Original Article

Anti-Hemorrhagic Effect of Horsetail, Ortie, Alfalfa, Chêne, and Aleppo oak in an Experimental Model of Rats - a Potential Theoretic Approach for Traumatic Bleeding

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Abstract

Background: Prompt bleeding control in civil accidents, incidents, and combat casualties is critically essential. Preparing efficient, portable, and low-cost local anti-hemorrhagic products with minimum side effects is one of the main challenges of using them in hemorrhage control. Anti-hemorrhagic effect of some medicinal plants, including Horsetail (H), Ortie (O), Alfalfa (A1), Chêne (C), and Aleppo oak (A2), were evaluated in the femoral arterial bleeding rat model.

Materials and Methods: After plant extraction by the maceration method, forty male rats received general anesthesia, and the left femoral artery was surgically transected. Bleeding was treated with direct gauze pressure, in both the control (without treatment) and test groups added with the mixture of five herbal extracts at 200 g/l concentration (M-200), the mixture of five herbal extracts at 400 g/l concentration (M-400), and individual extracts at 400 g/l concentration). Bleeding stoppage time (BST), blood loss volume (BLV) was defined and some blood coagulation tests were assessed.

Results: There was no statistically significant difference of BLV between mix-200 and control groups, though it was significantly lower for mix-400 than that the control and mix-200 groups ($P<0.05$). The bleeding was statistically lower for group C compared to groups H, O, A1, and A2 ($P<0.05$). However, the difference between groups A2, O, H, and A1 was not significant ($P>0.05$). The results of BST showed no statistically significant difference between the mix-200 and control groups, while it was significantly shorter for the mix-400 group than the control and mix-200 groups ($P<0.05$). BST was shorter in group C than groups H, O, A1, and A2 ($P<0.05$).

Conclusion: According to the results, Chêne extract, as well as a mixture of five mentioned herbal extracts at 400g/l concentration, were influential in bleeding control. Our results showed that the anti-hemorrhagic effect of the mentioned plant extracts was superior to the mixed form.

Keywords: Anti-hemorrhagic effect, Herbs, Horsetail, Ortie, Alfalfa, Chêne, Aleppo oak, Rat

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Introduction

Prompt bleeding control in the perioperative period, traumatic patients, and in critically ill patients is an integral and part of clinical care (1, 2), mandating efficient, portable, and low-cost local anti-hemorrhagic products with minimum side effects (3). A study of 81 casualties on the battlefield found that 81.5% of them died from bleeding and 30.9% from head trauma (2). Different anti-hemorrhagic agents- also called hemostatics- and products have been developed to assist in bleeding control, including direct pressure, cyanoacrylates, zinc chloride, silver nitrate, aluminum chloride, gelatin, cellulose, thrombin, platelet gel, individual coagulation factors, hydrogen peroxide, Chitosan, Zeolite, Kaolin, Smectite, and also herbal extracts, each having its merits and shortages (1, 4-8). Depending on the wound status, bleeding severity, and the patient's clinical and coagulation status, each implies mechanisms, including wound contraction, shrinking coagulation factors, tissue adhesion, and releasing pro-coagulant agents to the hemorrhage site (1, 9-11).

Herbal plants have been applied extensively to treat several disorders (12-15). Horsetail (*Equisetum arvense*) a well-known medicinal plant used for remedies since ancient Greek and Roman times, has been traditionally used as a diuretic, as an anti-bleeding, for wound healing (16, 17). Alfalfa (*Medicago sativa* or *lucerne*) a perennial plant belonging to the Fabaceae family, has been used in traditional medicine to treat various disorders with potential therapeutic effects i.e. as an antimicrobial, anti-inflammatory, anticancer, and antioxidant compound (18, 19). Aleppo oak (*Quercus infectoria* or *Mazu*) traditionally used in Indian and Asian medicine, is beneficial in all internal hemorrhages, with other potential effects like antimicrobial, local anesthetic, and, anti-inflammatory effects (20-22). Chêne species have been described in traditional medicine to have astringent, antiseptic, wound healing, and hemostatic properties (23). Ortie has been widely used, including the practice of soaked leaves for internal/external bleeding, diabetes, skin dermatitis, containing a large amount of some coagulation factors and vitamins with related evidence on blood clotting prevention and other effects on the liver (24-27).

However, the combined effects of Horsetail, Ortie, Alfalfa, Chêne and, Aleppo oak on the hemostatic system and bleeding control have not been investigated; therefore, this animal bleeding model was designed to assess this cumulative effect.

Methods

Preparation of plant samples

Aerial parts (leaves, flowers, and stems) of Horsetail, Ortie, Alfalfa, Chêne, and Aleppo oak plants were collected from pastures and fields. After drying in a dry and dark place at a temperature of 32 °C, the plants were crushed using an electric grinder.

Extraction was performed by the variable alcohol maceration method. For this purpose, 30 g of dried plant powder was added to 120 ml of 96% ethanol and placed on a shaker for 24 h (Heidolph Unimax 2010, Rotor, Kief, Shaker Germany, Schwabach) at a speed of 90 rpm. The pulp was then removed from the extract using sterile gas. Using Whatman filter paper (Whatman 0.5 mm, USA, SANFORD) and Buchner funnel, under suction conditions by vacuum pump, the extract was filtered entirely and, excess material was removed. By rotary vacuum distillation machine (rotary evaporator, Heidolph WD 2000, Brinkmann, Canada), the extract was concentrated under negative pressure at 40 °C, and the alcoholic solvent was wholly separated from the extract. Then, to dry the extract, it was incubated at 30 °C for 16 h, and the dried extract was dissolved in PBS (dimethyl sulfoxide) to a volume of 20 ml. The extracts were stored in sterile containers with lids in the refrigerator at 4 °C until the experiments were performed (28, 29).

Animals: First, this plan was presented and approved by the Medical Ethics Committee of AJA University of Medical Sciences to ensure that the principles of working with animals are observed, including the correct method of care, transportation, feeding and anesthesia, surgery, and disposal of animal carcasses (IR.AJAUMS.REC.1399.168).

Forty male Wistar albino rats within 200-250 gram in weight were selected. All rats were fed the same diet. Blood samples were taken, and hematology

and coagulation tests were measured to ensure uniformity in the rats' population.

Rat arterial bleeding model: Rats were anesthetized after transfer to the operating room by intraperitoneal administration of a combination of xylazine 2% (10 mg/kg) and ketamine 10% (100 mg/kg) (30). After transfer to the operating table, the left inguinal area of all animals was weighed. Then, after the final scrub and under aseptic conditions, a skin incision was made in the inguinal area. After thinning the subcutaneous tissues and muscles of the area, the femoral artery was exposed. The posterior part of the femoral artery was then closed using a Mosquito hemostat, and the femoral artery was transected in the area anterior to the homeostatic site using a surgical blade.

Evaluation of hemostasis: This study was performed in two stages. In the first stage, the groups were: control group (sterile gauze without extract), M-200 group (sterile gauze impregnated with five plant compositions with a concentration of 200 g/l), and the M-400 group (sterile gauze impregnated with five plant compounds with a concentration of 400 g/l). Then, according to the best response received from the first stage, the plants of the same effective concentration were evaluated individually. Therefore, the second stage groups included sterile gauze impregnated individually with five plants with a concentration of 400 g/l.

For this purpose, bleeding stoppage time was determined, and blood loss volume (BLV) was defined by weighing the blood-soaked gauzes and calculating the rats' pre-and post-bleeding weight differences.

Statistical analysis: IBM SPSS Version 15 (SPSS Inc.; IL, USA, ®) was used for data analysis. One-way analysis of variance (*ANOVA*) with *Tukey's test* was used to analyze the data between treatments. Results were reported as mean ± standard deviation (SD) with a confidence level of 95%.

Results

Hematological parameters: Statistical analysis of hematological parameters showed that there was no significant difference between the groups (Table 1).

Coagulation tests: Based on coagulation tests, including PT, APTT, and INR, there was no significant difference between the groups showing the studied rats' homogeneity (Table 2).

Bleeding control: Based on the results of the blood loss volume, there was no statistically significant difference between mix-200 and control groups ($P>0.05$), but it was significantly lower for mix-400 than that of control and mix-200 groups ($P<0.05$). On the other hand, it was statistically lower for group C compared to groups H, O, A1, and A2 ($P<0.05$) (Table 3). However, the difference between groups A2, O, H, and A1 was not significant ($P<0.05$) (Table 4). In complete agreement with blood loss volume, the results of bleeding stoppage time showed no statistically significant difference between the mix-200 and control groups ($P>0.05$), but it was significantly shorter for the mix-400 group than that of control and mix-200 groups ($P<0.05$) (Table 5). Furthermore, bleeding stoppage happened faster in group C than groups H, O, A1, and A2 ($P<0.05$) (Table 6).

Discussion

Effects of various medicinal plants on the hemostasis process (in vitro and in vivo) have been studied in different studies, and sometimes contradictory results have been reported. Accordingly, the combination of Horsetail, Ortie, Alfalfa, Chêne, and Aleppo oak plants in this study induced hemostasis in experimental femoral artery bleeding in the rat model. These plants can grow and are accessible in different geographical regions in Iran (8, 12). These plants were selected based on their astringent, antibacterial, and healing properties containing vitamin K and calcium as the basic ingredients for the blood coagulation cascade; to the best of our knowledge, there have been no previous studies assessing the hemostatic effects of plants mentioned here. Therefore, there is a lack of data for the comparison of the results (13, 14, 18, 24, 25).

Table 1: Hematological parameters of rats.

Groups	RBC($\times 10^6$/uL)	Hb(g/dL)	Hct (%)	Plt($\times 10^3$/uL)
Control	6.74 ± 0.37	11.30 ±0.63	35.83 ± 1.46	219.00 ± 16.04
M-200	6.37 ± 0.92	12.76 ± 0.23	40.26 ± 1.50	217.00 ± 17.61
M-400	6.8 ± 0.29	11.80 ± 0.50	37.90 ± 1.34	207.66 ± 7.26
H	6.33 ± 0.49	11.50 ±0.77	36.16 ± 3.12	227.21 ± 20.03
O	5.79 ± 0.56	12.26 ± 0.38	38.63 ± 1.37	223.33 ± 14.84
A_2	6.69 ± 0.26	11.63 ± 0.31	38.93 ± 1.42	224.66 ± 16.76
C	6.75 ± 0.18	12.46 ± 0.93	37.60 ± 1.58	239.17 ± 12.37
A_1	5.70 ± 0.55	12.30 ± 0.26	38.06 ± 0.92	230.20 ± 16.01

Abbreviations: M-200, herbal composition at a concentration of 200 g/l; M-400, herbal composition at a concentration of 400 g/l.

Table 2: PT, APTT and INR test results in rats.

Groups	PT (Second)	APTT (Second)	INR
Control	1.68 ± 2.13	27.16 ± 1.77	1.10 ± 0.12
M-200	18.76 ± 2.02	27.51 ± 1.87	1.11 ± 0.11
M-400	19.08 ± 3.08	26.85 ± 5.35	1.13 ± 0.10
H	18.03 ± 0.95	34.45 ± 1.00	1.06 ± 0.05
O	18.10 ± 0.60	34.53 ± 0.48	1.07 ± 0.03
A_2	16.96 ± 0.29	33.44 ± 2.33	1.00 ± 0.02
C	18.03 ± 0.88	34.20 ± 1.44	1.05 ± 0.05
A_1	16.96 ± 0.63	35.16 ± 0.98	1.00 ± 0.07

The methanolic extract of the Ageratum conyzoides L. plant of the Asteraceae family has been evaluated on the process of clot formation in vitro and in vivo in rats. According to the results of this study, both bleeding time and clot formation time, and PT test duration have been reduced (31, 32). In the other study, hemostatic properties of the n-butanol extract of Artemisia anmua L. from the Asteraceae family have been investigated, reducing the time of clot formation in laboratory conditions have been reported (33). Meanwhile, it has been demonstrated dose-dependent effects of aqueous extract of the Brownea grandiceps Jacq. on the blood coagulation process which demonstrated pro-coagulant activity and reduced PT time at low concentrations, but inhibitory properties alongside increased PT, APTT, and TT tests result at high concentrations (34-36). These results support our findings.

Table 3: Comparison of blood loss volume between the combined groups; Different letters in each column indicate a significant difference.

	Mean±SEM
Groups	**Blood Loss volume (gr)**
Control	3.00 ± 0.56 [a]
Mix-200	2.16 ± 0.03 [a]
Mix-400	0.83 ± 0.21 [b]

Table 4: Comparison of blood loss volume between the individual groups; Different letters in each column indicate a significant difference.

Groups	**Blood Loss volume (gr)**
H	5.26 ± 2.87 [a]
O	4.10 ± 0.55 [ab]
A_2	3.40 ± 0.52 [ab]
C	0.43 ± 0.06 [b]
A_1	2.96 ± 0.03 [ab]

Table 5: Comparison of bleeding stoppage time between the combined groups; Different letters in each column indicate a significant difference.

	Mean±SEM
Groups	**Bleeding Stoppage Time (Minute)**
Control	5.33 ± 0.66 [a]
Mix-200	5.00 ± 0.57 [a]
Mix-400	2.33 ± 0.88 [b]

The effect of Camellia sinensis (L.) on bleeding control was investigated. In that study, it was observed that after tooth extraction and placing ethanolic extract of the plant on the site, the number of bleeding decreases; while it has been shown that Capsella bursapastoris (L.) had reduced the amount of postpartum hemorrhage; meanwhile other studies demonstrated that both aqueous and ethanolic extracts of Chromolaena odorata (L.) leaves from the Asteraceae family had reduced bleeding time, clot formation time, and duration of PT and APTT tests results (36-38). However, the latter studies were in favor of our results.

In 2013 Wang et al. observed a reduction of both hemorrhage time in rats as well as in blood clotting time under the effect of n-Butanol extract from the rhizome of the Paris bashanensis plant of the family

Table 6: Comparison of bleeding time between the individual groups; Different letters in each column indicate a significant difference.

Groups	Bleeding Stoppage Time (Minute)
H	5.66 ± 0.31 [a]
O	4.00 ± 0.00 [a]
A_2	5.33 ± 0.88 [a]
C	1.66 ± 0.41 [b]
A_1	4.33 ± 0.88 [a]

Melanthiaceae (37). Another study by Ediriweera et al. in 2011 showed the pro-coagulant activity of Scoparia dulcis L. extract of almond at high concentrations (39).

In a study in 2016, Dasgupta et al. evaluated the hemostatic properties of the aqueous extract of parsley leaves from the Compositae family and found that it reduced PT test duration (40). In one study, the hemostatic properties of Tridax procumbens aqueous extract in both in vitro and in vivo conditions were assessed and it was demonstrated that the bleeding time and the duration of laboratory coagulation tests had been reduced after treatment by this extract (41). However, in another study on pollen Typha latifolia L. from the Typhaceae family, it was demonstrated that this extract was able to decrease bleeding time in mice and also laboratory PT and APTT tests duration, and to increase coagulation factor XII activity (42). These studies are also in favor of our findings.

In some previous studies, horsetail has shown astringent effects and has been used to control bleeding from the mouth, nose, and vagina (16, 17, 43). While in our study, this plant was not able to control bleeding effectively. In another research, the hemostatic properties of Ortie on mice tail bleeding model was evaluated and it was indicated that alcoholic extract of this plant could reduce clot formation time and mice tail bleeding duration (43). However, in our study, the extract of this plant was not adequate to control bleeding from the rat's femoral artery which might be due to the extraction method, study population, or bleeding location.

In other studies, sterile gauze impregnated with Chêne ethanolic extract and ferric sulfate, 15.5% was applied to control bleeding from the bony crypt; based on these observations, the hemostatic effect of Chêne extract in 4 and 5 minutes is controversial (23, 44-46). In contrast, Chêne extract significantly led to a bleeding stop in our study.

Other studies have demonstrated that the animals treated with the hydroalcoholic extract of alfalfa do not show significant changes in their platelet count, PT, and PTT indices, the amount of total protein and plasma fibrinogen increased (3, 47-49). Accordingly, we did not observe a significant difference between the alfalfa and control groups in terms of coagulation test results and the amount of blood lost. However, the study method was different.

Conclusion

According to the results, Chêne extract, and a mixture of five mentioned herbal extracts at 400 g/l concentration, were effective in bleeding control. Our results showed that the anti-hemorrhagic effect of the mentioned plant extracts was different when using the individual one compared to the mixed form. Furthermore, according to the better effect of mixed extract at the concentration of 400 g/l than that of 200 g/l, it can be concluded that the anti-hemorrhagic effect of the plants mentioned above might be dose-dependent, and using the higher concentrations will probably result in better effects. According to the literature data, it is possible that plant extracts exert

their inductive or inhibitory effect on the bleeding control by local constriction of the bleeding site or affecting the blood coagulation system. However, determining the exact mechanism of action of the mentioned extracts requires further studies in the future.

Acknowledgment

None.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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