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ARTEMISIA DRACUNCULUS, *PUNICA GRANATUM* AND *BERBERIS VULGARIS* INHIBITORY EFFECTS ON PLATELET ADHESION AND COAGULATION FACTORS IN RATS

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ABSTRACT

Excessive platelet activity is one of the most important factors responsible for the incidence of cardiovascular diseases and, also, play important role in coagulation cascade. In this study, the comparative effects of methanol extracts of three herbs on adhesion of the activated platelets to fibrinogen coated plates and clotting factors were investigated. *Artemisia dracunculus*, *Punica granatum* and *Brberis vulgaris* are used as blood anti-coagulatory plants in Iranian folk medicine. Platelets were prepared and incubated with different concentration of the test samples (50-200 µg/ml) for 60 min. The treated and un-treated platelets were then activated with thrombin (0.25U/ml) and their adhesions to fibrinogen coated plates were investigated. Based on obtained data, the methanol extract of *Artemisia dracunculus*, *Brberis vulgaris* and *Punica granatum* at a concentration of 200 µg /ml reduced platelet adhesion to coated wells by 35%, 25% and 20%, respectively. In addition, the effects of the crude extracts of each plant on atherogenic lipoproteins were also examined. The results indicated that the LDL and cholesterol concentration were dramatically reduced by 56% and 36%, respectively, by *B. vulgaris*. This result provided the scientific basis for the traditional use of *A.dracunculus* and *B. vulgaris* in treatment of cardiovascular related disorders.

INTRODUCTION: Platelets, derived from mega-karyocytic cell, are anucleated blood elements. Platelet adhesion to extracellular matrix proteins such as immobilized fibrinogen, vonWillbrandfactor, fibronectin and collagen is one of the initial steps of platelet activation for haemostasis maintenance [1].

Haemostasis is the most important body's defense to control hemorrhage which if carried too far, it can block blood vessels with thrombi and lead to unstable angina and myocardial infarction [2]. As soon as an injury occurs, platelets become activated by the generated thrombin, adenosine diphosphate (ADP) and collagen and then, they adhere to extracellular matrix proteins through various glycoprotein (GP) receptors expressed by the platelets [3]. Platelets, after activation by agonists, express cell membrane glycoprotein (GPIIβ/IIIα) for adhesion to fibrinogen. Both in the formation of the blood clot and in cell adhesion, fibrinogen plays a central role in blood coagulation [4].

Under unphysiological conditions, inadequate platelet function might occur which leads to bleeding whereas plethora platelet activation plays an important role in arterial occlusion and progression of atherosclerosis that eventually leads to thrombosis [2]. Agonist activation of platelets will induce membrane exposure of phosphatidylserine (PS).

The exposed PS accelerates the prothrombinase complex reactions of the coagulation cascade, leading to the creation of thrombin, the most potent platelet agonist (5,6). Thrombin, in turn, activates different factors that play vital role in coagulation cascade [7]. Phytochemicals, mainly the phenolic compounds and coumarins of the fruits and vegetables, are believed to reduce the risk of cancer and cardiovascular diseases [8]. Flavonoids, a broad class of low molecular weight secondary plant phenolics possessing the flavan nucleus, are widely found in fruits and vegetables as well as many medicinal plants [9]. Most of the beneficial health effects of flavonoids are attributed to their antioxidant and chelating abilities. By virtue of their capacity to inhibit LDL oxidation, flavonoids have demonstrated unique cardioprotective effects [10-12].

In addition, it has been shown that high flavonoid intake is associated with lower coronary heart mortality, lower incidence of myocardial infarction in elderly and reduced risk of coronary heart disease in postmenopausal women [10, 13]. Coumarins, members of polyphenols, are prescribed for prevention of thromboembolism in patients with anti-phospholipid syndrome and stroke risk [14]. These compounds act by suppressing the synthesis of functional vitamin K-dependent coagulation factors II, VII, IX and X, and thus, causing relevant functional deficiencies [14] which are clinically measured in term of PT and aPTT factors.

Artemisia dracunculus L. (Asteraceae), *Punica granatum* L. (Punicaceae) and *Berberis vulgris* L. (Berberidaceae) are well known herbal remedies against cardiovascular diseases [15-17] mainly in Iran in addition of being traditionally used for other medical disorders. For example *A. dracunculusus* is used for treatment of headaches, epilepsy and dizziness [17]. *P. granatum* is used against cancer and diabetic cells. *B. vulgris* is used against gastrointestinal, blood, endocrine, skin and Immune system.

The purpose of the present study was to find scientific basis for the traditional use of these plants while evaluating their relative anti-coagulating potencies.

MATERIAL AND METHODS :

Chemicals: Human fibrinogen, thrombin, bovine serum albumin (BSA), p-nitrophenyl Phosphate and Triton 2X-100 were purchased from Sigma Chemical Co. (USA). Chloroform was obtained from Merck (Germany). The blood samples obtained from N-Mary rats provided from the animal house of University of Tehran (Tehran, Iran). Sterile 96-well microtiter plates with flat-bottom wells were obtained from Nunc (Denmark).

Plant Material: *P. granatum* for this study was the Malas variety from Tehran province (Iran). After transferring the fruits to the laboratory, their peels were manually removed. The aerial sections of *A. dracuculus* were collected from suburb of Arak at the end of spring (2009). The *A. dracuculus* and *P. granatum* materials were dried, far from direct sunlight and then were powdered and kept in a closed container in a cold room. *B. vulgaris* fruit obtained from local market and authenticated at the Herbarium of Faculty of Pharmacy, Medical Sciences University of Tehran, where the voucher specimen is deposited under No. 6507.

Extraction: *A. dracunculus* and *P. granatum* powders were extracted with methanol at 30°C four times and the collected extracts were filtered for removal of particles. The extracts were concentrated by a rotary evaporator and the residues were lyophilized. The *B. vulgaris fresh* fruits were extracted with boiling water and concentrated. The thick syrup was dried by a freeze dryer which yielded an adhesive residue. The freeze-dried residue of each sample was stored at -20°C for further investigation. The extraction yields were 15%, 8% and 5% for *B. vulgaris*, *P. granatum* and *A. dracunculus*, respectively.

Isolation of Blood Platelet: Blood samples were collected into plastic tube containing ACD solution (78 mM citric acid, 117 mM sodium citrate, 111 mM dextrose) in a 5:1 (v/v) ratio. Then platelet rich plasma (PRP) obtained by centrifugation (200 × g for 15 min at room temperature). PRP centrifuged for 20 min at 800 × g to yield platelet pellet.

Thereafter, the platelet pellet was gently resuspended in Ca^{+2}/Mg^{+2}- free modified Tyrod's Buffer (140 mM NaCl, 10 mM glucose, and 15 mM Tris/HCl, pH 7.4). Platelets were washed three times with the same buffer and visually examined by an invert microscope to ensure the absence of other blood cells. The platelets were then suspended in Ca^{+2}/Mg^{+2}- free modified Tyrode's Buffer at a final concentration of

3.9×10^8 platelets/ml [18]. Platelets were counted manually by using a Neubauer chamber.

Adhesion Assay: Adhesion assay was done according to Bellavite *et al*., (1994). Briefly, the 96-well microtiter plates were coated (overnight at 4°C) by adding 50 µl/well of human fibrinogen (2 mg/ml in phosphate buffer saline (PBS)). Wells were aspirated and washed twice with PBS. The nonspecific adhesions were blocked by incubation of wells with 200 µl of PBS containing 1% BSA (1 h at 37°C). Then the plates were washed three times with PBS, inverted, followed by gentle tapping over towel papers to remove the last residual droplets [19, 20].

The platelet adherence was evaluated by determining the acid phosphatase activity of the attached platelets [3]. The Platelets, incubated for 1 h with different concentrations of the crude extract (50, 100, 200 µg/ml at 37°C) or untreated (control), were activated by thrombin (0.25 U/ml). To achieve this, 50 µl of platelet suspension was added to each fibrinogen coated well and the plate was incubated at 37°C for 1 h without shaking to allow platelets to adhere. The microplates were then washed three times with PBS as mentioned above.

Subsequently, 140 µl of the substrate solution containing 1 mg/ml p-nitrophenyl phosphate in 100 mM sodium citrate/100 mM citric acid and 0.1% (w/v) Triton X-100 (pH 5.4), was added to each well. For the estimation of total platelet count, 50 µl of PRP was mixed with 140 µl of the substrate solution and for the blank 50 µl of the platelet-poor plasma (PPP) was used (20). Lysis of platelets was achieved by Triton X-100 without adverse effects on acid phosphatase activity (21). After incubation at RT for 60 min with gentle rocking motion, the reaction was stopped by addition 100 µl NaOH (2N). The reaction product, p-nitrophenole, was measured using a microplate reader (power wave X2, Bio Tak, USA) at 405 nm. The percentage of adherent cells was calculated on the basis of a standard graph constructed by using known number of platelets.

Animals Treatments: N-Mary rats, weighing 75-85 g, with bedding of wood shavings, were allowed free access to tap water ad libitum and fed standard rat chow. They were housed in cages at 20-24±1°C under 12 h/12 h light-darkness cycle. All procedures for animal experiments were in accordance with the animal ethics committee of university of Tehran. Animals were divided into 4 groups: group 1 (control, n=5) was daily oral fed with 1 ml of distilled water, group 2 (n=5), group 3 (n=5) and group 4 (n=5) received the dried samples of *A. dracunculus, P. granatum* and *B. vulgaris* at doses of 75 mg/kg rat body weight (RBW) dissolved in 1 ml distilled water, respectively, for 7 consecutive days. Sixteen hour after the last dose, venesection was performed from each rat into plastic tubes containing sodium citrate (3.8% w/v) in 9:1 (blood to anticoagulant) ratio for PT and aPTT assays.

Anticoagulatory Assay: Plasma was prepared from each blood sample following centrifugation for 10 min at 1,500 × g and stored at -40°C before use [22]. The plasma PT and aPTT were measured by laboratory experts using standard methods (Fisher Diagnostics, Middletown, USA).

Biochemical Analyses: The sera levels of total cholesterol, triglycerides, and low density lipoprotein cholesterol (LDL) were determined using enzymatic kits (Pars Azmoon, Tehran, Iran). Alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were assessed as a measure of hepatic cell damage using the corresponding commercial kits (Pars Azmoon, Tehran, Iran).

Statistical analysis: All values are expressed as mean ± SD. The significance of differences between the means of the tests and controls were calculated by unpaired Student's t-test, and p values less than 0.05 were considered significant.

RESULTS:

Platelet Adhesion Assay: In order to evaluate the inhibitory effect of each extract on the platelet adhesion property, the platelets were treated with each extract and the responses were recorded by determining the number of platelets attached to the well based on acid-phosphatase activity. As it is evident from **Fig. 1**, the adhesion have been decreased by almost 35%, 20% and 25% for the methanol extracts of *A. dracunculus*, *P. granatum* and *B. vulgaris*, respectively, at a concentration of 200 µg/ml.

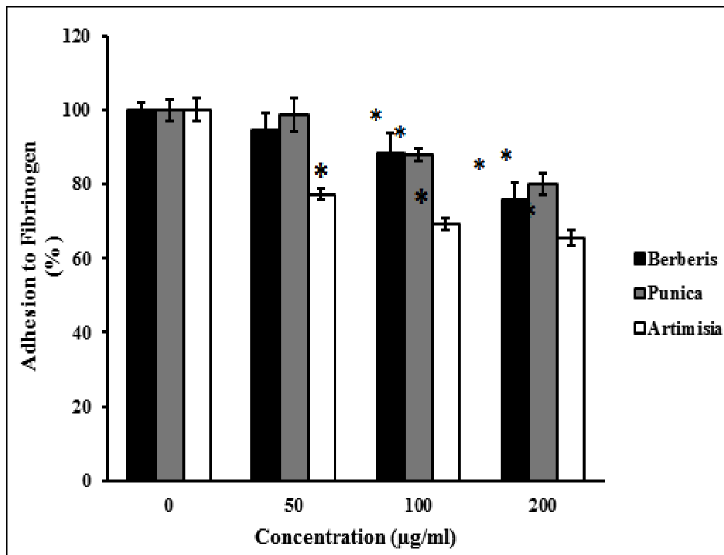
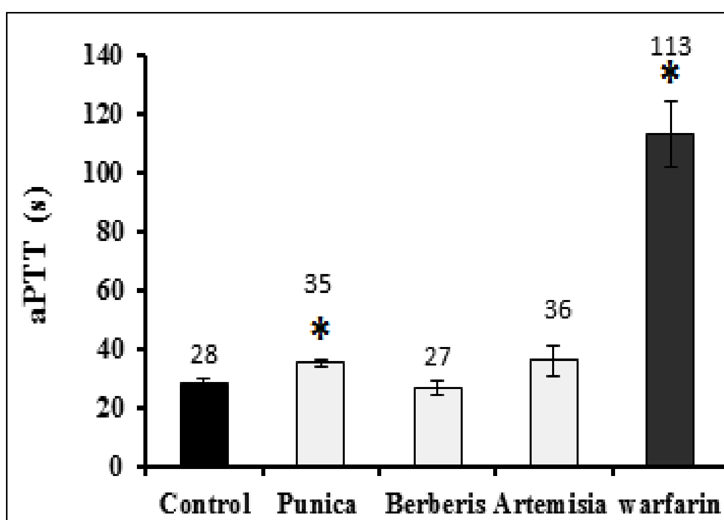


FIG. 1. EFFECT OF EACH CRUDE EXTRACT AT DIFFERENT CONCENTRATIONS (50-200 μg/ml) ON ADHESION OF THROMBIN ACTIVATED PLATELETS TO FIBRINOGEN-COATED WELLS. The extent of platelet adhesions to fibrinogen coated wells (%) was determined by measuring the absorption at 405 nm. Each value represent the mean ±SD (n=3). $^{*}P < 0.05$.

Anticoagulant activity: Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured and the following results were obtained for the drug-untreated control rats: 13 s for PT and 28 s for aPTT. However, after 7 days of drug treatment, at a dose of 75 mg/kg, the PT values were increased in rats treated with either the *A. dracunculus* or *P. granatum extracts*, however, aPTT value was only augmented by *P. granatum extract* relative to the control group (**Fig. 2**). At lower doses of each extract (25 and 50 mg/kg) PT and aPTT did not significantly change. The aPTT and PT values did not remarkably change in rats treated with *B. vulgaris*. The warfarin was used as the positive control.



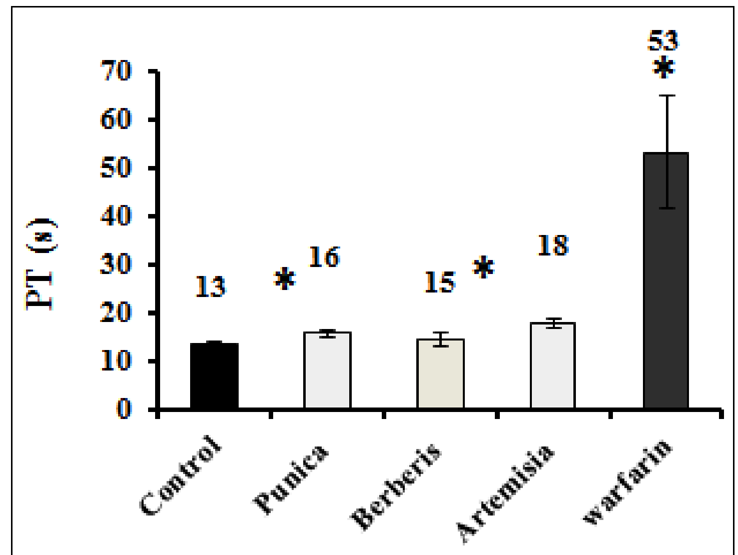
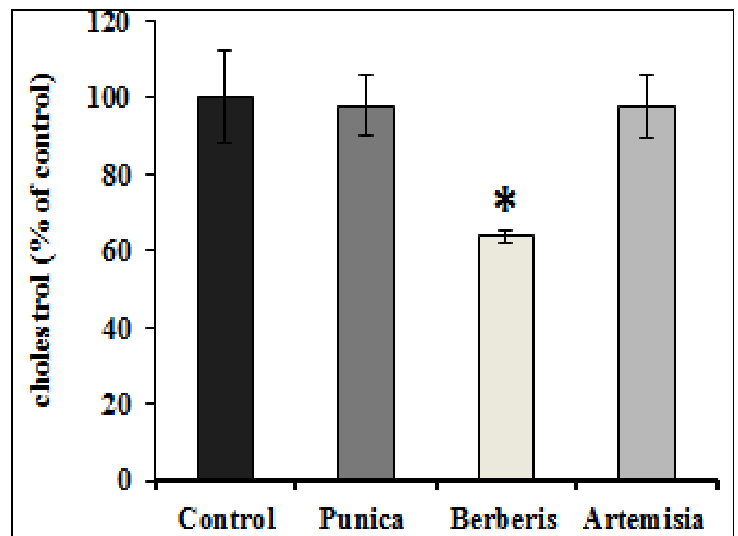
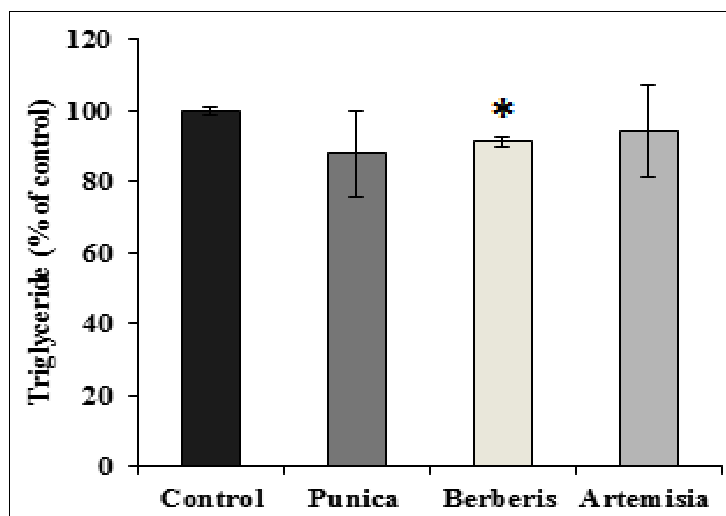


FIG. 2: EFFECTS OF EACH CRUDE EXTRACT AT 75 mg/kg b.w. ON RATS' BLOOD PT AND aPTT VALUES. Rats were fed 1 ml of one of each plant crude extract dissolved in distilled water or distilled water only (Control) for 7 consecutive days. Sixteen hours after the last dose, venesection was performed and then, PT and aPTT were measured. Each value represent the mean ±SD (n=5). $^{*}P < 0.05$.

Effects of plant extracts on Serum Lipoprotein Profile: In rats treated with *B. vulgaris* extract, the LDL and cholesterol concentration were dramatically reduced by 56% and 36%, respectively, relative to control group. The triglycerides sera levels didn't significantly change relative to the control (plant untreated) group (**Fig. 3**). In addition, the sera levels of ALT, AST, and ALP, as a measure of liver function under the influence of plant extracts, did not significantly vary compared to those of control group (data not shown).





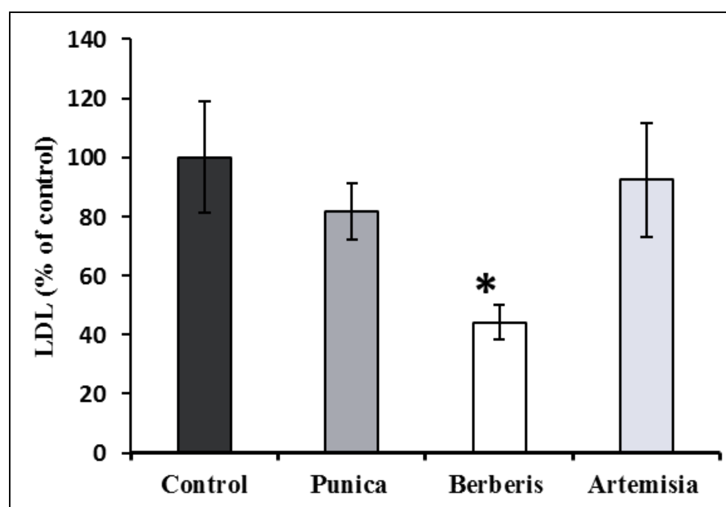


FIG. 3: EFFECTS OF EACH PLANT EXTRACT (75 mg/kg b.w) ON SERUM LIPOPROTEIN PROFILE. The rats have been treated with extract for 7 consecutive days at the indicated doses. Each value represents the mean ±SD (n=5) of triplicate measurements. LDL= low density lipoproteins. $^{*}P < 0.05$.

DISCUSSION: In the blood vessels, platelets play integral roles in formation of haemostatic plugs and thrombi formation at sites of vessel damage ([6]). Medical treatments of the platelet-based diseases relay mostly on natural plant derived and/or the semisynthetic compounds [23, 24]. Regular consumption of foods rich in phytochemicals such as allicin, polyphenols and anthocyanins have been considered responsible for the lower incidence of cardiovascular diseases [25, 26]. The presence of various phytochemicals such as flavonoids, alkamids, coumarins and isocoumarins in *A. dracunculus* [27]; anthocyanins, hydrolyzable tannins punicalagin, ellagic and gallic acid in *P. granatum* [16] and isoquinoline alkaloid, coumarin, carotenoid, phenylpropanoid and flavonoid in *B. vulgaris* have been reported [15].

Consequently, the antiplatelet and anticoagulant activities of these herbs might be attributed to their components especially flavonoids, Isoquinoline alkaloid and coumarins. Accordingly, several studies have shown that polyphenols, flavonoids and coumarins significantly inhibit platelet adhesion, aggregation and secretion [28]. It is believed that due to structural similarity to vitamin K, coumarins act as anticoagulant, impairing the cyclic interconversion of vitamin K and its 2, 3 epoxide (vitamin K epoxid) leading to inhibition of vitamin K dependent factors (VII, IX, X and prothrombin), the essential participants in the coagulation cascade [29].

In the present work, the anti-platelet activities of each of the methanolic crude extracts of *A. dracunculus, P. granatum* and *B. vulgaris*, on rat platelet adhesion to fibrinogen-coated plates were evaluated. Our data showed that treatment of the platelets with different concentration of each plant extract significantly reduced their attachment behavior to the fibrinogen coated plates (Fig. 1. a, b, c).

However, our results indicated that *A. dracunculus* was more active than *P. granatum* and *B. vulgaris*. Beside of this effect, *A. dracunculus* caused a significant increase in PT that can be attributed to various coumarin derivatives present in the crude extract of *A. dracunculus*. On the other hand, *B. vulgaris* significantly decreased (by almost 56% and 36%) bad lipids (LDL and cholesterol) contents which are believed to play major roles in thrombosis [30].

Our data showed that treatments of rats with either of the three plant crude extracts did not affect the sera levels of hepatic enzymes, indicating the absence of adverse effects on liver by the plant extracts under the experimental conditions used.

CONCLUSION: Our data clearly indicated the scientific basis behind traditional use of *A. dracunculus* and *P.granatum* extracts as anti-thrombotic agents. However, our data indicated that the cardiovascular beneficial effects of *B. vulgaris* might be due to its strong effects on attenuation of LDL level rather than on its interrupting role on vitamin K cascade. Further delicate work is required to establish the exact mode of action of *B vulgaris*.

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