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EFFECTS OF 7- METOXYCOUMARIN ON COAGULATION SYSTEM IN RATS

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ABSTRACT

Platelets play a vital role in haemostasis, development and progression of atherosclerosis and thrombosis. Platelets adhere to the injured vessel walls and cause platelets plug production that leads to blood clotting. In platelet hyperactivity, these processes continue till the generation of thrombosis. To attenuate these processes, flavonoid and coumarin groups of compounds, as anticoagulant agents, have been used. *Artemisia dracunculus* L (Asteraceae) has long been used as an anticoagulant medicinal plant in Iran. This herb possesses various compounds especially coumarins and flavonoids, which might account for its antiplatelet and anticoagulant activity. One of the *Artemisia dracunculus* L active compounds was characterized using ^{1}H NMR and GC-MS. Then, the antiplatelet and anticoagulant effects of this compound were investigated. In vitro data clearly demonstrated that the active compound, at 60 µg/ml, significantly reduced platelet adhesion by 38% compared to the control. In addition, at a dose of 20 mg/kg, prothrombin time (PT) and activated partial thromboplastin time (aPTT) were increased by almost 3 and 2 folds, respectively, compared to the rats of the control group. Our results clearly provide scientific basis for the traditional use of the plant besides of introducing a valuable anticoagulant agent for further pharmaceutical evaluations.

Keywords: Prothrombin Time, Platelet, 7-Metoxycoumarin, Fibrinogen

INTRODUCTION

Human platelets are small anucleated cells derived from megakaryocytes and typically alive for 10 days. These multifunctional cells are implicated in many pathological processes including thrombosis and haemostasis, inflammation including promotion of atherosclerosis and clot retraction **[1]**. Upon vessel wall damage, platelets endure a highly regulated set of functional responses comprising adhesion, spreading, activation, release reactions, aggregation and exposure of a procoagulant surface which function to occlude the site of damage to prevent blood loss **[2]**. Interaction of platelets with vessel wall is mediated by a series of membrane receptors and by adhesive proteins such as fibrinogen, collagen and laminin **[3]**. Platelet adherence to the vascular matrix including fibrinogen is occurred through GPIIb/IIIa receptor. These receptors are inactive on the surface of unstimulated platelets. However, platelet stimulation by agonist such as collagen and/or thrombin causes the relocation of the cytoplasmic membrane phospholipids to the plasma membrane **[4, 5]**. This transition of phospholipids probably is instrumental in providing an optimal surface for facilitating coagulant activation reaction leading to thrombin generation and subsequent fibrin deposition **[6]**. Thrombin, in turn, activates different factors that play important role in coagulation cascade **[7]**. Regarding these facts, inhibition of platelet functions would decrease thrombin, fibrin and eventually clot formation, with subsequent lower risk of coronary thrombosis.

Phytochemicals, mainly the phenolic compounds and coumarins of fruits and vegetables, have been proposed as the major bioactive compounds responsible for their health benefits **[8]**. Flavonoids are secondary plant phenolics found ubiquitously in fruits and vegetables as well as many medicinal plants **[9]**. It has been demonstrated that the intake of fruits and vegetable is associated with reduced risk of cardiovascular diseases. In addition, it has been shown that high flavonoid intake is associated with lower coronary heart mortality, lower incidence of myocardial infarction in older men and reduced risk of coronary heart disease in postmenopausal women **[10, 11]**. In modern medicine coumarins, such as warfarin and phenprocoumon, are prescribed for prevention of thromboembolism in patient with stroke risk, mechanical heart valves, susceptibility to venous thrombosis and anti-phospholipid syndrome **[12]**. These compounds act by suppressing the synthesis of functional vitamin K-dependent coagulation factors II,

VII, IX and X, and therefore generating a functional deficiency of these proteins **[13]**.

Artemisia dracunculus L (tarragon) is a small shrubby perennial herb belonging to Asteraceae family from which various compounds such as alkamid, coumarins **[14]** and isocoumarins **[15, 16**] have been isolated. Anticonvulsant and antiepileptic effects of *A. dracunculus* in Iranian folkloric medicine had been evaluated **[17]**. Due to the sedative nature of the plant, it has also been used as a sleep aid **[18]**. In addition, the extract of the plant's leaves have been evaluated for its effect(s) on lipid and coagulatory parameters in rats **[19]** and platelets functions **[20]**. In this study, the anticoagulant responsive constituent has been isolated and its structural and functional activities have been established, using both in vitro and in vivo model systems.

MATERIALS AND METHODS

Materials

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 purchased 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

The leaves powder of *A. dracunculus* was extracted four times with methanol and the accumulated crude extract was concentrated under reduced pressure. The residue was then purified by several consecutive preparative silica gel TLC plates using chloroform / ether (7:3) as the developing solvent. Five major bands were observed, under UV light, on the plates. The bands were scratched and extracted with chloroform four times. Each accumulated extract was then concentrated under reduced pressure at room temperature. Each purified compound was then subjected to antiplatelet activity assay. The compound with highest activity was chosen for structural elucidation and further biological evaluations. The structure of the pure compound was established using spectroscopy techniques including ^{1}H NMR, and GS-MS.

Animals Treatments

N-Mary rats, weighing 75-85 g, were housed in cages at 20-24±1°C under 12 h/12 h light-darkness cycle, with bedding of wood shavings and were allowed free access to tap water ad libitum and fed standard rat chow. All procedures for animal experiments were in accordance with the animal ethics committee of university of Tehran. Animals

were divided into 3 groups: group 1 (control, n=5) was daily fed orally 1 ml of distilled water, group 2 (n=5) and 3 (n=5) received the active compound at doses of 10 and 20 mg/kg rat body weight (RBW) dissolved in 1 ml distilled water, for 7 consecutive days. Sixteen hours after the last dose, we performed venesection from each rat into plastic tubes containing sodium citrate (3.8% w/v) in 9:1 (blood to anticoagulant) ratio for PT and a PTT assays.

Isolation of blood platelet

Each blood sample was collected into 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) was obtained by centrifugation (200 × g for 15 min at room temperature). The yielding PRP was again centrifuged for 20 min at 800 × g to get 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 **[2]**. Platelets were counted manually using a Neubauer chamber.

Adhesion Assay

The 96-well microtiter plates were coated (overnight at 4°C) with 50 µl/well of human fibrinogen (2 mg/ml in phosphate buffer saline (PBS)). Wells were aspirated and washed twice with PBS and 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 200 µl of PBS, inverted and followed by gentle tapping to remove the last residual droplets **[4, 21]**. The platelet adherence was evaluated by determining the acid phosphatase activity of the attached platelets **[22]**. The Platelets, treated for 1 h with different concentrations of the active compound (20, 40, 60 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 200 µl of 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]**. Triton X-100 caused instantaneous lysis of platelet and did not affect acid phosphatase activity. After incubation at room temperature for 60 min with gentle rocking motion, the reaction was stopped by addition of 100 µl of 2N NaOH. 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.

Anticoagulatory Assay

Plasma was prepared from blood samples of each group (n = 5) following centrifugation for 10 min at 1,500 × g and stored at -40°C before use. 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, high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL) of each group (n = 5) 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.

REZULTS

Structure Elucidation of the Pure Compound

The purity of the isolated compound from the crude extract of *A. dracunculus* was established by GC-MS. The structure elucidation was achieved based on ^{1}H NMR, electron ionization mass spectrometry (EI-MS) and comparison to the literature data. The ^{1}H NMR spectrum showed the following peaks = a singlet at δ 3.90 for OCH_3, two doublets at C 6.25 and δ 7.65 with J = 9 Hz for H-3 and H-4, respectively, a doublet at δ 7.34 for H-5 with J = 8.2 Hz, a doublet of doublet at δ 6.9 with J = 8.2, and J = 2.3 Hz for H-6 (for the *ortho* coupling with H-5 and *meta* coupling with H-8). A doublet at δ 6.85 with J = 2.5 Hz for H-8. The major peaks in MS spectrum were m/z 176 (C_{10} H_8 O_3),

(100), the base peak; m/z 148 (68), 133 (68), 105 (10), 77 (17), 63 (12) and 51 (16). The ^{1}H NMR and MS data were in accordance to the literature data [23].

Construction of Acid Phosphatase Calibration Graph

A set of experiments were arranged to establish the optimal condition for the measurement of platelet number based on acid phosphatase activity. The blank's absorbance was low (0.09–0.11 O.D. units) at 405 nm, while in the presence of increasing numbers of platelets the assay procedure gave net absorbance value in the range of 0.2–1.02 OD units. Based on our data, a linear relationship between the OD values at 405 nm and the number of platelets was obtained as shown in **Figure 1**.

Platelet Adhesion Assay

In order to evaluate the inhibitory effect of active compound on the adhesion property, the platelets were pretreated with the purified compound and the responses were inspected by an invert microscope. According to **Figure 2**, the active compound, at 60 µg/ml, significantly reduced the adherence of the drug-treated thrombin activated platelets to the coated plates relative to the control drug-untreated thrombin activated platelets. This observation was further confirmed by determining the number of platelets attached to the well based on acid-phosphatase activity. As shown in **Figure 3**, the active compound, at 60 µg/ml, reduced the number of attached platelets by almost 38% relative to the drug-untreated control platelets.

Anticoagulant Activity

Prothrombin time (PT) and activated partial thromboplastin time (aPTT), which evaluate tissue factor pathway (extrinsic factors) and contact activation pathway (intrinsic factors), respectively, were measured among the drug-treated and un-treated rats and the following results were obtained for the drug-untreated control rats: 12.6 s for PT and 32 s for aPTT. However, after 7 days of drug treatment, at a dose of 20 mg/kg, the PT and aPTT values were increased by almost 3 and 2 folds, relative to the control group **(Figure 4)**, respectively. Treatment of rats with a dose of 10 mg/kg did not induce any change in PT and aPTT values (data not shown). On the other hand, higher than 25 mg/kg doses, were toxic to rats.

Effect of Active Compound on Serum Lipoprotein Profile and Hepatic Enzymes

As it is evident from **Figure 5a**, feeding the drug to rats has led to a 34% reduction in sera level of LDL. However, significant changes in the sera levels of triglyceride, HDL and cholesterol were not observed relative to the drug-untreated control group **(Figure 5a)**.

Regarding the results presented in **Figure 5b**, it could be concluded that the active compound is devoid of any significant hepatotoxicity at the dose level (20 mg/kg) used.

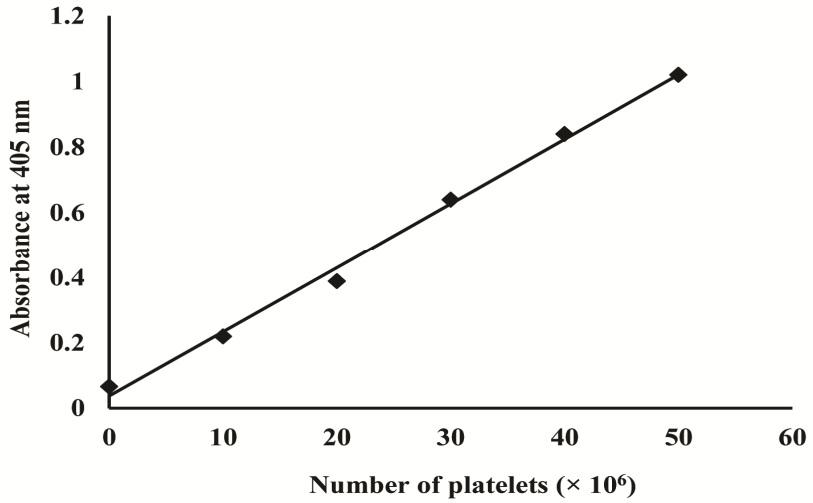

Figure 1: Calibration Graph Linking Absorbance at 405 nm to the Number of Platelets. Different Number of Platelets Were Dispensed in Uncoated Wells Then Incubated With a Solution Containing Acid Phosphatase Substrate, p-Nitrophenyl Phosphate, and T-X100 as the Detergent. Changes in Absorbance Were Recorded at 405 nm. Absorbance Values are the Average of Triplicate Determinations (R^2=0.9953)

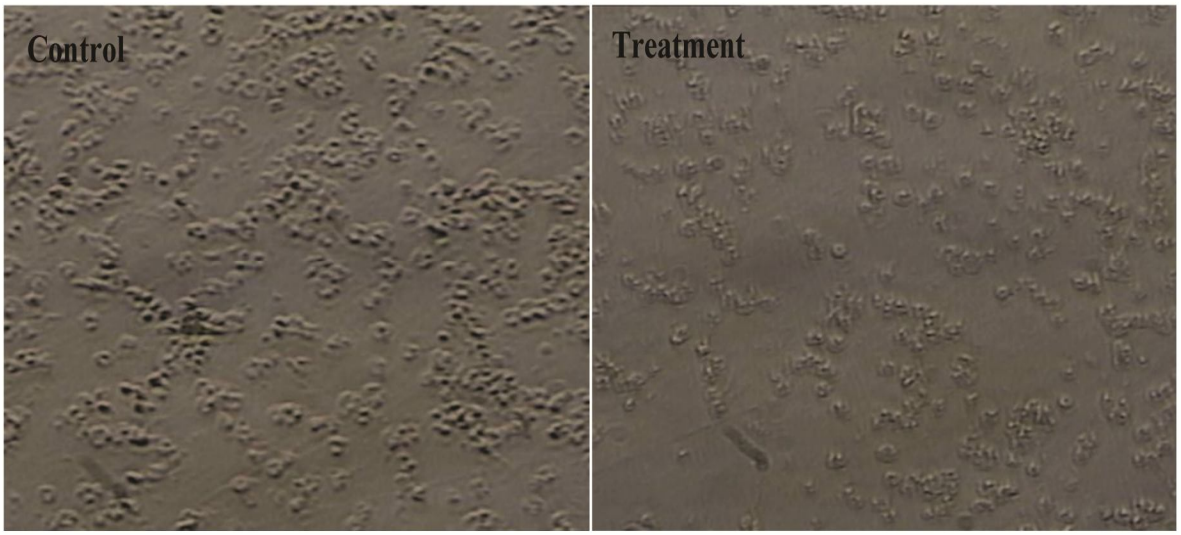

Figure 2: Effect of Active Purified Compound on Platelet Adhesion to the Fibrinogen Coated Wells. Platelets were Treated With 60 µg/ml of Active Compound for 1 h Followed by Activation With Thrombin for 30 Min; Platelets in the Absence (Control) or in the Presence of the Compound (Treatment) Were Examined with an Invert Microscope

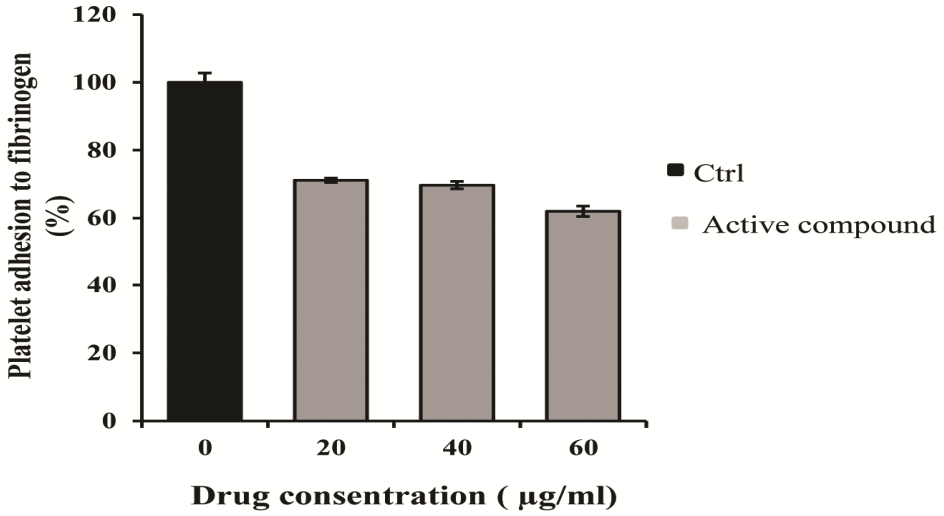

Figure 3: Effect of Different Concentration of the active Compound on Platelet Adhesion. Blood Platelets Were Incubated with Different Concentrations of Active Compound and Activated With Thrombin. After Incubation With Substrate for 1 h, Reaction Was Stopped and the Extent of Platelet Adhesion (%) was Evaluated by Measuring Absorbance at 405 nm. Each Value Represent the Mean±SD (n=3). Values are Statistically Different From Control (P<0.05)

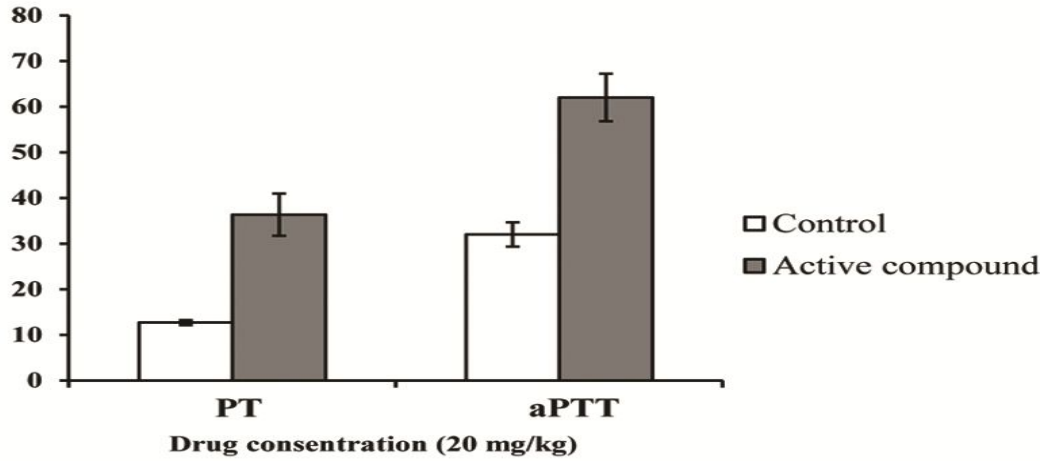

Figure 4: Effects of Active Compound on PT and aPTT in the Rat System. Rats Were Fed 1 ml Active Compound (1.6 mg/ml in Distilled Water) or distilled Water (Control) for 7 Days Consecutively. Sixteen Hours After the Latest Dose, Venesection was Performed from each Group (n = 5) and Then, PT and aPTT Were measured. Each Value Represent the Mean±SD (n=5). Values are Statistically Different From Control (P<0.05)

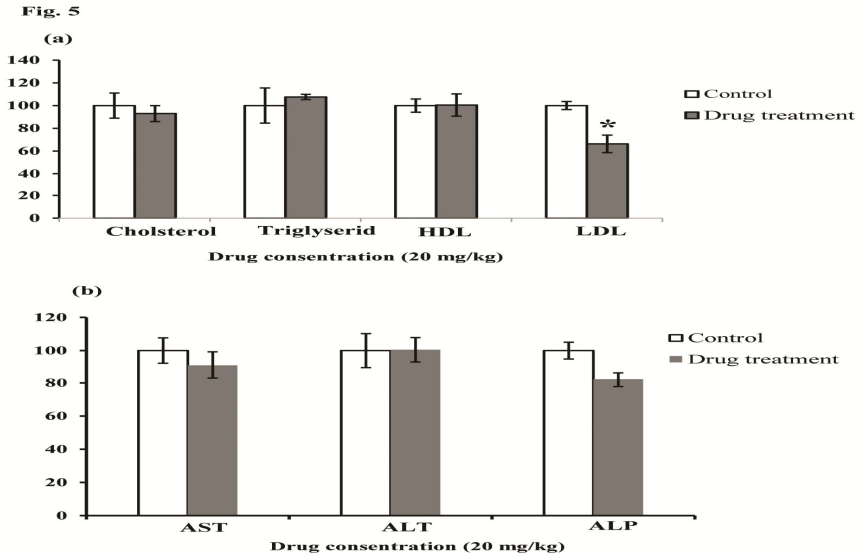

Figure 5: Effects of Active Compound on Biochemical Factors. After Seven Days of Consecutive Drug Feeding (20 mg/kg b.w.), Each Rat (n = 5) blood Sample Was Collected and Serum Was Separated. Then the Serum Lipoprotein Profile (a) and Hepatic Enzymes (b) were Measured. Results Shown as Mean ± SD (n = 5) of Triplicate Measurements of Each sample. *(P<0.05). HDL = high density Lipoproteins; LDL = Low Density Lipoproteins; ALP = Alkaline Phosphatase; ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase

DISCUSSION

Platelets play a central role in coagulation and thus have crucial effect on the body's first line of defense (haemostasis) against uncontrolled hemorrhage **[24]**. Excessive platelet activation, as occurs in various diseases and/or conditions, can lead to thrombosis, whereas insufficient platelet function can cause bleeding. Thus, evaluation of platelet function is important for estimating the risk of bleeding or thrombosis under numerous conditions **[25]**. Medical treatments of the platelet-based diseases rely mostly on natural plant derived as the semisynthetic compounds **[26]**. So far, many phytochemicals with anti platelet and cardioprotective effects including flavonoids, polyphenols, anthocyanins, isoflavonons, isothiocyanates, organosulfur and terpenoids have been evaluated **[27, 28]**. On the other hand, regular consumption of foods rich in phytochemicals such as allicin, polyphenols and anthocyanins have been considered responsible for the lower incidence of cardiovascular diseases **[29, 30]**. Coumarins are present in significant amounts among compositae family members with vast therapeutic potentials **[31]**. The presence of various phytochemicals such as flavonoids, alkamids, coumarins and isocoumarins have been reported in *A. dracunculus* which might account for its well-known antiplatelet, anti-hypercholesterolemia, antiepileptic, anticonvulsive and antidiabetic activities of *A.*

dracunculus **[17, 21]**. The antiplatelet and anticoagulant activities of this herb might be attributed to its various flavonoids, coumarins and isocoumarins constituents. It is believed that coumarins inhibit platelet function via different mechanisms including: scavenging reactive oxygen species, inhibiting cyclic nucleotide phosphodisterases and prostaglandin syntheses **[32]**. On the other hand, because of the structural similarity to vitamin K, coumarins act as anticoagulant, interfering with the cyclic interconversion of vitamin K and its 2, 3 epoxide (vitamin K epoxid). This will lead to inhibition of vitamin K-dependent factors (VII, IX, X and prothrombin) posttranslational carboxylation at glutamate residues on the N-terminal region of these factors which is an essential step in the coagulation cascade **[13]**. In addition atherogenic lipoprotein (LDL), which plays crucial roles in initiation and progression of atherosclerosis and thrombosis **[33]**, was diminished by the 7-metoxycoumarin. This might lead to a decline in thrombus formation by unidentified mechanism(s).

CONCLUSION

Our results indicated that the anticogulating activity of the purified compound was associated with a significant reduction on the adherence properties of the thrombin activated platelets. This response might account for augmentation of PT and aPTT values of the drug-treated rats. Furthermore and based on the sera levels of the hepatic enzymes, it might be concluded that the plant extract is devoid of hepatic cytotoxicity among the experimental rats Overall, the observed pharmacological activities might candidate the purified compound as a new anticoagulant agent pending further pharmaceutical evaluation.

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