

Calculation of the clotting time was carried out based on the method developed by Li and White (Huang et al., 2010; Vatandoost et al., 2022c). On the 13th day, the tail tip was punctured using 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.

2.6. Statistical analysis

GraphPad Prism Software (version 9) was used to measure the analysis data. The significance between the two groups was evaluated using an analysis of variance (One-Way ANOVA) followed by Tukey multiple, Games Hawel comparison test, and transformation test. All results are presented as the means ± SEM and are statistically significant at a p value < 0.05.

3. Results and Discussion

3.1. Platelet count test

The results of platelet number (PLT) showed that the mean platelet number in negative and positive control mice are 6.4×10^4 and 35×10^4 cell/µL, while this average in treated mice is 74, 75 and 90 cell/µL for MS300, TV100, and combined extract, respectively. although there isn't any significant change between treated groups, there was a significant increase of 11 and 15 fold for individual and combined groups compared with the control, respectively. Furthermore, up to 2.5 fold incresed PC was observed in treated groups than the positive control (Fig. 2a).

Both individual and combined extracts had a significant increase effect on platelets number. These extracts' flavonoid, phenolic and alkaloid compounds have been the possible cause of increasing platelets. It is supposed that quercetin (El-Newary et al., 2017) is one of the possible reasons for the increase of platelets, through affecting the thrombopoietin (TPO). This hormone, as the main regulator of platelet production, binds to its receptor on the surface of platelets and

Fig. 1. The photograph of **A**) *Thymus vulgaris* and **B**) *Mediacgo sativa*.

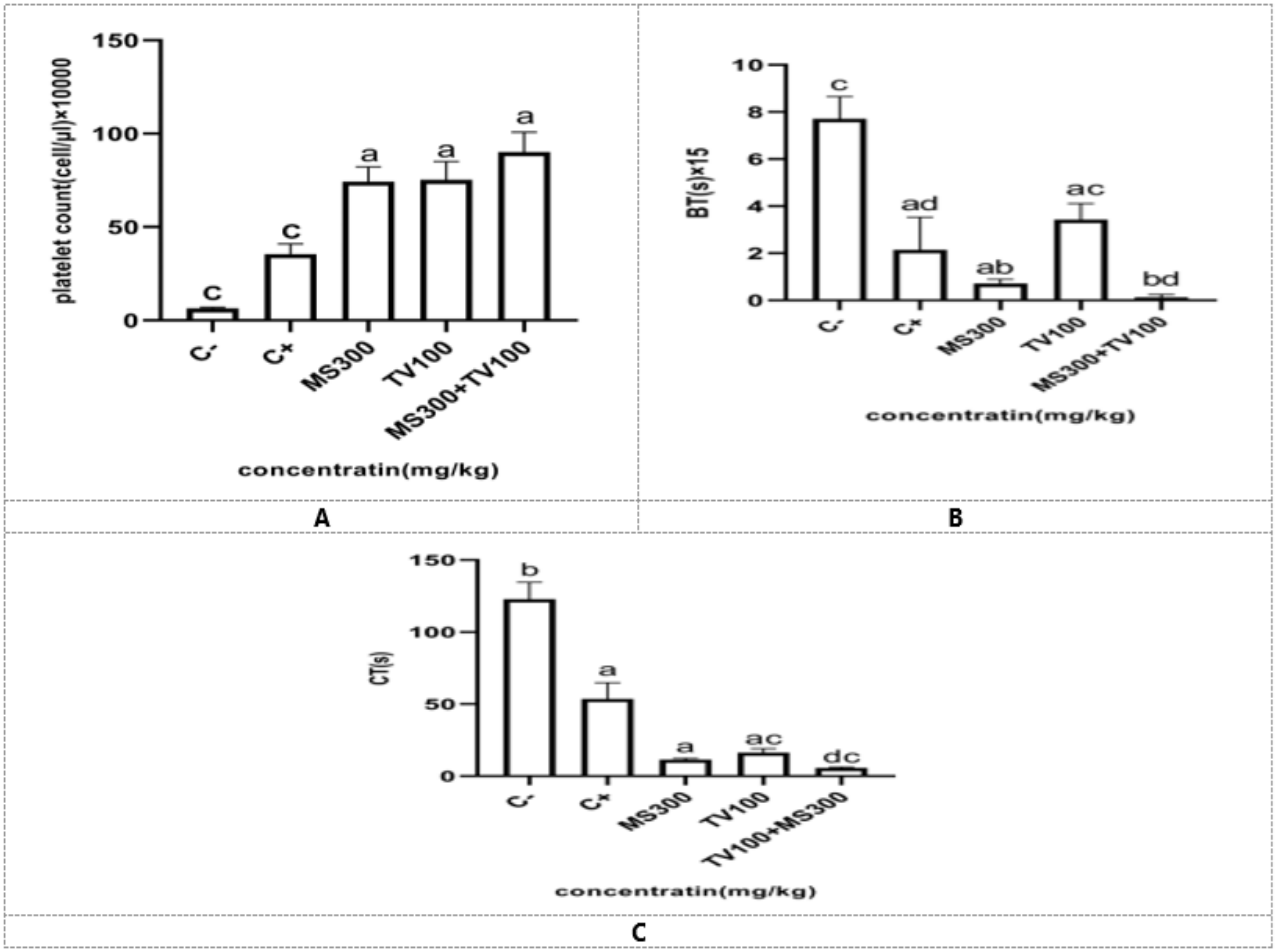

Fig. 2. *In vivo* coagulant effect of the *T. vulgaris* and *M. sativa* extract on blood coagulation parameters. A siginifacant increae in the number of platelets (a) and a significant decreas in bleeding time (b) and clotting time (c) was observed. The data are the means ± SD of three individual experiments, and the significance of the data was shown with different letters.

megakaryocytes, stimulating platelet production. Coumarins can also stimulate the release of interleukin-1-B from human mononuclear cells (Stuhlmeier et al., 1991) which increases the expression of TPO and the production of platelets (Beaulieu et al., 2014). Phenolic acids such as rosmaric acid (Javed et al., 2013) and caffeic acid (Kuete, 2017) can stimulate interleukin-6 secretion from IFN-γ (Radtke et al., 2003), causing an increase in megakaryocyte maturation to platelets. Inhibition of nitric oxide (NO) production in macrophage cells by caffeic acids (Uwai et al., 2008) and coumarin (Peana et al., 2006), suppression of NO production from peritoneal macrophages by Vitamin A and reduction of NO production by retinoic acid in keratinocytes (Bécherel et al., 1996) can lead to an increase in platelets. Polysaccharides can also increase platelets by affecting the runt-related transcription factor 1 (RUNX-1) (Yang et al., 2010) and stem cell factor (SCF) genes (Tajika et al., 1998) that induce megakaryocyte maturation. Since both *T. vulgaris* and *M. sativa* extracts contain compounds such as rosmaric acid, caffeic acid, linalool, coumarin, Vitamin A, tannic acid and quercetin can increase platelet number.

3.2. BT test

The mean value of BT in negative and positive control is 101 and 31 seconds, respectively, while this average time is 11, 51, and 2 seconds in M300, T100, and combined extract, respectively (Fig 2b). There was a significant decrease in BT of the treated groups with M300 and combined extracts compared with the control. In contrast to TV100 which seems not effective, combined extract causes a 50-fold decrease in BT. The BT test, which is related to platelet aggregation and vasoconstriction, is one of the most common tests for the identification of primary homeostasis disorders (Lind, 1991; De Caterina et al., 1994). This test indicates the formation of plaque hemostasis, which depends on sufficient platelet number and adhesion and reduced blood fluidity (Barber et al., 1985). The bleeding time was reduced in the treatment groups compared with the control groups. It is supposed that the inhibition of vasodilators such as nitric oxide can reduce BT. The B vitamins and compounds such as caffeic acid, retinoic acid, vitamin A, linalool, and carvacrol (Asgari, 2013; Kaeidi et al., 2020; Kaidi and Rhahmani, 2020) reduce the production of nitric oxide, result in vasoconstriction, platelet aggregation, and bleeding prevention.

3.3. CT test

The results indicated that the mean value of CT in M300, T100, and the combined extract was 11, 16, and 5 seconds respectively which significantly reduced than control (124 sec). The combined extract caused a 25-fold decrease in CT compared to negative and even 10-fold compared to positive control (Fig. 2c).

The CT test reflects the function of common and intrinsic pathways and platelet aggregation. The coagulation time in the CT test in the treatment groups was significantly reduced compared with the control. It is supposed that beta-carotene increases the number of red blood cells (RBCs), causing high hematocrit and activating platelet aggregation (Pietrzak and Grela, 2015). RBCs are effective in inducing platelet aggregation by releasing a significant portion of their adenosine diphosphate (ADP). Released ADP from RBCs has a 60% and 28% contribution to the reduction of individual platelets and adhesion of platelets, respectively. It has been reported that alkaloids can reduce blood CT by inducing epinephrine (adrenaline) secretion (Singh and Singh, 1975), increasing the FV amount. Both *T. vulgaris* and *M. sativa* extracts contain gallic acid and other flavonoid compounds, which increase the expression of TNF-α (Karimi et al., 2013; Mancini et al., 2015; Bilen et al., 2016; Zagórska-Dziok et al., 2020). TNF-α causes platelet activation by interacting with its receptor on the surface of platelets. Another effective factor may be linoleic acid which causes the accumulation of platelets by producing arachidonic acid (Lee et al., 2003). In fact, arachidonic acid is converted to prostaglandin H2, and this to thromboxane α2 which is the cause of platelet aggregation and vasoconstriction. Tannins are another possible reason for the reduction of clotting time. Tannins can precipitate blood proteins such as albumin, and this process of protein deposition induces the synthesis of thromboxane α2 to increase platelet aggregation and thus accelerate the formation of platelet aggregation at the site of damaged vessels (Fauzi et al., 2018). Vitamin E (alpha tocroferol) is another reason for the coagulant effects found in *T. vulgaris* and *M. sativa* extracts and inhibits urokinase activity in fibrin plates in a concentration-and time-dependent manner (Ogston, 1982). Urokinase binds directly to plasminogen and produces plasmin, which subsequently dissolves the fibrin clot (Bassampoor, 2002; Bahraini, 2020). Increasing the amount of fibrin decreases the clotting time (Hashemi Tayer et al., 2013). In addition to the common factors between the two extracts that cause CT to be reduced, there are exceptions. reducing CT in *M. sativa* may caused by malic acid, malonic acid, oxalic acid and succinic acid compounds (Xuan et al., 2003; Huang et al., 2010; Hashemi Tayer et al., 2013). The reason of decrease in clotting time in the *T. vulgaris* treatment group is the increase in blood LDL caused by the compounds in the extract. LDLs cause the formation of atherosclerotic plaques in the walls of arteries (Abasalizadeh et al., 2017), followed by vascular thrombosis. The deposition of lipids, especially LDL, and their oxidation in the macrophages of the vessel wall cause the greatest change in the inner layer of the arteries (Hamidpour, 2014). On the other hand, the infiltration of LDL into these cells and their oxidation causes more activation of coagulation factors and increase in platelet accumulation. Oxidized LDL (OxLDL) stimulates the production and accumulation of platelet microvesicles and increases platelet aggregation. *T. vulgaris* extract contains polyphenols and polyphenols can stabilize the structure of LDL particles through interaction with apoprotein B (Chen et al., 2005). Stabilization and increase of LDL increases the formation of OxLDL and subsequently OxLDL activates platelets through surface receptors, changes their shape and ultimately increases platelet aggregation (Hamidpour, 2014). Also, phenols can have synergistic effects with other active ingredients

in the extracts. Although vitamin C helps to stabilize LDL by inhibiting the breakdown of lipid peroxides, the synergistic effects of the extract compounds can be another reason for increasing platelet aggregation and reducing CT. It seems that the synergistic effect of vitamin C and flavonoids in *T. vulgaris* extract can increase platelets and subsequently decrease CT. The antioxidant activity of flavonoids through synergism with vitamin E and C and the synergistic effects between genistein (a type of flavonoid) and ascorbic acid confirm these reasons (Chen et al., 2005). During the combined effects of these two plants, the amount of CT decreased significantly compared to the positive and negative control ($p < 0.05$).

4. Concluding remarks

In addition to current therapies for bleeding disorders, herbal medicine can be one of the alternative methods. Although the procoagulant effect of some herbs was investigated, there are various reports of the superior therapeutic effects of combined extracts compared to individual extracts in the treatment of various diseases. Considering probabilistic coagulation compounds in the *T. vulgaris* and *M. sativa*, an animal study was performed to inquire the effect of the combined extract on mice. Considering the combination of *T. vulgaris* and *M. sativa* extract in the combined extract, the amount of effective compounds on clotting time, including tannins (Klotoé et al., 2012; Deng et al., 2019) , alkaloids (Raaof et al., 2013a), vitamin K (Poston, 1964), succinic acid, malic acid, oxalic acid, malonic acid, arachidonic acid (Lynch et al., 1958), linoleic acid and gallic acid increases (Crescente et al., 2009). Each of these compounds reduces clotting time by affecting each blood coagulation pathway.It concludes that each plant extract has unique and different effects in different coagulation pathways. Moreover, the combined extract had superior hemostatic effects compared to the indivitual extract, indicating the unique performance of the combined extract compared to individual treatment methods. On the other hand, it seems aromatic structures, hydroxyl alcoholic and phenolic groups of herbal compounds play a role in interaction with the coagulation factors. Therefore, it can be used as a blood-coagulant herbal medicine with antiseptic properties, although it seems necessary to investigate clinical trials.

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Authors contributions

Conceptualization: Zahra Mashkani and Jafar Vatandoost; Methodology, Data analysis, Investigation, Writing-original draft: Zahra Mashkani and Jafar Vatandoost; Supervision: Jafar Vatandoost; Animal advisor: Toktam Hajjar; Chemical advisor: Behnam Mahdavi. All authors read and approved the final version of the paper.

Conflict of interest

The authors declare that there is no conflict of interest.

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Ethical considerations

The protocols were approved by the Hakim Sabzevari University's Animal Ethics Committee (IR.HSU.REC.1399.002).

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