

**Antiplatelet and Antithrombotic Activities of
Artemisia Dracunculus L. Leaves Extract**

Leila Shahriyary, Razieh Yazdanparast*

*Institute of Biochemistry and Biophysics, P. O. Box 13145-1384, University
of Tehran, Tehran, Iran.*

Summary

The present study focused on evaluating the effects of a methanol crude extract of *Artemisia dracunculus* (tarragon) leaves on human platelet function *in vitro* and several markers of platelet coagulation, including the prothrombin time (PT), the activated partial thrombin time (APTT) and bleeding time (BT) using wistar rats and mice for *in vivo* pulmonary thrombosis. Human platelets were incubated with different concentrations of the test extract and then activated with thrombin. Adhesion, aggregation and spreading to the laminin-coated plates were evaluated by scanning electron microscopy (SEM). In the control samples, most of the adhered platelets showed extensive spreading on laminin-coated plates. Pretreatment of platelets with the plant extract had inhibitory effect on the secretion of adenine nucleotides and malondialdehyde (MDA) formation. On the other hand, oral administration of the extract protected the mice against thrombotic death or paralysis induced by collagen and epinephrine in a dose-dependent manner. In addition, the extract had significant effects on the coagulation parameters. Mouse tail bleeding time was significantly prolonged among the treated mice. Our results showed that the methanol crude extract of tarragon has anticoagulant and antithrombotic activity. These findings provide scientific basis for traditional use of tarragon for treatment of thrombotic diseases.

Keyword: Adenine nucleotides; Antiplatelet; Antithrombotic; *Artemisia drancunculus* L.; Malondialdehyde

*Corresponding Author:

R. Yazdanparast

Inst. Biochem. Biophys

P. O. Box 13145-1384, University of Tehran

Tehran – Iran, Tel: +98-21-66956976, Fax: +98-21-66404680

E-mail: yazdan@ibb.ut.ac.ir

Introduction

Adhesion of platelets to adhesive proteins in the subendothelial matrix and aggregation are crucial events in haemostasis and arterial thrombi. Following vessel wall damage, platelets are activated in response to a number of physiological agonists such as collagen, thrombin and arachidonic acid [1]. Upon activation, modulation in platelet functions including adhesion, spreading, release reactions, aggregation and clot formation will occur [2]. On the other hand, platelet activation and aggregation play the main role in the process of arterial thrombosis [3], heart attacks and stroke. Therefore, inhibition of platelet activation may constitute an ideal strategy for antiplatelet therapy and prevention of peripheral cardiovascular diseases [1]. Currently available drugs such as aspirin (acetylsalicylic acid, ASA), clopidogrel and glycoprotein IIb/IIIa receptor inhibitor inhibit some steps in the activation process, including adhesion, release reaction and aggregation [4]. Epidemiological studies have shown that platelet function modulation can also occur by the polyphenols and flavonoids obtained from the ingested food stuff [1, 5]. Thus, plant extracts can reduce the risk of clot formation and cardiovascular diseases [6].

Artemisia dracunculus (tarragon) is a small shrubby perennial herb belonging to Asteraceae family. Tarragon contains a number of monoterpenoids [7], alkaloids, coumarins [8] and isocoumarin [9] among many other bioactive compounds. Due to these vast arrays of bioactive compounds, *A. dracunculus* has long been used medicinally for treatments of various illnesses. It is usually used as: antifungal, antibacterial [10-12], anticonvulsant [13], antiplatelet [14] and antidiabetic [7]. Rats fed with hyperlipidemic diet showed a 15% reduction in serum cholesterol and a 25% reduction in triglycerides levels when treated with an extract of *A. dracunculus* [15]. In addition, the extract of *A. dracunculus* appears potentially useful for decreasing the incidence of coronary diseases due to its significant effects on prolonging the blood coagulating times [15]. This property may construct the scientific basis for the traditional Persian history of use as a natural cleanser of the blood [15,16].

To explore the mode of action of the plant extract on blood coagulation, we have previously shown that the methanol crude extract and its chloroform fraction have reduced by a significant measure the adhesion and aggregation of the treated human platelets to the laminin-coated plates and decreased the extent of platelet protein secretions [17]. To expand our knowledge, in the present study, we evaluated the effects of the methanol crude extract on human platelet spreading on the laminin-coated plates, and the line of adenine dinucleotide (ADA) upon their activation. We also measured the levels of MDA and lactate dehydrogenase secretions as a measure of platelet destruction upon treatment. In addition, we examined the anticoagulant and antithrombotic activity of the crude extract by the coagulation assays and thrombi formation using *in vivo* models.

Materials and Methods

I. Chemicals

Laminin, thrombin, collagen, bovine serum albumin (BSA) and trichloroacetic acid (TCA) were purchased from Sigma Chemical Co. (USA). Nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), methanol and organic solvents were obtained from Merck (Germany). Adenosine triphosphate (ATP) was obtained from Aldrich Chemical Co. Ltd. (England). The human blood samples obtained from the Iranian Blood Bank (Tehran, Iran). Sterile 24-well plates with flat-bottom wells were obtained from Nunc (Denmark).

II. Plant material and Extraction

The aerial sections of *A. dracuncululus* were collected from suburb of Arak at the end of spring (2006). The plant materials were dried, far from direct sunlight. The dried leaves were powdered and kept in a closed container in a cold room. The plant powder (500 g) was extracted four times with methanol. The accumulated extract was concentrated under reduced pressure and then the final volume was adjusted to 500 ml using distilled water.

III. Animals

Male wistar rats, 5-7 months old with a weight of 200-250 g and male mice approximately 25-30 g were purchased from institute of Biochemistry and Biophysics (University of Tehran, Tehran, Iran). They were housed under conventional conditions and were allowed free access to food and water before experiments. Meanwhile, all experiments were carried out according to the guidelines for the care and use of experimental animals approved by state veterinary administration of University of Tehran.

IV. Isolation of blood platelets

The human blood samples were obtained from healthy adult volunteers and collected into ACD (78 mM citric acid, 117 mM sodium citrate, 111 mM dextrose) solution in a 5:1 (v/v) ratio and platelets were isolated by differential centrifugation of blood (20 min at 200×g). Platelet-rich plasma (PRP) was separated and centrifuged for 20 min at 1000×g to sediment platelets. The resulting pellet was gently resuspended in Ca⁺²/Mg⁺² free modified Tyrode's buffer (140 mM NaCl, 10 mM glucose, and 15 mM Tris/HCl, pH 7.4). The resulting platelets were subsequently washed three times with the same buffer. Blood platelets were suspended in Ca⁺²/Mg⁺² free Tyrode's buffer at a final concentration of 10⁹ platelets/ml [18].

V. Methodology:

1. Lactate dehydrogenase (LDH) assay

Damage to the plasma membrane of platelets was evaluated by measuring the release of the intracellular lactate dehydrogenase (LDH). Human platelets were treated with the plant extract at different concentrations for various time intervals and then centrifuged for 4 min at 1312×g. The supernatant was kept for LDH assays as the extracellular (released) source of LDH. The platelets were then lysed with 1% Triton X-100. 150 µl of each sample was collected and added individually to 2500 µl of the substrate solution

containing 0.24 mM NADH and 500 μ l of 9.76 mM pyruvate. The change (decrease) in the absorbance of each reaction mixture was recorded at 340 nm for 3 min [19]. Total LDH activity was the sum of the released and the intracellular LDH activities. The released LDH activity was expressed as a percentage of the total LDH activity [20].

2. Platelet adhesion assay by scanning electron microscopy

Each well was incubated for 1-2 h at 22°C with 50 μ l of laminin solution (10 mg/ml in phosphate buffer saline, PBS). Wells were aspirated, treated with 200 μ l of PBS containing 1% BSA for 1 h, and then washed three more times with 200 μ l of PBS [21]. Suspensions of human platelets were preincubated with the plant methanol crude extract at a concentration of 500 μ g/ml for 120 min followed by activation with thrombin. Then, the platelets were allowed to adhere to the plates for 60 min at 22°C, the wells were washed twice with phosphate buffer. Platelets were fixed by adding 100 μ l 2% glutaraldehyde in 0.053 M cacodylate buffer for 45 min. The wells were washed with phosphate buffer and postfixed with 1% osmium tetroxide in cacodylate for 30 min. The wells were dehydrated using sequentially acetone and then liquid CO₂. Platelets were sputter with gold and they were examined in a Zeiss DSM-960A scanning electron microscope (Germany) [22].

3. Measurement of secreted adenine nucleotides

The platelet suspensions were preincubated with different plant extract concentrations (100-500 μ g/ml) for 120 min followed by thrombin activation. Each sample was then centrifuged for 20 min at 1100 \times g and the concentrations of adenine nucleotides in the supernatants were determined as followed: To an aliquot of each supernatant an equal volume of HClO₄ (0.6 M) was added and the precipitated proteins were removed by centrifugation at 2700 \times g for 10 min. The absorbance of each supernatant was recorded at 260 nm. The amount of adenine nucleotides was calculated with respect to the absorbance of ATP standard solution in HClO₄ (0.6 M). The liberated adenine nucleotides were expressed as nanomoles/mg of the cell lysate protein [18].

4. MDA determination

Malondialdehyde formation was assayed in platelets by the method of Wachowicz [23]. The method is based on a spectrometric measurement of the purple color generated by the reaction of TBA with MDA. For this purpose, the plant-treated platelets were activated with thrombin (0.25 U/ml). Samples were then transferred to an equal volume of 20% w/v trichloroacetic acid in 0.6 M hydrochloric acid. The protein-precipitate was separated by centrifugation (1200 \times g, 10 min). One volume of each supernatant was then mixed with 0.2 vol of 0.12 M Thiobarbituric acid in 0.26 M Tris, pH 7 and immersed in a boiling bath for 15 min. After cooling to room temperature, the absorbance was measured at 532 nm. The concentration of MDA was calculated based on the absorbance coefficient of the TBA–MDA complex ($\epsilon = 1.55 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$) and it was expressed as nmol/10⁹ cells.

5. Acute pulmonary thromboembolism

The antithrombotic assay was performed by mouse pulmonary thrombosis test according to the method of DiMinno and Silver [24]. The extracts of *A. dracuncululus* (equivalent to

50, 100, and 200 mg of plant leaves powder/kg body weight) was administered orally. After 90 min a mixture of collagen (114 µg/mouse) and epinephrine (1.83 µg/mouse) was injected into the tail vein to induce pulmonary thrombosis. The number of dead or paralyzed mice was recorded up to 15 min, and the percentage of protection was calculated as follows: $[1-(\text{death or paralyzed})/\text{total}] \times 100$.

6. Anticoagulation assay

The extract of *A. dracuncululus* was administered orally to a group of rats for 2 weeks once a day at different doses (equivalent to 100-500 mg of plant leaves powder/kg body weight). All experiments were performed 4 h after the final administration. Citrated blood samples of rats were collected by cardiac puncture and then centrifuged at 1000 rpm for 10 min to obtain platelet poor plasma. Coagulation parameters were measured using an automated coagulation counter. Bleeding time (BT) was estimated by cutting the tail-tip as described by Chan et al [25]. In brief, the tail of each anesthetized rat was cut 2 mm from the end and bleeding time was measured from the moment the tail was cut until the bleeding completely stopped.

7. Statistical analysis

Statistical analyses were performed using Student's *t-test*. All data are presented as means \pm SD. The statistical significances were achieved when $P < 0.05$.

Results

1. Lactate dehydrogenase (LDH) assay

The cytotoxicity effect of the methanol extract on platelet was evaluated by LDH assay. The LDH activities were approximately 2.8%, 2.9% and 3.0% for the control and the platelets treated with two different concentrations of the plant extract (250, 500 µg/ml) for 120 min, respectively. No significant increase in LDH release was observed relative to the control samples (Table. 1).

Table 1. Effect of *A. dracuncululus* methanol crude extract on platelet lactate dehydrogenase release.

Time (min)	Released LDH (%)		
	control	extract (250 µg/mL)	extract (500 µg/mL)
0	2.1 \pm 0.8	2.0 \pm 0.2	2.1 \pm 1.0
60	2.6 \pm 0.4	2.5 \pm 0.8	2.6 \pm 0.2
120	2.8 \pm 0.5	2.9 \pm 0.2	3.0 \pm 0.3

All values statistically different from the control ($P < 0.05$).

2. Platelet adhesion assay by scanning electron microscopy

The binding of platelets to laminin-coated wells was examined by scanning electron microscope. A microscopic image of the platelets attached to the culture plates is shown in Fig. 1. The SEM image of the control sample (Fig. 1A, B) after 1h incubation in the laminin-coated wells clearly demonstrate sheetlike underlayers of extensively spreaded platelets while the plant extract-treated platelets (Fig. 1C, D) did not show such morphologies. In addition, adhesion of human platelets to the laminin-coated surfaces is significantly reduced and much less aggregates were formed following treatment with the plant extract.

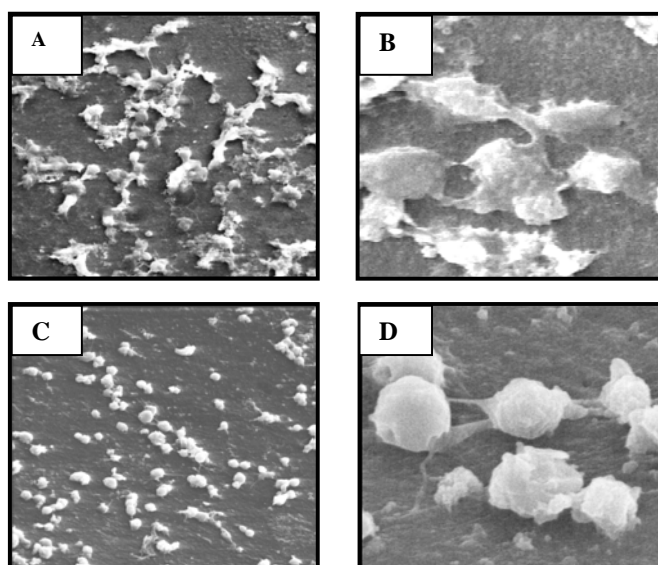


Fig1. Scanning electron micrographs of platelet adhesion to the laminin-coated wells. Wells were coated with laminin and then washed with PBS. Platelets were preincubated with the test extracts and activated with thrombin (0.25 U/ml). Then platelets were incubated on the laminin-coated wells for 60 min. Platelets in the absence of the extract (A, B), in the presence of 500 µg/ml of the crude extract (C, D). After washing out nonadherent platelets, the wells were supplemented with 200 µl of PBS and examined with an SEM.

3. Measurement of secreted adenine nucleotides

In order to examine the effect of the methanol extract on adenine dinucleotide release, the human platelets were preincubated with various concentrations of the extract (100-500 µg/ml) followed by thrombin activation (0.25 U/ml). Based on Fig.2, the adenine nucleotide secretion decreased by almost 30% at a concentration of 500 µg/ml of the crude methanol extract of *A. dracuncululus*.

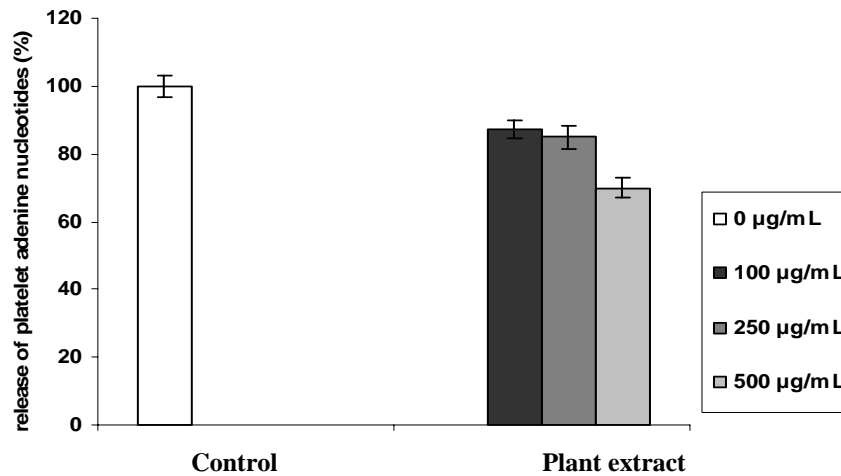


Fig 2. Effects of the methanol crude extract of *A. dracunculus* at different concentrations, on the release of adenine nucleotides from thrombin-activated platelets (0.25 U/mL). Each value represents the mean \pm SD (n=3). All values are statistically different from the control ($P < 0.05$).

4. MDA determination

We also evaluated the effect of *A. dracunculus* on MDA production induced by thrombin in human platelets. Our observation showed that the plant extract inhibited the MDA formation in a dose-response manner by 13.3%, 40% and 66.7% at the plant extract concentrations of 100, 250 and 500 µg/ml, respectively (table 2).

Table 2. Effect of *A. dracunculus* methanol crude extract on the MDA production in human platelet.

MDA	Concentration of the extract (µg/mL)			
	Control	100	250	500
nanomoles/ 10^9 cells	96.7 \pm 3	83.8 \pm 0.5	58 \pm 1	32.2 \pm 7

Each value represents the mean \pm S.D (n=3). All values are statistically different from the control ($P < 0.05$).

5. Acute pulmonary thromboembolism

The effect of *A. dracunculus* leaves extract in preventing death or paralysis in mice induced by thrombolytic agent, was evaluated in affected mice (Table.3). Oral administration of the extract showed a significant dose-dependent protective effect. The percentage of thrombosis inhibition was 30, 50 and 80% at doses equivalent to 50,100 and 200 mg of the plant powder/kg body weight, respectively.

Table 3. Effect of methanol extract of *A. dracunculus* on pulmonary thrombosis in mice.

Sample	Dose (mg/kg)	No. dead or paralyzed/ No. tasted	Protection (%)
Control	-	9/10	10
extract	50	7/10	30
	100	5/10	50
	200	2/10	80

Each sample was administered orally 90 min before the intravenous injection of epinephrine (1.83 µg/mouse) and collagen (114 µg/mouse). All values are statistically different from the control ($P < 0.05$).

6. Anticoagulation assays

The anticoagulant activity of the extract was investigated by APTT and PT assays. The methanol extract was able to prolong APTT and PT in a dose-dependent manner (Table 4). At 500 mg/kg body weight, the anticoagulant activity was almost 1.5 (41 s) times greater than that of the control (27 s) in terms of APTT. The anticoagulant effect of the extract was also evaluated by the PT test, which showed that the anticoagulant activity was about 1.6 (27 s) times greater than that of the control (16 s). The rat transection bleeding time was measured to determine the antihemostatic effects of the methanol extract. These results showed that the methanol extract caused dose-dependent increase in the tail bleeding time. In the control group, the bleeding time was 136.2 s while the extract caused an increase in bleeding times to 145, 186.8 and 300 s at doses equivalent to 100, 200 and 500 mg plant powder/kg body weight, respectively (Table 4).

Table 4. Effect of methanol extract of *A. dracunculus* on rat plasma coagulation and tail bleeding time.

Sample	Dose(mg/kg)	APTT (s)	PT (s)	BT (s)
Control	-	27 ± 0.2	16 ± 0.1	136.2 ± 0.2
extract	100	29 ± 0.3	17 ± 0.4	145 ± 0.5*
	200	32 ± 0.2	20.7 ± 0.2	186.8 ± 0.1
	500	41 ± 0.5*	27 ± 0.3	300 ± 0.1

Each sample was administered orally once a day for 14 days. Each value represents the mean ± SD (n=3). * $P < 0.01$ compared with control.

Discussion

Platelets become activated in response to many factors such as damaged vessels walls and various platelet agonists which are released from the stimulated platelets [26]. Upon activation, the platelets undergo a morphologic change from smooth disks into spiny spheres and adhere to the exposed subendothelial connective tissues, aggregate and release several biologically active compounds such as ADP, ATP, calcium and serotonin [26]. Adenine nucleotides exist in platelets in two different forms. One form is stored in the granules which are released from the platelets upon activation and the soluble form which is cytoplasmic and has mainly metabolic function [23]. It has been shown that a correlation exists between the level of ATP and the ability of the platelets to perform shape change, adhesion and aggregation [27]. Platelet adhesion to adhesive proteins is accompanied by the synthesis and release of platelet activators such as ADP and thromboxane A₂ [18]. The formation and release of thromboxane A₂ (TXA₂) is a main step in the platelet responses to a variety of agonists. TXA₂ is an eicosanoid formed via the cyclooxygenase pathway [28].

In this study, the morphological features of activated platelets upon exposure to different doses of the plant extract were evaluated. Based on the acid phosphatase assay, our previous studies have demonstrated that the platelet adhesion to laminin-coated wells is inhibited by 51% following preincubation with the methanol crude extract (200 µg/ml). Platelet adhesion to laminin is mediated by specific platelet surface receptors such as Integrins (VLA-6) or GPIIb-IIIa [29]. Inhibition of platelet adhesion by *A. dracuncululus* extract probably affects the affinity of the platelet receptors for the surface coated proteins. Figure 1 clearly indicates that the activated platelets have adhered and spreaded on the laminin-coated surfaces and they had irregular shape with multiple cytoplasmic projections. Exposure to the plant extract, however, has reduced the platelet spreading and adhesion. The treated platelets on the laminin surface had rounded shapes with minimal projections and pseudopodia extensions. On the other hand, Fig.1 clearly indicates that the treated platelets had formed much less aggregates relative to the untreated samples. Following platelet treatments with the methanol crude extract, at a dose of 500 mg/ml, the ADP and MDA secretion have both decreased by 30% and 66.7%, respectively relative to the controls. At the applied doses in this investigation, no cytotoxic adverse effects were observed (based on LDH release) by the plant extract-affected platelets relative to control samples.

Several studies have shown that polyphenols and coumarins significantly inhibit platelet function and some coumarins inhibit the activity of vitamin K-dependent γ -carboxylase involved in the activation of coagulating factors [33]. Accordingly, the anticoagulating activity of tarragon, measured in our study in terms of APTT, PT, BT, might be mainly due to its coumarin and polyphenolic contents. *Artemisia* species are known to be very rich in coumarins [9, 30-32]. To elucidate the exact mechanism, further studies regarding the structure elucidation of the active compound(s) and evaluation of their biological activities in the pure states are essential. These studies are in progress in our lab and will be published upon completion.

Conclusions

The present results indicated that the *A. drucunculus* crude extract effectively inhibited platelet function induced by thrombin treatment and it also showed strong antithrombotic and anticoagulant activities. These observations provided the scientific basis for traditional use of this plant for some of the blood associated disorders.

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