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THROMBOLYTIC POTENTIAL OF *PUNICA GRANATUM* – A STUDY IN THE RAT MODEL

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Punica Granatum; Thrombolysis, Rat Model, Thrombotic Factors

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ABSTRACT: Although the antioxidant and thrombolytic potential of the "super fruit" pomegranate has been well established and documented in *in vitro* models, its efficacy in an animal model has not been recognised. Therefore in the present study, the aril and rind of *Punica granatum* were scrutinized for their role in clot lysing competence in a thrombus induced rat model. Visual manifestation, coagulation time parameters and the level of the thrombotic factors confirmed the thrombolysis. The extracts were compared with the standard drug streptokinase that was administered in rats.

INTRODUCTION: Cardiovascular diseases (CVD) remain the grim reaper's primary calling card. CVDs and other related disorders are the major cause of death in the populace all over the world [1]. Mortality and morbidity from CVDs, both coronary heart disease (CHD) and stroke, have declined in most high income countries and presently the largest burden has shifted to middle and low income countries [2]. Vitamin K antagonists were the mere choice for long-term anticoagulation until recent times. Novel oral anticoagulants have reduced medication and food interactions compared to warfarin and they eliminate the need for frequent monitoring [3]. All available thrombolytic drugs still have considerable limitations. Hence, a quest for an alternative reliable source remains the need of the hour.

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Previous studies in our laboratory have proved the *in vitro* clot lysing ability of the fruit pomegranate. So, in an attempt to study the *in vivo* clot lysing potency of the fruit, carrageenan induced rat tail model was chosen. Advantages of the carrageenan induced thrombosis model in rats and mice are: (i) simple induction in small laboratory animals, (ii) easy observation and quantification all the time without killing the animals [4]. Another advantage is that it allows easy evaluation of drug efficacy by comparing the length of the infarct region in tail of rats.

MATERIALS AND METHODS:

Collection and preparation of extracts:

The fruit sample was collected from the Coimbatore city and certified by the Botanical Survey of India, Coimbatore. The voucher specimen was collected and maintained. The aril and rind of fresh fruits were collected, washed and homogenized using distilled water for the preparation of aqueous extract. It was then filtered using Whatmann No 1 filter paper and used for further study.

Evaluation of *in vivo* clot lysis in rat models:
In vivo studies were carried out by the oral administration of aril and rind of *Punica granatum* to examine the clot lysing ability and antioxidative effect to the experimental rats.

Maintenance of experimental animals:
Male Swiss Albino rats were procured from Bangalore and were housed in microloan boxes in a controlled hygienic environment at temperature 25 ± 2°C and 12 hr dark/light cycle. The study was conducted after obtaining institutional animal ethical committee's clearance (IAEC No: KMCRET / Ph.D / 12/ 2014 – 15). As per the standard practice, the rats were segregated and quarantined for 15 days before the commencement of the experiment. They were fed on standard healthy diet and water *ad libitium.* The acute toxicity studies carried out confirmed that the extracts are not toxic even at the concentration of 5000 mg/kg.

Grouping of animals:
The rats were divided into five groups with six rats in each. The groupings are as follows:

Groups	Description	Extract / Drug Dose
I	Control	-
II	κ – carrageenan only	-
III	κ – carrageenan + Streptokinase	30,000 IU
IV	κ – carrageenan + PgAA	250 mg/kg
V	κ – carrageenan + PgRA	250 mg/kg

PgAA – *P. granatum* Aril Aqueous ; PgRA - *P. granatum* Rind Aqueous

Thrombus induction:
Experimental rats were anaesthetized with i.p injection of ketamine hydrochloride (100 mg/kg). To attain effective clot formation κ – carrageenan (1 mg/kg) dissolved in saline was injected into the rat tail vein at a site 12 cm from the tip of the tail with a ligation. After a period of 10 minutes, the ligature was removed. The length of the infarct was monitored for thrombus formation. Once thrombus was formed, the animals were treated with respective extracts and monitored for the reduction in the length of the thrombus in rat tail for 30 days.
At the end of the study, the rats were sacrificed after an overnight fasting. The blood of the animals was collected by heart puncture and the serum separated was used for the estimation of haematological parameters associated with thrombolysis.

Evaluation of the thrombotic parameters:
The plasma samples were analysed for the D-dimer content as described by Marder and Francis [5], for the fibrinogen by coagulometric method, tPA as described in Roche. The serum samples were analysed for the CPK content [6] and C - reactive protein [7].

Estimation of bleeding time and clotting time:
At the end of the period, bleeding time was determined for each animal using Duke's method while clotting time was determined by Ivy's method as reported by Ibu and Adeniyi [8]. For bleeding time, the tip of the tail of each rat was cut to cause bleeding. A stopwatch was started as soon as animal began to bleed. A blotting paper was used to wipe off blood every 15 seconds. As soon as bleeding ceased the stopwatch was stopped and the time recorded as bleeding time for that particular animal. For the clotting time, a drop of blood from the tail of each rat was placed on a clean glass slide and a stopwatch was started at the same time. A pin was passed across the drop of blood once every 15 seconds. As soon as threads of fibrin were noticed, the stopwatch was stopped and the time recorded as the clotting time for that particular rat.

Determination of euglobulin clot lysis time [9]:
Venous blood was collected into chilled tubes containing trisodium citrate as an anticoagulant and placed on ice. The sample was then centrifuged at 4°C and the plasma sample was collected, diluted with acetic acid and incubated on ice for 15 minutes. A precipitate was formed (the euglobulin fraction of plasma) which contains plasminogen, plasminogen activators (primarily t-PA) and fibrinogen. The supernatant was collected by centrifugation in refrigerated centrifuge at 4°C.

The supernatant was discarded and the precipitate was dissolved in buffer. This was then clotted with thrombin and the time to clot lysis was determined by inspection every 15 minutes. A control plasma sample collected at the same time must be run in parallel.

Determination of Activated partial thromboplastin time [10]:

Blood samples were collected from anaesthetized rat into a 1ml-microfuge tubes containing an anticoagulant sodium citrate solution (3.8%) in (9:1) ratio. The citrated blood samples were centrifuged at 1500g for 15 min at room temperature. Supernatant plasma was carefully transferred to tube for estimation by turbidity method. Fibrin formation results in an increase in turbidity, which is detected by photometer at 470 nm. 25µl plasma and 25µl charged phospholipids (kaolin) was added and incubated exactly for 5 minutes. 25µl prewarmed calcium chloride was added and the result was displayed in seconds.

RESULTS AND DISCUSSION

Visual manifestation of thrombus:

The efficacy of κ – carrageenan in thrombus induction was evaluated by comparing the infarct length of the control group with that of the induced one. The length of the infarct was significantly reduced when administered with both streptokinase and fruit extracts. Results of the infarct length before and after lysis are recorded in **Table 1** and **Fig.1**. Photographs of the infarcted tails of rats induced with κ – carrageenan and those treated with pomegranate extracts and standards are given in **Fig. 2**.

TABLE 1: INFARCT LENGTH FORMED AND LYSED BY *PUNICA GRANATUM* EXTRACTS

Groups	Treatments	Tail length (cm)	Infarct tail length (cm)	
			Before lysis	After lysis
I	Control	15.0	NA	NA
II	κ – Carrageenan only	15.5	15.1	NA
III	κ - Carrageenan + Streptokinase	15.5	15.0	3.5
IV	κ – Carrageenan + PgRA	15.0	14.3	12.0
V	κ – Carrageenan + PgAA	15.0	14.5	5.0

PgAA – *Punica granatum* Aril Aqueous PgRA – *Punica granatum* Rind Aqueous

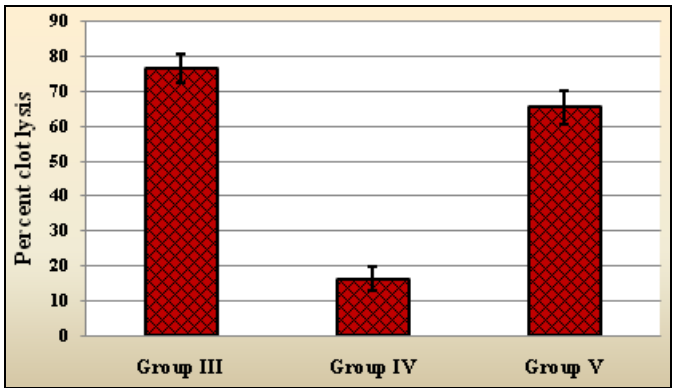
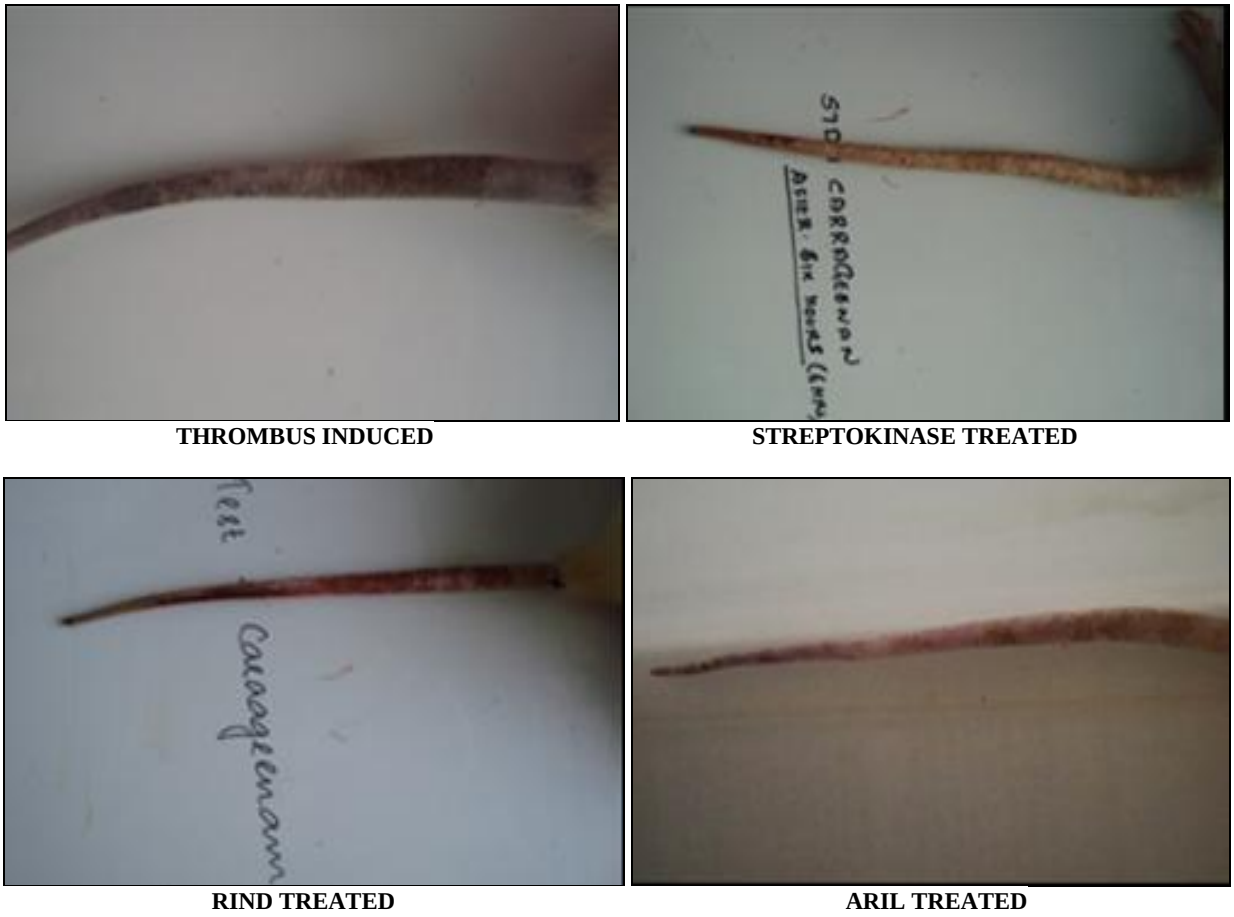


FIG. 1: PERCENT CLOT LYSIS BY *PUNICA GRANATUM* EXTRACTS IN EXPERIMENTAL RATS

Group III – Streptokinase treated Group IV – Rind treated Group V – Aril treated

CONTROL



THROMBUS INDUCED **STREPTOKINASE TREATED**

RIND TREATED **ARIL TREATED**

FIG. 2: VISUAL EVIDENCE FOR *IN VIVO* THROMBOLYSIS

Rats with tail length more than 15 cm were chosen for the study. Thrombus formation was induced with a frequency of 100% by ligating the tail for a period of 10 minutes and thereby altering the normal blood flow. Hagimori *et al*. [11] have reported that 1mg/kg kappa-carrageenan injection in combination with 10 minutes ligation at the tail increased the frequency of thrombosis to almost 100% in rats. Carrageenan induced thrombosis model was used because of its advantages of simple induction and easy to observe nature. It is widely used as a model in peripheral obstructive diseases[12]. A distinct clot formation was observed in rats when injected with κ – carrageenan which was then lysed with streptokinase and fruit extracts. The mean length of the infarct was found to be significantly shorter in rats administered with the standard (streptokinase) and fruit extracts, after lysis when compared with the infarct length formed before lysis. Effect of the aqueous extract of aril of *Punica granatum* was highly comparable with the streptokinase. The rind of pomegranate showed least clot lysis when compared to all the other extracts.

Coagulation time parameters:

Clot formation and subsequent lysis was found to profusely affect bleeding time, clotting time, activated partial thromboplastin time and euglobulin clot lysis time. Hence the rats in different groups were analysed for the above mentioned time parameters and the results are as recorded in **Table 2** and **Fig. 3** and **4**.

The bleeding time, clotting time and APTT were found to decrease on the onset of thrombus formation and increased during thrombolysis. Activated partial thromboplastin time is a global marker indicative of changes in the intrinsic and common pathway of thrombus formation. Achneck *et al*. [13] affirmed that lower APTT is associated with high levels of certain coagulation factors and corresponds to a procoagulant tendency. A marked increase in the bleeding and clotting time was observed in κ- carrageenan induced rats when

administered with *Douchi* fibrinolytic enzyme from *Bacillus subtilis* [12]. Similar pattern was found in the present study when the thrombus induced rats were treated with streptokinase and pomegranate extracts. Euglobulin clot lysis time (ECLT) measures the overall fibrinolytic pathway. Shortened euglobulin clot lysis time marks an increased fibrinolytic pathway and hence an increased ECLT in streptokinase and extract treated groups is substantiated.

TABLE 2: COAGULATION TIME PARAMETERS IN EXPERIMENTAL RATS

Groups	Treatments	Bleeding time	Clotting time	APTT (s)	ECLT (min)
I	Control	8 min 25 s	4 min 8 s	40	95
II	κ – Carrageenan	7 min 5 s	3 min 28 s	28	405
III	κ - Carrageenan + Streptokinase	18 min 35 s	5 min 37 s	54	65
IV	κ – Carrageenan + PgRA	9 min 35 s	4 min 50 s	48	90
V	κ – Carrageenan + PgAA	12 min 22 s	5 min 25 s	47	75
	Normal values	3 to 10 min	2 to 5 min	30 – 50	90 - 240

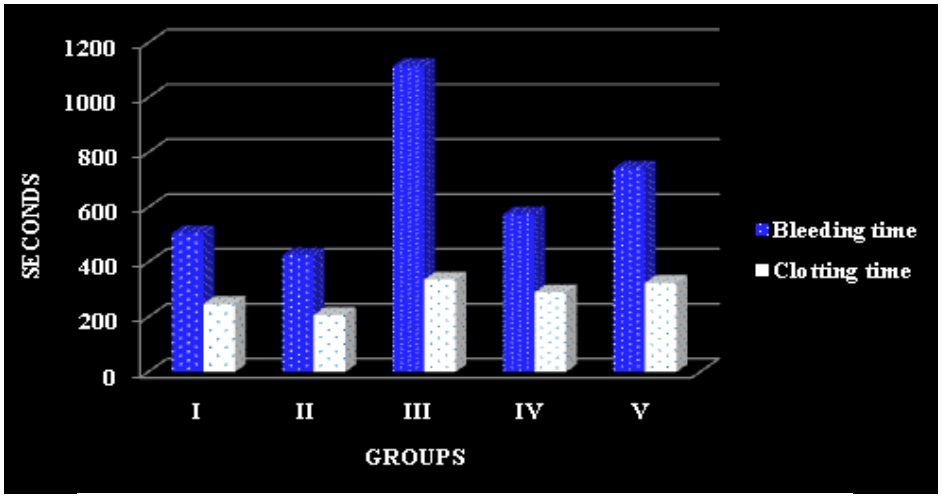


FIG. 3: BLEEDING AND CLOTTING TIME IN EXPERIMENTAL RATS

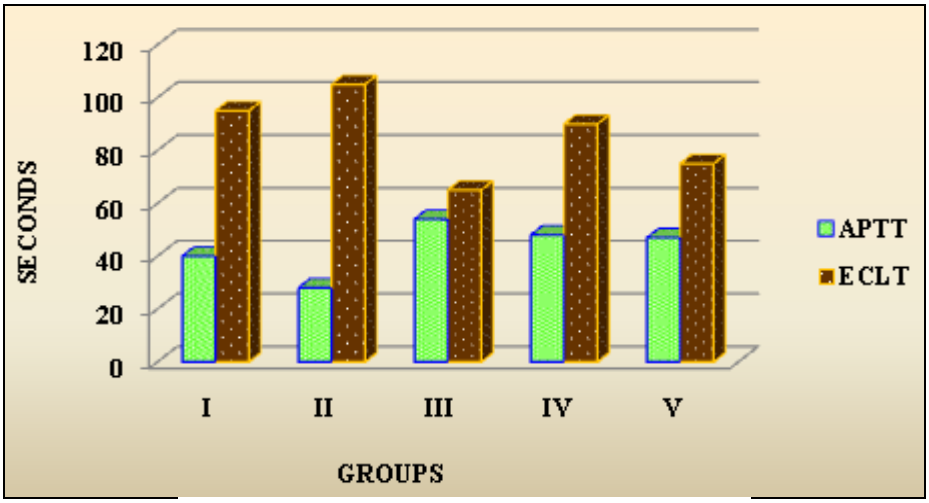


FIG.4: APTT AND ECLT IN EXPERIMENTAL RATS

ECLT – minutes APTT – seconds

Thrombotic parameters:

Laboratory tests of blood coagulation and fibrinolytic processes could potentially be used with thrombolytic therapy to detect and monitor the presence of a plasma proteolytic state; to predict bleeding complications, reperfusion and reocclusion. Hence coagulation parameters namely D – dimer, fibrinogen, hs – CRP (high sensitive C - reactive protein), tPA (tissue plasminogen activator) and CPK (Creatine phosphokinase) were analysed and the results are as tabulated in **Table 3**.

TABLE 3: LEVEL OF THROMBOTIC FACTORS IN EXPERIMENTAL RATS

Groups	Treatments	D- dimer (ng/ml)	Fibrinogen (mg/dl)	hs-CRP (mg/l)	tPA (ng/ml)	CPK (U/l)
I	Control	239.5 ± 41.75	342 ± 53.85	0.90 ± 0.04	13.1 ± 4.1	79.54 ± 9.3
II	Thrombus induced	583.4 ± 71.60[a]	602 ± 81.50[a]	3.60 ± 0.09[a]	17.6 ± 1.7[a]	109.82 ± 6.8[a]
III	Streptokinase Treated	841.3 ± 57.5[a]	925 ± 92.46[ab]	1.97 ± 0.12[ab]	28.0 ± 2.5[ab]	349.86 ± 18.5[ab]
IV	PgRA Treated	590.8 ± 47.6[ab]	630 ± 64.50[a]	2.90 ± 0.35[ac]	14.2 ± 2.6[abc]	168.10 ± 7.2[abc]
V	PgAA Treated	751.5 ± 82.9[abc]	834 ± 32.13[abc]	2.25 ± 0.80[ab]	25.6 ± 3.2[abcd]	278.20 ± 10.4[abcd]
Normal values		< 500 – negative > 500 – positive	150 – 400	< 1.0 – Low risk 1.0 > and < 3.0 – Moderate risk >3.0 - High risk	1-15	128

PgRA – *Punica granatum* rind PgAA – *Punica granatum* aril

The levels of D-dimer, fibrinogen, creatine phosphokinase and hs-CRP were slightly increased when thrombus was formed which peaked during the treatment with standard drugs and fruit extracts. Rats treated with streptokinase exhibited maximum d-dimer, fibrinogen and hs-CRP generation. Many studies have been reported on the generation of D-dimer during infarction treatment. Bäumer *et al*. [14] demonstrated that D-dimer generation increased when treated with tPA, a thrombolytic agent.

Lelakowski *et al*. [15] showed that thrombotic factors such as D-dimer, fibrinogen and hs-CRP levels increased on the incidence of venous thromboembolism and after pacemaker implantation. The D-dimer level in patients with deep vein thrombosis was higher when compared with patients without DVT [16]. Knipper *et al*. [17] found that the increased CPK level provided an indication of effective thrombolysis.

tPA is the primary initiator of thrombolysis wherein it plays an important role in removing the thrombus formed. Our study reveals that the level of tPA is greatly increased on the commencement of thrombolysis by fruit extracts and standards. The visual evidence in addition to the increased tPA levels proves the efficiency of *Punica granatum* in lysing the clot. The fruit extracts might have triggered the production of tPA apart from directly lysing the clot and hence the increase in plasma tPA levels. This also renders a possibility of decreased levels of PAI-1 as tPA is known to inhibit the inhibitor of thrombolysis.

CONCLUSION: With a plethora of shortcomings and negativity of the available thrombolytic therapies, these modest findings of the present study gain significance in employing the fruit *Punica granatum* for efficient clot lysis with further insight into the mechanism of action.

REFERENCES:

1. Aja PM, Ibekwe VI, Ekpono EU and Okechukwu UPC: Effect of ethanol extract of Cajanus cajan leaf on plasma lipid level in albino rats. International Journal of Current Research and Academic Review 2015; 3(1): 161-167.
2. Gupta R: CSI at global forum on cardiovascular disease prevention in clinical practice at ESC-EuroPrevent 2013. Indian Heart Journal 2014; 65: 639-642.
3. Raja AS and Geyer B: Emergency department management of patients on novel oral anticoagulant agents. Emergency Medicine Practice 2013: 15(10): 1-18.
4. Kishore K: In vitro and in vivo screening methods for antithrombotic agents. American Journal of Phytomedicine and Clinical Therapeutics 2013; 1(5): 497-506.
5. Marder VJ and Francis CW: Plasmin degradation of cross-linked fibrin. Annals of the New York Academy of Sciences 1983; 408: 397-406.
6. Lott JA and Stang JM: Serum enzymes and isoenzymes in the diagnosis and differential diagnosis of myocardial ischemia and necrosis. Clinical Chemistry 1980; 26: 1241-1250.
7. Wadsworth C: A rapid spot immunoprecipitate assay method applied to quantitative C reactive protein in pediatric sera. Scandinavian Journal of Immunology 1977; 6:1263-1273.
8. Ibu JO and Adeniyi KO: A Manual of Practical Physiology, Published by Jos University Press, Jos. 1989; 126.
9. Nordby E, Arnesen H, Andersen P and Godal HC: The euglobulin clot lysis time: A rapid and sensitive method for the assay of fibrinolytic activity after venous stasis. Scandinavian Journal of Haematology 1980; 25: 407-411.
10. Nagashima M, Yin ZF, Zhao L, White K, Zhu Y, Lasky N, Halks-Miller M, Broze GJ Jr, Fay WP and Morser J: Thrombin-activatable fibrinolysis inhibitor (TAFI) deficiency is compatible with murine life. Journal of Clinical Investigation 2002; 109: 101–110.

11. Hagimori M, Kamiya S, Yamaguchi Y and Arakawa M: Improving frequency of thrombosis by altering blood flow in the carrageenan-induced rat tail thrombosis model. Pharmacological Research 2009; 60(4): 320-323.
12. Yuan J, Yang J, Zhuang Z, Yang Y, Lin L and Wang S: Thrombolytic effects of douchi fibrinolytic enzyme from Bacillus subtilis LD-8547 in vitro and in vivo. BMC Biotechnology 2012; 12(36): 9 pages.
13. Achneck HE, Sileshi, B, Parikh A, Milano CA, Welsby IJ and Lawson JH: Pathophysiology of bleeding and clotting in the cardiac surgery patient. Contemporary Reviews in Cardiovascular Medicine 2010; 122: 2068-2077.
14. Bäumer W, Herrling, GM and Feige K: Pharmacokinetics and thrombolytic effects of the recombinant tissue-type plasminogen activator in horses. BMC Veterinary Research 2013; 9(158): 6 pages.
15. Lelakowski J, Domagata TB, Ryddlewska A, Januszek R, Kotula – Horowitz K, Majewski J, Ząbek A and Malecka B: Relationship between changes in selected thrombotic and inflammatory factors, echocardiographic parameters and the incidence of venous thrombosis after pacemaker implantation based on our own observations. Archives of Medical Science 2012; 8(6): 1027-1034.
16. Nata S, Hiromatsu S, Shintani Y, Tomokazu O, Akashi H and Tanaka H: D-dimer value more than 3.6 µg/ml is highly possible existence deep vein thrombosis. Kurume Medical Journal 2013; 60(2): 1-5.
17. Knipper P, Maladry D and Mitz V: In situ thrombolysis. Correlation between creatine phosphokinase increase and local thrombolysis. An experimental study in the rat. Annales de Chirurgie Plastique Esthétique 1996; 41(4): 374-375.

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