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SHORT COMMUNICATION

Hemostatic Activity of *Cistus creticus* Extract in Wistar Albino Rats

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Abstract
Aerial part preparations of *Cistus creticus* L., Cistaceae, are traditionally used in Anatolia as a hemostatic agent. The air-dried aerial parts were extracted with methanol. Gels were prepared, followed by the hemostatic activity evaluation using Wistar albino rats *in vivo* tail tip amputation model. The chemical characterization of the total extract was performed by high-performance thin-layer chromatography. The methanol extract was loaded to a hydrogel base to complete a concentration of 2.5 and 7.5%. As a result, the 2.5% extract gel was found more effective on both bleeding time and amount. Hyperoside and tiliroside, which may be the components responsible for the hemostatic properties of the analyzed plant material, were dereplicated as major constituents.

Keywords HPTLC · Topical application · Blood stopper · Coagulating activity · Flavonoid glycoside · Tail bleeding

Introductory Remarks

Cistus creticus L. belongs to the Cistaceae family and grows in Mediterranean countries which include the Southern European and Northern African coasts along with the Levantine coast of Western Asia. Various preparations are known in traditional medicine and used mainly as an antispasmodic, tonic, anti-diabetic, anti-inflammatory, hemostatic, carminative, and among other uses (Yeşilada et al. 1995; Honda et al. 1996; Yeşilada et al. 1997a, b; Tuzlacı and Aymaz 2001; Attaguile et al. 2004; Koçyiğit and Özhatay 2006). The purpose of this study was to prepare a gel from the *C. creticus* methanol extract and to prove its hemostatic activity on Wistar albino rats. To the best of our knowledge, this is the first study on *in vivo* hematological evaluation of *C. creticus* extract gel.

Materials and Methods

The aerial parts of *Cistus creticus* L., Cistaceae, were collected on 28 May 2018 from Çınarcık, Yalova, Turkey. Dr. Yavuz Bülent Köse identified the plant material and voucher specimens which were deposited in the Herbarium of the Faculty of Pharmacy, Anadolu University (ESSE 15549). The air-dried aerial parts (500 g) were crushed and extracted with methanol (1 l) by maceration in a dark place at room temperature (3× 24 h). After filtration and evaporation *in vacuo*, the extract yield was 19.9% dry weight. For the HPTLC analysis, the extract was dissolved in methanol (10 mg/ml) and used as a test solution. HPTLC analysis was performed on glass HPTLC plates coated with silica gel 60 F_{254} (Merck). Ethyl acetate-dichloromethane-formic acid-acetic acid-water (100:25:10:10:11, v/v/v/v/v) was used as a developing solvent system. Sample test solution (10 mg/ml) (5 μl) and 0.2 mg/ml of each standard solutions of hyperoside (**1**), tiliroside (**2**), and quercitrin (2 μl) were applied on the plates and developed

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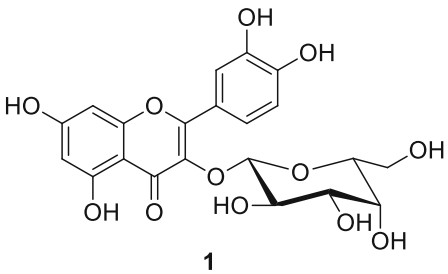
according to well-known procedures (Guzelmeric et al. 2015). Detection was performed at 366 nm and with NP-PEG 400 reagent solutions (Guzelmeric et al. 2015).



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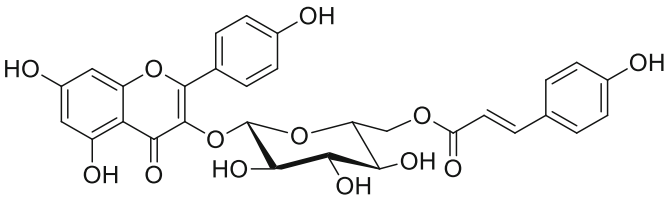

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To prepare the base gel, a previously reported methodology was used with slight modifications (Ayla et al. 2019). Two different gels with 5% and 7.5% extract concentrations were prepared. Viscosity and pH value were measured by using a viscometer and a pH meter, respectively. Formulations were evaluated for 3 months at 5 ± 3 °C, 25 ± 2 °C, and 40 ± 2 °C.

Wistar albino rats were procured from Istanbul Medipol University, Meditam, Istanbul, Turkey. The rats were hosted in cages with food and water *ad libitum*, at 20 ± 1 °C. Approval of the study protocol was obtained from the Ethical Committee of Istanbul Medipol University (Approval Number: 2019-08). This research was conducted in accordance with internationally accepted principles for laboratory animals' use and care such as the European Community guidelines (EEC Directive of 1986; 86/609/EEC). Rats were processed according to the laboratory animal ethical guidelines. Thirty-five animals were assigned into 5 groups of 7 rats each as follows: group 1: control (untreated); group 2: blank gel; group 3: 2.5% *C. creticus* extract gel; group 4: 7.5% *C. creticus* extract gel; and group 5: Ankaferd® group. Anesthesia of rats was achieved by intraperitoneal injection of 80–100 mg/kg ketamine and 10 mg/kg xylazine.

In vivo bleeding test was performed in rats by tail tip amputation method. Accordingly, the distal 1 cm of the tail of the rat was placed prone under anesthesia and cut transversely using a scalpel. After bleeding occurred, each wound was irrigated with an equal volume (1 ml) sample. Gentle compress was then applied to the wound for 1 min with the help of gauze. Clotting time was determined by a stopwatch as the time elapsed from the onset of bleeding until the occurrence of permanent hemostasis. To determine the amount of bleeding, the difference was recorded as the amount of bleeding (g) before and after each gauze application. Each rat was followed for 30 min to evaluate the bleeding status. At the end of the study, experimental animals were sacrificed with carbon monoxide gas under anesthesia (Sogut et al. 2015). *In vivo* study outcomes were given as means ± standard error of the mean (mean ± SEM). Statistical analyses were evaluated with GraphPad Prism 7.0 program. Statistical significance between groups was analyzed by one-way ANOVA followed by Dunnett's post hoc test. Values for $p < 0.05$ were considered statistically significant.

Results and Discussion

Chemical analysis of the *C. creticus* methanolic extract was performed by HPTLC, where hyperoside (**1**) and tiliroside (**2**) were detected as the main components, while quercitrin was absent. The results of the HPTLC analysis are shown in Fig. 1. Consequently, these components may be considered as the main components responsible for the hemostatic effect of

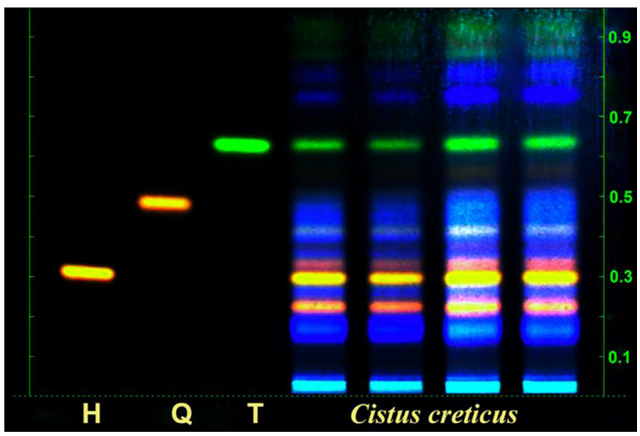

Fig. 1 HPTLC chromatogram of standard compounds and aerial parts of *Cistus creticus* methanol extracts. Detection was performed at 366 nm with NP-PEG 400 reagent solutions. Abbreviations: H, hyperoside (**1**); T, tiliroside (**2**); and Q, quercitrin

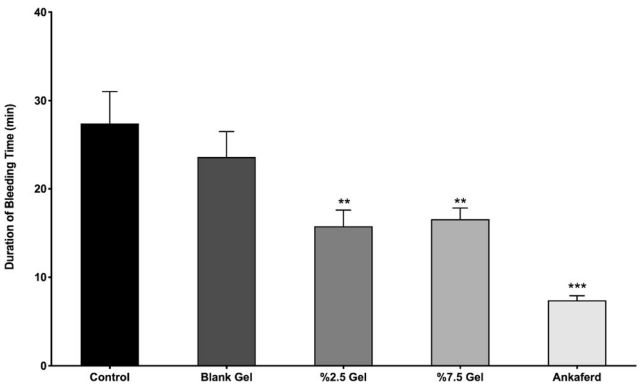

Fig. 2 Comparison of mean bleeding time of all groups. Data are expressed as mean ± SEM. ***$p < 0.001$ and **$p < 0.01$ compared with the control group. Ankaferd® blood stopper was used as a positive control for a topical hemostatic agent

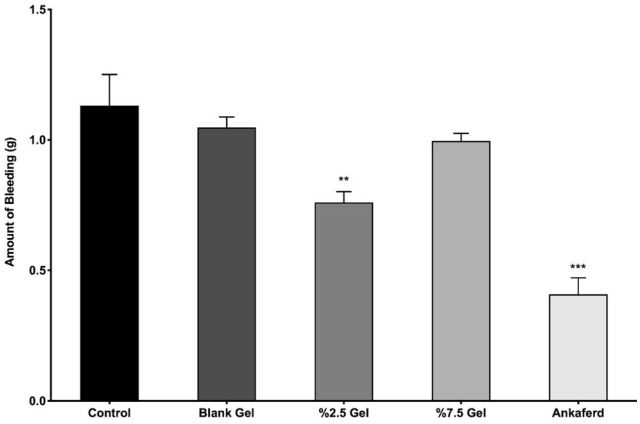

Fig. 3 Comparison of mean bleeding amount of all groups. Data are expressed as mean ± SEM. ***$p < 0.001$ and **$p < 0.01$ compared with the control group. Ankaferd® blood stopper was used as a positive control

C. creticus. It is a well-known fact that plant extracts rich in flavonoids have a high antioxidant capacity and accordingly exhibit various biological activities (Raza et al. 2017; Grochowski et al. 2018).

The physicochemical properties of the extract gels were also tested. The pH value of the 2.5% extract gel was determined as 7.545, and that of the 7.5% extract gel was 7.209. The viscosity of the gel containing 2.5% extract was 4.867 cP and the viscosity of the gel containing 7.5% extract was 126.667 cP. As the amount of extract increased, the viscosity value also increased. The stability studies indicated no significant change in the pH, viscosity, and appearance of the formulations by the end of 3 months.

The hemostatic activity of the gel was statistically evaluated by calculating the *in vivo* experimental bleeding model by bleeding time (min) and bleeding amount (g). The bleeding amount was described as the collected blood after tail tip amputation. The bleeding amounts in each group are shown in Fig. 2. The mean bleeding times achieved were 1.13 ± 0.1 g for the control group, 1.05 ± 0.04 g for the blank gel group, 0.76 ± 0.04 g for the 2.5% extract loaded gel group, 0.99 ± 0.02 g for the 7.5% extract loaded gel group, and 0.40 ± 0.06 g for the Ankaferd® group. The Ankaferd®—positive control—($p < 0.001$) and 2.5% formulation ($p < 0.01$) groups significantly decreased the bleeding amounts comparing to the control group. There were no significant differences between the blank gel group, 7.5% extract loaded gel group, and control group. Ankaferd® blood stopper, a registered traditional herbal medicine (*Thymus vulgaris*, *Glycyrrhiza glabra*, *Vitis vinifera*, *Alpinia officinarum*, and *Urtica dioica*), was used as a positive control for a topical hemostatic agent (Iynen et al. 2011). Tail bleeding times (Broze et al. 2001) were used to evaluate hemostasis in murine models and the results in each group are shown in Fig. 3. The bleeding time of untreated rats (control group) was measured as 27.4 ± 3.6 min. When the blank gel applied group (23.6 ± 2.9 min.) was compared with the control group, the bleeding time presented no obvious difference ($p < 0.05$). In addition, the tail bleeding time of rats treated with Ankaferd® was 7.4 ± 0.5 min, which was significantly shortened ($p < 0.001$) compared to the control group. Compared to the control group, 2.5% and 7.5% extract loaded gel groups (15.8 ± 1.8 min and 16.6 ± 1.2 min, respectively) significantly ($p < 0.01$) reduced tail bleeding time. The fast decrease in the bleeding time could be due to vasoconstriction or platelet aggregation (Ebrahimi et al. 2020). When the effects of the gel formulation prepared at 2.5 and 7.5% were compared to the control group, it was found that the 2.5% extract loaded formulation was more effective on both bleeding time and bleeding amount. Therefore, this activity was concentration-independent. In relation to the hemostatic effect, hyperoside was reported to possess blood coagulating activity (Ku et al. 2013). Flavonoids (active redox agents) are widely spread in nature and fully recognized as antioxidants or scavengers. The pharmacological potential of the *C. creticus* is probably due to the capability of polyphenols and flavonoids to interact with important cellular processes in which key enzymes such as cyclooxygenase, lipooxygenase, phospholipase A2, NADH-oxidase, or glutathione reductase are involved. Consequently, the hemostatic activity of the analyzed plant may be attributed to its major flavonoids (active redox agents) which have been fully recognized as antioxidants or scavengers and the hemostatic potential of the analyzed plant material is probably due to the capability of flavonoids to interact with key enzymes of important cellular processes.

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Authors' Contribution AEK: conceptualization, formal analysis, investigation, and writing of the original draft. AÇ: formal analysis, investigation, and project administration. MEO: conceptualization, formal analysis, investigation, resources, and writing of the original draft. EG, NÜO, FT, EY, and FD: technical supervision and resources. All authors: critical revision of the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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