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Antithrombotic effects of ethanol extract of *Crataegus orientalis* in the carrageenan-induced mice tail thrombosis model

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ABSTRACT

Introduction: *Crataegus* species (common name is Hawthorn) are medicinal plants, which have flavonoids, triterpene acids, proanthocyanidins, and organic acids as main constituents, used in the treatment of cardiovascular diseases. One of the main causes of multiple cardiovascular diseases is intravascular thrombosis and current agents, which are used for the treatment and prevention of thrombosis, have some side effects. Therefore, new antithrombotic and thrombolytic agents are still needed.

Materials and Methods: Antithrombotic function of ethanol extract of *Crataegus orientalis* (COE) leaves was investigated in carrageenan-induced mice tail thrombosis model. Mice were injected with 40 µl (1%) carrageenan (Type I) dissolved in physiological saline by intraplantar administration in the right hind paw. After carrageenan injection, the extract was administered at the doses of 100, 200, and 300 mg/kg. Heparin was used as a positive control (10 and 100 IU). The length of tail-thrombosis was measured at 24th, 48th, and 72nd hours.

Results and Conclusion: 100 mg/kg COE and 10 IU heparin were not significant when compared to control groups at the time interval (24–72 h) that results was obtained. At 24th hour, both 200 and 300 mg/kg of COE showed a significant antithrombotic activity ($p<0.05$ and $p<0.01$, respectively). However, 200 mg/kg COE lost its significance and there was a decrease in the significance values of 300 mg/kg COE ($p<0.05$) at 48 and 72 h. From these results, it was concluded that COE significantly inhibited carrageenan-induced mice tail thrombosis *in vivo*.

1. Introduction

Hawthorn (*Crataegus* spp.), belonging to the Rosaceae family, consists of small trees and shrubs. Common names for hawthorns may include, mayblossom, quick thorn, whitethorn, haw hazels, gazels, halves, hagthorn, and bread and cheese tree [1].

The main constituents of hawthorn are flavonoids, proanthocyanidins, organic acids and some amines. Some *Crataegus* constituents are predicted to be good antioxidants. The leave, flower and fruit constituents responsible for free radical scavenging activity are especially epicatechin, hyperoside and chlorogenic acid. They are also among the best antilipoperoxidants [2–4].

Hawthorn extract is a popular herbal medicine which is widely and traditionally used for preventing and treating cardiovascular diseases including angina, hypertension, arrhythmias, and congestive heart failure. It has been reported that 20 species of hawthorn are used as herbal drugs in many countries such as China, Germany, France, and England [5]. In addition, *Crataegus oxycantha* (a common hawthorn) tincture and its isolated flavonoids from hawthorn have also been found to inhibit the formation of thromboxane A_2 (TxA_2) *in vitro* which is a potent inflammatory mediator and also causes platelet aggregation [6].

Thrombosis is a very complicated physiological process participated by many factors. Blood vessel injury, platelet adhesion, and aggregation are responsible for the initial stage of artery thrombus formation which is followed by blood blockage that usually causes vein thrombus. One of the main causes of multiple cardiovascular diseases is intravascular thrombosis [7,8]. Occlusion of either arterial supply or venous drainage causes ischemic necrosis of the area which leads to infarction. 99% of infarctions are caused by thrombotic or embolic events. In addition, formation of thrombus may play an important role in the progression of atherosclerotic plaques. Atherosclerosis and its thrombotic complications are the major cause of morbidity and mortality in the cardiovascular diseases. Since the

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discovery of acetylsalicylic acid, heparin and warfarin, the use of antiplatelet and anticoagulant agents in the prevention and treatment of cardiac disease, especially at the early treatment of acute myocardial infarction, has become widespread. Based on these, it has become evident that formation of thrombosis in cardiovascular disease is a central mechanism which is responsible for much morbidity and mortality [9,10].

Carrageenan-induced tail thrombosis model is one of the models that have been used to evaluate antithrombotic and thrombolytic agents, such as heparin and aspirin. It allows observing the progression of thrombosis visually and directly in a time-dependent manner. Moreover, this model is simple and non-invasive to use on small laboratory animals without causing severe stress [11,12]. Based on these advantages; the aim of the present study was to investigate the antithrombotic function of ethanol extract of *Crataegus orientalis* (COE) leaves in carrageenan-induced mice thrombosis model.

2. Materials and methods

2.1. Materials

Carrageenan Type I was purchased from Sigma (St. Louis, USA). Heparin (low molecular weight heparin) was purchased from Sanofi-aventis (Istanbul, Turkey). DMSO was obtained from Merck (Darmstadt, Germany).

2.2. Plant material

Crataegus orientalis M. Bieb. var *szovitsii* Pojark. leaves were collected from Gaziantep in Turkey, in 27 May 2002. The plant material was identified by Prof. Dr. Ali A. Donmez, from the Department of Biology, Faculty of Science, of Hacettepe University. Authenticated voucher specimen (A.A.Dönmez10693) was deposited in the Herbarium of Faculty of Biology, Hacettepe University.

2.3. Preparation of the ethanol extract of Crataegus orientalis leaves

Leaves were air-dried at room temperature and under shade, and then powdered. Plant material was extracted in 50% ethanol using a Soxhlet apparatus for 18 h. The ethanol extract was lyophilized resulting in the crude dry extract (11.82 g, 23.15% yield).

2.4. Animals

Inbreed Swiss albino mice (30–40 g) were obtained from Anadolu University Experimental Animals Research Centre. Animals were maintained in a room with controlled temperature (22 ± 2 °C) for 12 h light/12 h dark cycle with free access to food and water. Animal care and research protocols were based on the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH publication No: 85-23, revised in 1985) and approved by the Local Ethics Committee of Anadolu University, Eskisehir.

2.5. Drugs administration

A total of 36 male and female mice were randomly divided into 6 groups ($n = 6$) and mouse gender was equally distributed throughout groups. Group 1 served as control with 20% DMSO; Groups 2, 3, 4 were, respectively *i.p* injected with 100, 200 and 300 mg/kg COE dissolved in 20% DMSO (80 mL saline water, 20 mL DMSO). Groups 5 and 6 were, respectively *i.p* injected with 10 and 100 IU heparin sodium as positive controls in order to evaluate the effects of doses of heparin.

2.6. Carrageenan-induced mice tail thrombosis model

One hour after test samples were administered intraperitoneally, each mouse was injected with 40 μl (1%) carrageenan (Type I) dissolved in physiological saline by intraplantar administration in the right hind paw. Mice were observed for the formation of thrombosis and thrombus lengths were measured and photographed at 24, 48 and 72 h.

2.7. Statistical analysis

The statistical analyses were carried out using GraphPad Prism version 5.0. Data obtained from experiment was expressed as mean values ± standard error of mean (S.E.M.) to show variation in groups. Statistical differences between the treatments and the control were evaluated by one-way ANOVA, followed by Tukey's multiple comparison tests. The results were expressed as the differences were considered significant when $p \leq 0.05$.

3. Results

About 6 hours after the subplantar injection of carrageenan, the thrombosis tail infarction became visible in the control group. In the groups that were administered doses of COE, the thrombosis formation was not as visible as control group and even thrombosis formation did not occur at 6th hour in the groups that were given higher doses of COE,. The lengths of thrombosis at 24, 48 and 72 h were shown in Fig. 1. 100 mg/kg COE and 10 IU heparin were not significant when compared to control groups at the time interval (24–72 h) that results was obtained. The effect was more obvious with the concentration increasing. At 24th hour, both 200 and 300 mg/kg of COE showed a significant antithrombotic activity ($p<0.05$ and $p<0.01$, respectively).

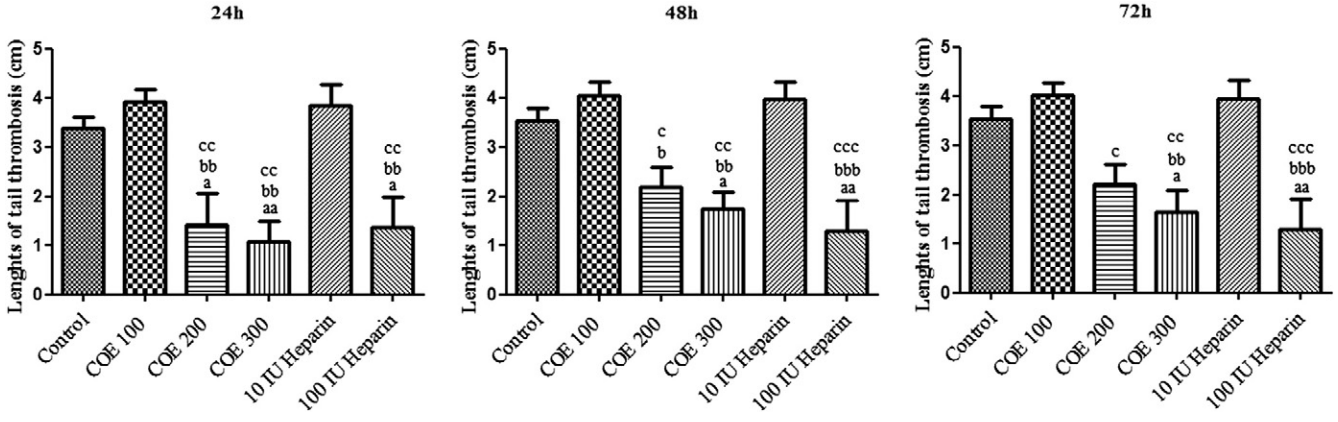

Fig. 1. Effects of COE and heparin in carrageenan-induced mice tail thrombosis at 24, 48 and 72 h. Values are presented as the mean ± S.E.M ($n = 6$). $^{aa}P<0.01$, $^{a}P<0.05$ significant difference from control; $^{bbb}P<0.001$, $^{bb}P<0.01$, $^{b}P<0.05$ significant difference from heparin 10 IU alone; $^{c}P<0.05$, $^{cc}P<0.01$, $^{ccc}P<0.001$ significant difference from COE100 mg/kg alone.

Table 1
The inhibition activity of COE against carrageenan-induced tail-thrombosis in mice.

Test Samples	Doses	Inhibition of thrombosis (%)		
		24 h	48 h	72 h
COE	100 mg/kg	_	_	_
	200 mg/kg	58.32	38.39	37.43
	300 mg/kg	68.62	50.61	53.91
Heparin	10 IU	_	_	_
	100 IU	59.79	63.31	63.31

However, 200 mg/kg COE lost its significance and there was a decrease in the significance values of 300 mg/kg COE ($p<0.05$) at 48 and 72 h. 100 IU heparin conserved its antithrombotic effect from the 24th hour to 72nd hour, any decrease or increase at the thrombosis lengths was not observed.

Findings obtained in the present study were indicated that COE significantly inhibited carrageenan-induced mice tail thrombosis *in vivo* and also inhibition activity increased along with the injected amount of COE (Table 1). The representative actual results of each group at 48 h were shown in Fig. 2 (representative results of 24 and 72 h not shown). 48 h after carrageenan injection the infarction in the tip end of mouse tail showed dissimilarity based on the amount of doses that were administered. The average thrombus length in Groups 1 and 2 were 3.5, and 4 cm respectively, whereas the average length of thrombus decreased gradually in Groups 3 (2.2), and 4 (1.8) with the increase amount of COE. After 1 week of carrageenan injection, thrombosis of tail blood led to extensive tail necrosis especially at groups 1, 2 and 5 (data not shown).

4. Discussion

Carrageenans are a member of polysaccharide polymers family. They are extracted from red seaweeds and they have a usage as gelling and thickening agents in the food and pharmaceutical industry [13]. The three main industrial types of carrageenans are κ (kappa), ι (iota), and λ (lambda) [14]. κ-carrageenans are the most potent thrombogens among others [15]. Therefore, the type I carrageenan was chosen to be use in this study as it contains high amounts of κ type. The carrageenan-induced tail thrombosis model was reported earlier by Bekemier et al. [13] and, in later years, it has been widely used for testing antithrombotics and thrombolytics by observing wine colored region in end of the tail and the length of pathological increases with the elapse of time. Carrageenans can be used to induce tissue inflammations and tail thrombosis in animal models (rat, mouse, and guinea pig models). When the type of carrageenan, which contains high amount of κ type, injected subcutaneously it induces tail thrombosis along with the inflammation in mouse paws [15–17]. Formation of thrombosis and inflammation are considerably related subjects since inflammation of blood vessels, causes thrombosis, on the contrary, blood clots in the veins, causes inflammation [18]. Based on the pathological studies some researchers estimate that local blood vessel inflammation and endothelial cell injury may lead to formation of thrombus in carrageenan induced mouse tail by releasing inflammation factors such as interleukin-1 and tumor necrosis factor. These factors may responsible for the destruction of normal endothelial cells which maintain hemagulitination and fibrinolysis [19].

For patients at risk of blood clotting, anticoagulants are used therapeutically. When heparin injected to those patients, it has immediate action and is employed for the prophylaxis of thromboses. Heparin binds to antithrombin III which inhibits serine proteases, including several clotting factors – most importantly, thrombin (Factor IIa) and Stuart Factor (Factor Xa). In contrast, low molecular weight heparin binds antithrombin and inactivates factor Xa but does not bind to thrombin with higher affinity. Furthermore, cyclooxygenase inhibitors, such as aspirin (acetylsalicylic acid), inhibit platelet aggregation by blocking TxA_2 synthesis which promotes vasoconstriction and causes blood flow to slow [20,21].

Hawthorn, belonging to the Rosaceae family, is mainly used for treatment of cardiovascular diseases as cardiotonic, antiarrhythmic, hypotensive, hypolipidemic [5]. Flavonoids and oligomeric proanthocyanidins (OPC), the main constituents of hawthorn, are responsible for most of the pharmacological activity [22]. The flavonoid content of *C. orientalis* leaves were investigated by Melikoglu et al. [23] and the main component was found to be hyperoside, along with apigenin, apigenin 7-glucoside, vitexin and vitexin 4'-rhamnoside. Cardiovascular risks may be reduced by flavonoids therefore they have received attention in medicinal use. Some flavonoids are responsible for the inhibition of platelet aggregation. The possible mechanism of flavonoids

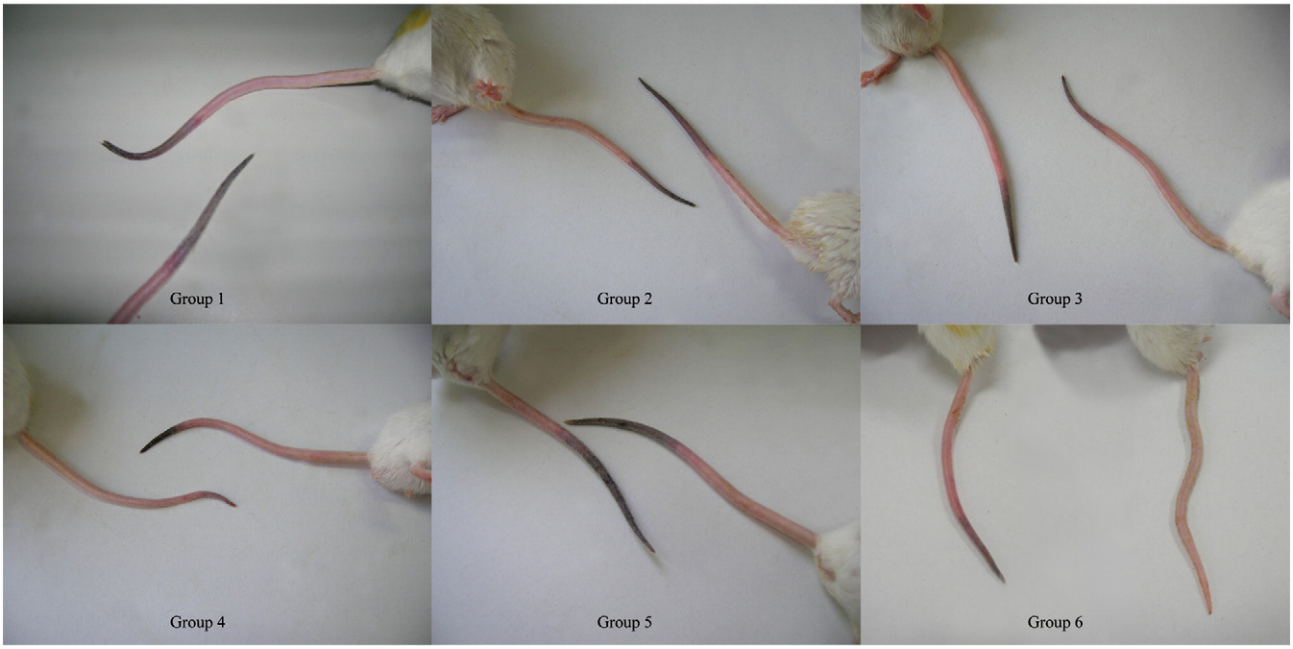

Fig. 2. Effects of COE and heparin on carrageenan induced mice tail thrombus length (48 h after carrageenan injection). Group 1: 20% DMSO; Group 2: COE 100 mg/kg; Group 3: COE 200 mg/kg; Group 4: COE 300 mg/kg; Group 5: 10 IU Heparin; Group 6: 100 IU Heparin. Data represents two of six animals for each group.

that has been postulated for their antithrombotic activity is their binding to cell receptors [24–26]. Thus, both adenosine receptors and vonWillebrand factor binding to platelet glycoprotein Ibα have been suggested to block by flavones [27,28]. Guerrero et al. [29] has been reported that some flavonoids such as apigenin, which is also a component of *Crataegus orientalis* leaves, effectively inhibited TxA_2-mediated responses in their *in vitro* platelet aggregation and secretion studies. It is not possible to suggest that the antithrombotic activity of COE is related to its apigenin content; however synergism among its component flavonoids could contribute to the inhibition of thrombus formation.

Some of the *Crataegus* species has been used as herbal medicines in many countries but the biological potency of genus *Crataegus orientalis* was not investigated sufficiently, especially in animal models. In order to evaluate antithrombotic effect of COE, carrageenan induced mice tail thrombosis model was used. DMSO (20%), which was used as a vehicle in this study, has been reported to exert antithrombotic effect by preventing tissue factor expression and activity, and also by impairing arachidonate-induced platelet aggregation via inhibition of COX-1 [30,31]. However, this effect of DMSO was eliminated by administrating 20% DMSO to control group. Therefore, it manifests that the antithrombotic effect of COE is not related to DMSO. This finding demonstrated that COE displayed strong antithrombotic activity in mice. Besides, the effect of COE at highest dose was almost similar to 100 IU heparin at 24 h, while better than 10 IU heparin. The antithrombotic activity of COE might be due to antiplatelet activity which need to further investigations.

It is possible to conclude that intraperitoneal administration of *Crataegus orientalis* has a significant antithrombotic effect. Nevertheless more studies are necessary to investigate its mechanism of action such as evaluation of the number of platelets, protrombin time, and the activated partial thromboplastin time. Meanwhile, this study indicates that the leaves of *Crataegus orientalis* ethanol extract suppress the formation of thrombosis in the carrageenan-induced mice tail thrombosis model and it could be a good candidate for the development of new antithrombotic medicine.

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