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Original Research Article

EFFECT OF COMBINATION DOSES OF CITRUS LIMON AND PUNICA GRANATUM JUICE ON BLOOD PARAMETERS IN RABBITS

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ABSTRACT:
Present study was performed on two combination doses of Citrus limon and Punica granatum i.e. 0.4ml/kg *Citrus limon* +5ml/kg *Punica granatum* (CPJ-1) and 0.2ml/kg *Citrus limon* +8ml/kg *Punica granatum* (CPJ-2) in healthy rabbits. CPJ-1 and CPJ-2 both showed significant increase in red blood cell count, hemoglobin, bleeding time and anticoagulation factors like protein C and thrombin antithrombin complex levels. While CPJ-1 cause significant increase in thrombin time and platelet count. However Fb was significantly decreased by CPJ-1. These results suggest that Citrus limon and Punica granatum has phytochemicals and other essential nutrients that possesses an anti-thrombin component, prevent thrombosis and plays cardio protective role.

Key words: Thrombin time, activated partial thrombin time, Fibrinogen concentration, Protein C, Thrombin antithrombin complex

INTRODUCTION:

Atherosclerosis is one of the leading causes of death, particularly in developed countries where a higher percentage of atherosclerotic deaths are observed. Inhibition of coagulation and thrombus formation are considered to be good strategies to prevent atherosclerosis and cardiovascular diseases [1, 2]. Various studies regarding coagulation, anticoagulant agents have been done [3,4]. Herbs, medicinal plants, fruits and vegetable extract or their components can be consumed, without risk to human health, due to their excellent antioxidant activity [5]. Flavonoids and polyphenol are mainly responsible for these antioxidant and anti-inflammatory activity. They also have role in prevention of cardiovascular diseases by reversing endothelial dysfunction[6,7] and increasing nitric oxide bioavailability or acting as antioxidant and anti-inflammatory [8,9,10].

Pomegranate (Punica granatum L .Punicaceae) is consumed worldwide, delicious and bears substantial amounts of

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phenolic compounds. These phenolic compounds account for its 92% antioxidant activity, including flavonoids i.e. anthocyanins, catechins and other complex flavonoids and hydrolysable tannins i.e punicalin, pedunculagin, punicalagin, gallic and ellagic acid [5]. Several studies have demonstrated the antimicrobial, anthelminthic, and antioxidant potential of its active ingredients, suggesting their preventive and curative role in gastro-mucosal injuries, cancer, ulcer and diabetes [11,12,13].

Lemon (Citrus limon [L.] Burm.f.) have antioxidant activity, due to the presence of abundant flavonoids including rutin, hesperidin, quercitrin, eriocitrin, narirutin, didymin and naringin,[14,15] vitamin C and carotenoids and could help to prevent atherosclerosis. It has significant economic value for its essential oil and is reported to be the source of magnesium, potassium, folic acid, limonoids and xanthoxyletin[16]. Number of studies has suggested its hypocholesterolemic, antifungal [17] and anti-cancer activity. Citrus limon has also shown usefulness as antidote against certain venom, due to its platelet inhibitory effect[09,18,19,20,21,22,23,24].

Various combination studies of herbal components and fruit juices show synergistic effects as compared to their sole effects in many system of health [25]. Present study was designed to evaluate the effects of two combination doses of Citrus limon and Punica granatum upon blood coagulation, platelet function, bleeding time and anticoagulation factors such as thrombin-antithrombin (TAT) complex and protein C (PC) in blood samples of healthy white rabbits.

MATERIALS AND METHODS

Combination of Citrus limon and Punica granatum juice:

Citrus limon and Punica granatum were purchased from local market, identified by center of plant conservation, University of Karachi. Voucher specimen no C.L 11-11 for Citrus limon and P.G 11-12 for Punica granatum were deposited in department of Pharmacognosy, University of Karachi. Fruit samples were peeled squeezed by hand and fresh juices so yielded were combined soon after filtration. All doses were given once daily for 60 days by gastric intubation.

Animals:

Study was carried out after the approval from Board of Advance Studies and Research, University of Karachi on blood samples drawn from fifty, white healthy rabbits of either sex. All animals had mean body weight of 1300 ± 50 grams. Body weight of the animals was noted weekly during the study. Rabbits were housed individually in steel rod bottom cages, under controlled condition of temperature 23 ± 2° C and humidity 50-60%. Diet and water was provided ad libitum.

Design of experiment:

Animals were divided into five groups with ten rabbits in each group. Two groups were given Citrus limon and Punica granatum juice orally in two combination doses i.e. 0.4ml/kg Citrus limon juice + 5ml/kg Punica granatum juice (CPJ-1) and 0.2ml/kg Citrus limon juice + 8ml/kg Punica granatum juice (CPJ-2). Third group served as control group and was given saline in the dose of 6 ml/kg. Fourth and fifth groups were given aspirin and warfarin respectively as standard antiplatelet and anticoagulant drugs. Aspirin was suspended in normal saline and administered in the dose of 150 mg/kg once

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daily for 6 days a week [26]. Warfarin was suspended in distilled water and was given for 6 days only, 5mg/kg for first 3 days and 10mg/kg next three days [27]. Blood samples were collected from ear vein in the EDTA containing tubes, trisodium citrate (3.8%) tubes in the ratio of 9:1 (v/v) and gel tubes at 30 and 60 day of dosing period. Humax 14 K centrifuge machine was used to separate plasma and serum. Plasma was separated by centrifugation at 2000 rpm for 10 min from blood samples collected in tri sodium citrate tubes while serum was separated by centrifugation at 2500 rpm for 10 minutes from blood samples collected in gel tubes.

Hematological parameters examination:

Huma Count (Human, Germany) fully automated hematology analyzer was used to examine red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), hemoglobin (Hb), Hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

Measurement of bleeding time:

Bleeding time (BT) was measured by cutting the ear tip as describe by [28,29, 30]. The ear was shaved, then small incision 5mm long and 1mm deep was made in the central ear artery using a template bleeding device. The incision sites were carefully blotted at 30 sec intervals with filter paper until bleeding ceased.

Measurement of Thrombin time, Prothrombin time, Activated partial thromboplastin time and Fibrinogen concentration:

Humaclot duo (Human, Germany) coagulometer was used to perform these tests. Principle of turbidimetric clot detection was used to assess coagulation endpoint by measuring change in optical density in plasma. 200ul plasma was incubated with 100ul thrombin reagent to measure thrombin time. To measure prothrombin time 100ul plasma was incubated with 200ul pre-warmed thromboplastin reagent. While activated partial thromboplastin time was measured by incubating 100ul plasma for 2 min at 37˚C, with 100ul aPTT-EL reagent, followed by adding 100ul $CaCl_2$. Incubation time before the addition of respective reagent for all these tests was 3 min at 37˚C. The timer was started with addition of reagent and time required for clot formation was recorded. Fibrinogen concentration (Fb) was measured by procedures described by McNerlan and Clauss [31, 32]. All parameters were determined using commercial kits by Human, Germany.

Platelet Function Assays:

Blood samples were drawn into tubes containing tri-sodium citrate 3.8% with 9:1 v/v ratio and processed within 2 hours. Blood samples were centrifuged at 100 rpm for 15 minutes in Humax 14 K, (Human, Germany) to obtain platelet rich plasma (PRP). While platelet-poor plasma (PPP) were prepared by further centrifugation of remaining blood at 1800 rpm for 10-15 minutes. Each PRP sample was standardized as needed (approx. 250 000/mm^3) with autologous PPP [33]. Platelet reactivity was traced for 10 minutes at 37°C [34]. The absorbance of the untreated PRP mixed with the aggregation reagent represent 0% aggregation, and the absorbance of PPP control represents 100% aggregation. Platelet aggregation was induced by addition of Adenosine diphosphate (20µM), Collagen (10µg/ml), Epinephrine (300 µM),

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Ristocetin (1500 µg/ml) and Arachidonic acid (500µg/ml). Platelet aggregation assay was performed with turbidimetric monitoring device, Helena AggRAM aggregometer (Helena Laboratories Corp, Beaumont, TX, USA), according to manufacturer's instructions. Pipette 450µl PRP into cuvettes incubate at 37˚C, insert PPP cuvette into appropriate channel and set to 100% aggregation, add 50µl of aggregating reagent. Resulting aggregation, measured as a change of light transmission, and was expressed as percentage of the PPP transmission value.

Protein C and Thrombin-antithrombin complex:

The activity level of PC and TAT complex in plasma was measured by commercial Protein C and TAT complex Elisa kit (Cusabio Biotech Co. LTD). Standard curves were prepared by standard plasma and absorbance was determined at 450nm. The activity level of TAT complex and Protein C in the samples was expressed as percentage related to the activity level of standard plasma. The entire tests were performed under NCCL guideline [35].

Liver function tests:

Serum samples were used for estimation of liver function by measuring SGPT (serum glutamic-pyruvic transaminase), γGT (Gamma glutamyltransferase) and total bilirubin concentration [36] by using standard kits of Human (Germany).

Histopathological examination:

Microscopic changes were observed by random selection of liver samples from each test and control animals. Samples were fixed in 10% formalin followed by dehydration in ascending grades of alcohol. Clearing by xylene and embedding in paraffin wax. Paraffin sections (5 µm thickness) were stained with hematoxylin and eosin (H & E) for histological examination [37].

Statistical analysis:

Data entry and analysis was performed using Superior Performance Statistical Software (SPSS) version 20. Data was presented as mean ± SD with 95% confidence interval. ANOVA followed by post hoc was performed for comparisons of values with control. Values of $p \leq 0.05$ were considered statistically significant and $p \leq 0.005$ highly significant.

RESULTS:

Table 1 shows the effect of combination doses of Citrus limon and Punica granatum on BT, TT, PT, aPTT and Fb. There was highly significant increase in BT by CPJ-1 combination both at 30 and 60 day, but it was increased significantly at 30 day and increased highly significantly at 60 day by CPJ-2 combination as compare to control. Whereas TT was increased and Fb concentration was decreased significantly by CPJ-1 at 60 day as compare to control, while aPTT was increase significantly at 60 day by CPJ-2 combination. However PT was not altered significantly.

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Table-1: Effect of combination doses of Citrus limon, Punica granatum and warfarin on coagulation parameters

GROUPS	Days	Parameters				
		BT (sec)	TT (Sec)	PT (Sec)	aPTT (Sec)	Fb (mg/dl)
Control	30	99.60±5.35	9.36±0.92	5.2±0.12	8.25±0.62	439.47±43.70
	60	101.66±5.8	9.41±1.02	5.32±0.12	8.33±0.61	437.50±43.14
CPJ-1	30	154.0±8.64**	10.85±0.43	6.22±0.19	12.19±1.04	397.73±51.77
	60	168.4±8.13**	12.7±1.11*	6.5±0.23	11.99±0.94	322.70±44.75*
CPJ-2	30	129.2±7.71*	9.8±0.32	5.88±0.05	11.57±1.54	385.63±33.42
	60	137.8±4.26**	10.6±0.6	6.18±0.13	12.49±0.95*	385.63±33.42
Warfarin	-	133.9±12.7*	16.58±1.74**	13.59±1.73**	15.73±1.77**	315.40±25.54*

n=10, Average values ± S.E.M.
*P ≤ 0.05 significant as compared to control
**P ≤ 0.005 highly significant as compared to control
CPJ-1: combination dose 0.4+5ml/kg/day ; i.e 0.4ml Citrus limon and 5ml Punica granatum
CPJ-2: combination dose 0.2+8ml/kg/day ; i.e 0.2ml Citrus limon and 8ml Punica granatum

Table 2 shows the effect of combination doses of Citrus limon and Punica granatum on hematological parameters. There was significant increase by CPJ-1 in RBC, Hb and significant decrease in red cell distribution width RDw at 30 and 60 day, whereas PLT were significantly increase at 60 day. There was highly significant increase by CPJ-2 in RBC and significant increase in Hb at 30 and 60 day. MCHC was significantly increased and RDw significantly decreased at 60 day. However Ht, MCV and WBC were not altered by both combinations.

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Table-2

Effect of combination doses of Citrus limon, Punica granatum and Aspirin on Hematological parameters

GROUPS	Days	PARAMETERS								
		RBC (x10³/cm)	WBC (x10³/cm)	Hb (g/dl)	Ht (%)	MCV (%)	RDW (%)	MCH (Pg/cell)	MCHC (%)	PLT (x10³/cm)
Control	**30**	3.62±0.34	3.22±0.42	9.72±0.37	27.96±3.70	61.0±0.63	16.04±0.22	19.55±0.64	29.82±0.65	271.40±27.57
	60	3.84±0.34	3.28±0.43	9.77±0.36	28.23±3.70	62.3±0.70	16.18±0.22	20.55±0.64	30.88±0.69	269.90±26.88
Aspirin	**30**	3.44±0.363	4.38±0.395*	5.31±0.18*	20.27±0.56*	63.6±0.73*	14.27±0.05**	21.71±0.79*	29.14±0.68	281.60±32.37
	60	3.64±0.370	4.43±0.511*	5.24±0.50*	20.56±0.51*	65.1±0.83*	14.22±0.08**	22.90±0.67*	30.47±0.75	324.60±15.27
CPJ-1	**30**	4.66±0.31*	3.63±0.30	15.03±1.74*	29.18±2.67	60.9±0.93	15.09±0.28*	19.96±0.77	30.89±0.64	336.0±39.50
	60	4.85±0.35*	3.71±0.32	14.38±1.75*	32.17±2.33	60.9±0.78	15.33±0.36*	20.36±0.66	31.36±0.59	392.0±78.14*
CPJ-2	**30**	5.11±0.37**	4.02±0.35	14.34±1.6*	31.37±2.54	62.1±0.56	15.66±0.49	19.46±0.61	31.21±0.66	312.40±24.48
	60	5.25±0.23**	4.22±0.41	14.39±1.36*	34.55±2.94	63.1±0.60	15.38±0.32*	20.26±0.50	33.32±0.58*	335.30±16.39

n=10, Average values ± S.E.M.
*P ≤ 0.05 significant as compared to control
**P ≤ 0.005 highly significant as compared to control

Table 3 shows the effect of combination doses of Citrus limon and Punica granatum on platelet aggregation. There was significant inhibition in platelet aggregation by CPJ-1 induced by adenosine diphosphate (ADP), collagen (Col), epinephrine (Epi) and arachidonic acid (AA) both at 30 and 60 day. However there was significant inhibition in platelet aggregation by CPJ-2 induced by collagen and Epi at 30 day, and by ADP, collagen, Epi and AA at 60 day.

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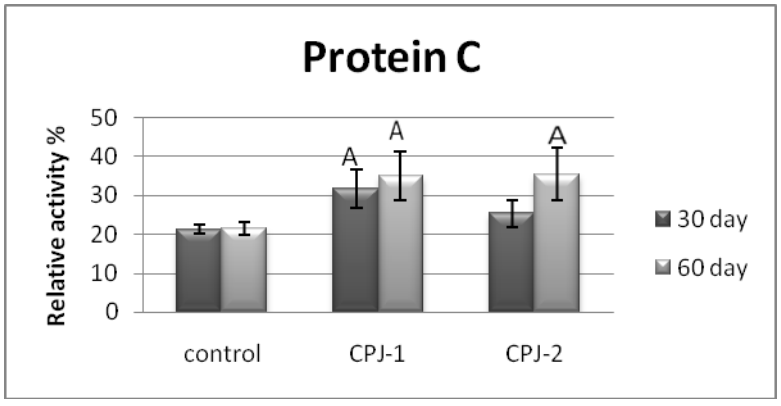
Table-3 Effect of combination doses of Citrus limon, Punica granatum and Aspirin on inhibition of platelet aggregation

GROUPS	Days	% Inhibition of Platelet Aggregation			
		ADP (20µM)	Col (10µg/ml)	Epi (300 µM)	AA (500µg/ml)
Control	**30**	42.20±2.13	18.15±0.92	13.88±0.98	69.81±8.91
	60	42.30±4.12	18.21±1.14	13.41±0.73	75.77±6.66
Aspirin	**30**	29.90±0.85*	10.43±0.77*	12.85±1.54	44.57±8.95*
	60	24.84±0.70**	10.72±0.93*	13.0±2.62	46.14±8.88*
CPJ-1	**30**	30.24±1.32*	13.84±1.27*	10.88±0.89*	48.47±8.62*
	60	30.08±2.42*	13.74±1.53*	8.95±0.6*	55.16±6.57*
CPJ-2	**30**	33.38±5.52	13.21±1.89*	10.78±0.37*	60.14±5.34
	60	31.61±3.42*	13.20±1.27*	9.32±0.46*	52.31±8.95*

n=10, Average values ± S.E.M.
*P ≤ 0.05 significant as compared to control
**P ≤ 0.005 highly significant as compared to control

Figure 1 shows the effect of two combination doses of Citrus limon and Punica granatum on PC. There was significant increase in PC at CPJ-1 both after 30 and 60 days. While significant increase in PC was also observed by CPJ-2 at 60 day.

Figure-1: Effect of combination doses of Citrus limon and Punica granatum on plasma activity of Protein C



n=10,

Columns with (A) are significantly different, P ≤ 0.05; as compare to control

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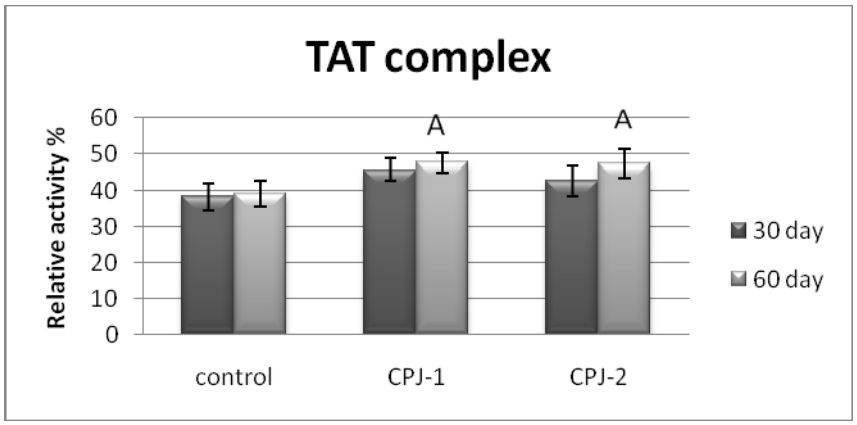
Figure 2 shows the effect of two combination doses of Citrus limon and Punica granatum on TAT complex. There was significant increase in TAT complex by both combinations at 60 day. However there was no significant change by any combination at 30 day.

Figure-2: Effect of combination doses of Citrus limon and Punica granatum on plasma activity of Thrombin antithrombin (TAT) complex



n=10,

Columns with (A) are significantly different, $P \leq 0.05$;

as compare to control

There were no significant changes in the levels of SGPT, γGT and total bilirubin at any combination as compared to control animals. No histological changes were also observed in hepatic tissues of treated groups at any dose (data not shown).

DISCUSSION :

Herbal medicines are now in great demand in the developing world for primary health care not only because they are inexpensive but because of better cultural acceptability and compatibility with the human body [38, 39]. Fruits and their juices have been increasingly studied due to their health promoting effects. Several studies have shown effectiveness of vegetables, fruits and their juices or extracts for the treatment or prevention of chronic diseases.

Present study was specifically designed to evaluate therapeutic efficacy of two combination of Citrus limon and Punica granatum on coagulation factors. Since abnormalities of coagulation are considered to be prognostic risk factor for bleeding [40]. Dietary pattern is one of many variables which may alter coagulation and fibrinolysis, since variations in the amount and type of fat in the diet have been shown to alter factor VII [41] fibrinogen, tissue plasminogen and plasminogen activator inhibitor type-1 [42].

Coagulation tests like BT, TT, PT, aPTT and Fb can better define the risk of bleeding [43].

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Hence in present study these parameters were used to monitor the influence of combination of Citrus limon and Punica granatum *on* coagulation process and thus their effectiveness in cardiovascular diseases (CAD). Results of present study did not reveal any significant effect of CPJ-1 and CPJ-2 on PT. Hence it shows that CPJ-1 and CPJ-2 have no effect on extrinsic coagulation factors, since prolong PT is due to deficiency of extrinsic coagulation factors, I, II, V, VII, and X [44]. Similarly no significant changes were observed in aPTT by CPJ-1 combination. It shows that CPJ-1 has no effect on intrinsic coagulation factors. Since prolong aPTT is due to deficiency of intrinsic coagulation factors I, II, V, VIII, IX, X, XI and XII [45, 46, 47]. However there was significant prolongation in aPTT by CPJ-2, it may be due to presence of rutin and hesperidin which are reported to prolong aPTT in vitro [48]. Hence it could be suggested that essential components of Citrus limon and Punica granatum may be responsible for these changes or coagulation factors [23].

Results of present study showed significant inhibition of platelet aggregation by both combinations CPJ-1 and CPJ-2. This might be due to flavonoids, vitamin C and coumarin compound-xanthoxyletin, reported to inhibit the cyclooxygenase and lipoxygenase pathways [14] or modify membrane fluidity of platelets [49]. There are evidences that flavonoids of Citrus limon and Puniuca granatum may inhibit platelet functioning [7]. Present study also reveals marked decrease in Fb by CPJ-1, hence it may be concluded that inhibition of platelet aggregation might be due to decrease Fb level. Since platelets need fibrinogen to activate their aggregation [50, 51]. More over there are several studies which show that platelet hyperaggregability and hyperfibrinogenaemia are associated risk factors for coronary heart disease [52, 53, 54, 55, 56].

Present study shows increase in PC and TAT complex by both combinations of Citrus limon and Punica granatum. TAT complex is one of the markers that measure the amount of inhibited thrombin [57] by combining with Anti-thrombin III [58]. On the other hand PC activation inhibit thrombin and all regulation signals generated by it, convert them into anticoagulation response via TAT complex and react with protein S to inactivate factors V and VIII [59,60, 61, 62]. Furthermore, both CPJ-1 and CPJ-2 did not cause any change in SGPT, γGT, total bilirubin concentration and did not cause any histo-pathological changes in liver. Hence it could be suggested that change in activity of anticoagulation factors probably was not due to liver damage, but may be due to due to impaired activity of thrombin. There are several studies which show decrease in PC and TAT complex in thrombotic diseases and cardio-vascular events [1, 63]. Hence it may be suggested that CPJ-1 and CPJ-2 may produce beneficial effects in the patients of thrombotic diseases by elevating PC and TAT complex. It could be concluded that impaired activity of thrombin not only causes elevation of PC and TAT complex, but may also results in prolonged TT, BT and decrease Fb by CPJ-1. Since thrombin plays an important role in platelet aggregation and conversion of fibrinogen to fibrin [64, 65, 66]. These results suggest that anticoagulant factors may play a crucial role in hyper coagulation.

Results of present study reveal significant increase in hematological parameters such as RBC and Hb by both combinations of Citrus limon and Punica granatum. However no

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significant change was observed in Ht. Hence it may be concluded that inhibition of platelet aggregation might be due to insignificant effect on Ht level by these combination of fruits juices. Since increase Ht enhances platelet adherence and aggregation [67].

Results of present study showed no significant change in platelet count by CPJ-1. Since normal platelet count is essential for normal blood coagulation [68]. However there was significant increase in platelets count by CPJ-1, hence it may be concluded that increased platelet count may be due to synergistic effect of flavonoids found in Citrus limon and Punica granatum on initiation phase that requires cAMP [69]. Thus leading to increases in platelet count.

In response to vascular injury platelets are recruited to the sub endothelium through a specific interaction between platelet GPIb–V–IX and sub endothelial-bound vWF [70]. There was no significant change in PT and aPTT by CPJ-1, which might be due to no significant effect of intrinsic as well as extrinsic coagulation factors. These effects may be due to increase synthesis of factor V and vWF. Hence CPJ-1 may produce its effect by initiating the above interaction between platelet receptors and coagulation factors V and vWF that ultimately leads to initiation of platelets (figure 3).

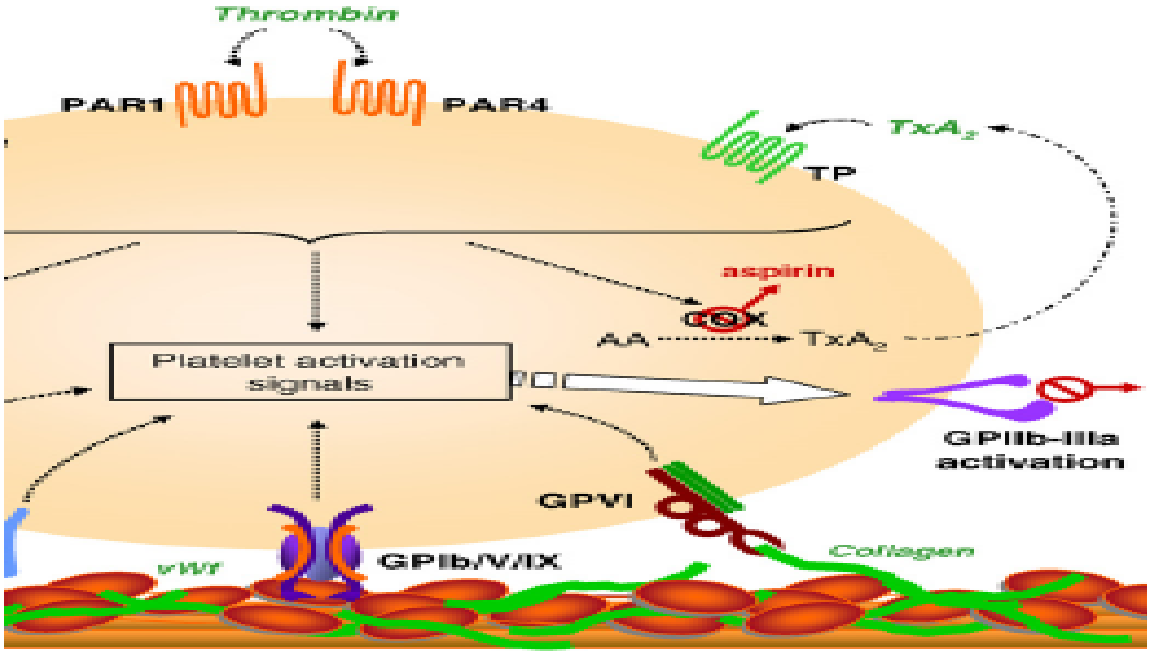

Figure -3: Possible sites of action of CPJ-1 on platelet initiation modified (Hamilton, 2008).

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Hence significant increase in platelet count by CPJ-1 may be beneficial in the management of Dangi fever suggesting the need to work on more different combination doses, to obtain desired increase platelet count. *Citrus limon*, *Punica granatum* combination CPJ-1 may retard the progression of atherosclerosis and prevent cardiovascular events due to their anticoagulant and anti- inflammatory effects.

Thus on the basis of data revealed in present study it may be concluded that anticoagulant activity of *Citrus limon*, *Punica granatum* combination CPJ-1 may be supported by its anti-inflammatory activity. Maximum anticoagulation, anti-platelet and anti-inflammatory effects observed at CPJ-1 combination, opens a new door for further investigations on different combination doses of these fruits.

References

1. Little WJ, Miller SC, Henry GR, McIntosh A.B, Fla N, Ky L. Antithrombotic agents: Implication in dentistry. Oral surg oral med oral pathol oral radiol endod. 2002; 93: 544-551.
2. Wang, X.M.Y.H., Steinbacher, T.E., Monticello, T.M., and Schumacher, W.A. Quantification of platelet composition in experimental venous thrombosis by real-time polymerase chain reaction. Thrombosis Res.2007; 119: 593-600.
3. Ahmed A, Khan RA, Mesaik MA. Anti-inflammatory effect of natural honey on bovine thrombin induced oxidative burst in phagocytes. Phytother. Res. 2008; 22: 1-8.
4. Riaz A, Khan RA, Ahmed, S.P. Assessment of anticoagulant effect of evening primrose oil. Pak J. Pharm. Sci. 2009; 22: 355-359.
5. Ismail T, Sestili, P, Akhtar S. Pomegranate peel and fruit extracts. A review of potential anti-inflammatory and anti-infective effects. Journal of Ethnopharmacology. 2012; 143: 397–405.
6. Heiss C, Kleinbongard P, Dejam A, Perre S, Schroeter H, Sies, H, Kelm M. Acute Consumption of Flavanol-Rich Cocoa and the Reversal of Endothelial Dysfunction in Smokers. J. of American Col. of Cardiol. 2005; 46 (7): 1276-1283.
7. McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R, Dwyer JT. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. Am J Clinical Nutrit. 2012; 95 (2): 454-464.
8. Kim HP, Son KH, Chang HW, Kang SS. Anti-inflammatory Plant Flavonoids and Cellular Action Mechanisms. J. of Pharmacol. Sci. 2004; 96: 229-245.
9. Chun OKY, Chung SJ, Claycombe KJ, Song WO. Serum C - reactive protein Concentrations Are Inversely Associated with Dietary Flavonoid Intake in U.S. Adults. Nutrition. 2008; 138: 753–760.
10. Grassi D, Desideri G, Ferri C. Flavonoids: Antioxidants against atherosclerosis. Nutrients. 2010; 2, 889-902.
11. Seeram NP, Henning SM, Zhang Y, Suchard M, Li Z, Heber D. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 hours . Journal of Nutrition. 2006; 136 (10): 2481-2485.
12. Fischer UA, Carle R, Kammerer DR. Identification and quantification of phenolic compounds from pomegranate (Punica granatum L.) peel, mesocarp, aril and differently produced juices by HPLC-DAD–ESI/MSn. Food Chemistry. 2011; 127; 807–821.
13. Riaz A, Khan, RA, Ahmed M. Glycemic response of Citrus Limon, Pomegranate and their combinations in Alloxan induced diabetic rats. Australian Jr. Basic and Applied Sciences. 2013; 7 (10): 215-219.
14. Nijveldt R, Nood EV, Hoorn DEC, Boelens PG, Norren KV, Leeuwen PAM. Flavonoids: a review of probable mechanisms of action and potential applications. Am. J Clin Nutr.2001; 74: 418–25.
15. Tripoli E, Guardia ML, Giammanco S, Di,

MD, Giammanco M. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chemistry. 2007; 104: 466–479.

16. Deyhim F, Garica K, Lopez E, Gonzalez J, Ino S, Garcia M, Patil BS. Citrus juice modulates bone strength in male senescent rat model of osteoporosis. Nutrition. 2006; 22: 559-563.
17. Viuda MM, Ruiz NY, Fernandez LJ, Perez AJ. Antifungal activity of lemon (Citrus lemon L.), mandarin (Citrus reticulata L.), grapefruit (Citrus Paradisi L.) and orange (Citurs sinensis L.) essential oils. Food Control. 2008; 19: 1130-1138.
18. Teng CM., Li, HL, Wu TS, Huang SC, Huang TF. Antiplatelet actions of some coumarin compounds isolated from plant sources. Thromb. Res. 1992; 66 (5): 549-57.
19. Berhow MA, Smolensky D. Development and substrate specificity of hesperetin-7-O-glucosyltransferase activity in Citrus limon tissues using high-performance liquid chromatographic analysis. Plant Sci. 1995; 112: 139-147.
20. Arias BA, Ramon LL. Pharmacological properties of citrus and their ancient and their medical use in the mediteraanean region. J. Ethnopharmacol. 2005; 97: 89-95.
21. Xu G, Liu D, Chen J, Ye X, Ma Y, Shi, J. Juice component and antioxidant capacity of citrus varieties cultivated in China. Food chemistry. 2008;106: 545-551.
22. Hernandez MV, Montes MMA, Elizalde GMP. Study of the thermal degradation of citrus seeds. Biomass. Bioenergy. 2009; 33: 1295-1299.
23. Gonzalez ME, Domínguez PR, Moreno DA, García VC. Natural bioactive compounds of Citrus limon for food and health. J.of Pharm. and Biomed. Anal. 2010; 51: 327–345.
24. Khan, Y., Khan, AR, Afroz S, Siddiq A. Evaluation of hypolipidemic effect of Citrus lemon. J. Basic. Applied Sci. 2010; 6: 39-43.
25. Riaz A, Khan RA, Ahmed, S.P. Assessment of anticoagulant effect of evening primrose oil. Pak J. Pharm. Sci. 2009; 22: 355-359.
26. Merchant MA, Modi DN. Acute and chronic effects of aspirin on hematological parameters and hepatic ferritin expression in mice. Indian J Pharmacol. 2004; 36: 226-230.
27. Warfarin dosing guideline, 2009, DISCLAIMER: guidelines were prepared by the Department of Surgical Education, Orlando Regional Medical Center. www.Surgical Critical Care.net
28. Johnstone MT, Andrews T, Ware JA, Rudd MA, George D, Weinstein M, Loscalzo J. Bleeding time prolongation with streptokinase and its reduction with 1-desamino-8-D-arginine vasopressin. Circulation. 1990; 82: 2142-2151.
29. Garcia MA, Gonzalez L, Lemini C, Rubio PC. Standardization of rat blood clotting tests with reagents used for humans. Proc. West. Pharmacol. Soc. 2001; 44: 153-155.
30. Li H, Cone J, Fong M, Kambayashi J, Yoshitake M, Liu Y. Antiplatelet and Antithrombotic Activity of Clostazol is potentiated by Dipyridamole in rabbits and Dissociated from bleeding time prolongation. Basic pharmacol. Cardiovas. Drugs and Therap. 2005; 19: 41-48.
31. McNerlan, SE, Crawford, VLS, Stout RW. Measurement of Fibrinogen in Frozen Plasma. Thrombos. Res. 1997; 88: 481-484.
32. Clauss A. Rapid physiological coagulation method in determination of fibrinogen. Acta Hematologica. 1957; 17 (4): 237-246.
33. Son DJ, Lee HW, Shin HW, Lee JJ, Yoo HS, Kim TJ, Yun YP, Hong JT. Enhanced release of sphingosine-1-phosphate from hypercholesterolemic platelets: Role in development of hypercholesterolemic Atherosclerosis. Prostagland. Leukotr. Essent. Fat. Acids. 2008; 78: 383-390.
34. Jeong YH, Hwang JY, Kim IS, Park Y, Hwang SJ, Lee SW, Kwak CH, Park, S.W. Adding Cilostazol to dual Antiplatelet therapy achieves greater platelet Inhibition than high maintenance dose clopidogrel in patients with acute myocardial infarction results of the adjunctive cilostazol versus high maintenance dose clopidogrel in patients with AMI (ACCEL-AMI) Study. Circ Cardiovasc Interv. 2010; 3: 17-26.

35. Wayne, P.A. Collection, transport and processing of blood specimens for coagulation testing and general performance of coagulation assays. Approved guideline H21-A2, 3rd Edition, National Committee for Clinical Laboratory Standards, 1998. (NCCLS Document H21-A3).
36. Fischbach F, Zawta B. Age dependent reference limit of several enzymes in plasma at different measuring temperatures. Klin Lab. 1992; 38: 555-561.
37. Diab KAE, ELmakawy A, Abd-Elmoneim OM, Sharaf HA. Assessment of genotoxicity and histopathological changes induced by polyethylene glycol (PEG6000) in male mice. J Cytology & Histol. 2012; 3 (153): 1-7.
38. Frenkel, M., Arye, E.B. The growing need to teach about complementary and alternative medicine: questions and challenges. Acad Med. 2001; 76: 251-4.
39. Pal SK., Shukla Y. Herbal medicine: current status and the future. Asian Pac J Cancer. 2003; 4 (4):281-288.
40. Stanworth SJ. The Evidence-based use of FFP and Cryoprecipitate for abnormalities of coagulation tests and clinical coagulopathy. American society of hematology. 2007; 179-185.
41. Bladbjerg EM, Marckmann P, Sandstrom B, Jespersen J. Non-fasting factor VII coagulant activity (FVII: C) increased by high fat diet. Thromb Hemostasis. 1994; 71: 755- 8.
42. Allman-Farinelli, M.A, Hall D, Kingham K, Pang D, Petocz P, Favaloro EJ. Comparison of the effects of two low fat diets with different α-linolenic:linoleic acid ratios on coagulation and fibrinolysis. Atherosclerosis. 1999; 142:159-1689.
43. Gentry PA. Comparative aspects of blood coagulation. The Veterinary Journal.2004; 168, 238-251.
44. Rao LV, Okorodudu AO, Petersen JR, Elghetany MT. Stability of prothrombin time and activated partial thromboplastin time tests under different storage conditions. Clinica Chimica Acta. 2000; 300: 13–21.
45. Khanin MA, Rakov DV, Kogan AE. Mathematical Model for the Blood coagulation Prothrombin Time Test. Thrombosis Res. 1998; 89: 227 – 232.
46. Mann KG, Butenas S, Brummel K. The Dynamics of Thrombin Formation. Atherioscler. Thombos. Vasc. Biol. 2003; 23: 17-25.
47. Kanahara M, Kai H, Okamura T, Wada T, Suda K, Imaizumi T, Sagawa K. Usefulness of high-concentration calcium chloride solution for correction of activated partial thromboplastin time (APTT) in patients with high-hematocrit value. Thrombos. Res. 2008; 121: 781–785.
48. Kuntic V, Filipovic I, Vujic Z. Effects of Rutin and Hesperidin and their Al (III) and Cu(II) Complexes on in Vitro Plasma Coagulation Assays. Molecules. 2001; 16: 1378-1388.
49. Furusawa M, Tsuchiya H, Nagayama M, Tanaka T, Nakaya K-ichi, Iinuma M. Anti-platelet and membrane-rigidifying flavonoids in brownish scale of onion. J. of health sci. 2003; 49 (6): 478-480.
50. Denninger MH, Jandrot-Perrus M., Elion J, Bertrand O, Homandberg GA, Mosesson MW, Gullin MC. ADP-induced platelet aggregation depends on the confirmation or availability of the terminal gamma chain sequence of fibrinogen. Study of the reactivity of fibrinogen Pairs 1. Blood. 1987; 70: 558-563.
51. Peterson AJ, Visentin PG, Newman JP, Aster SR. A recombinant soluble form of the Integrin ?IIbB3 (GPIIb-IIIa) assumes an active, ligand-binding conformation and is recognized by GPIIb-IIIa - specific monoclonal, allo-, auto-, and drug-dependent platelet antibodies. Blood. 1989; 92: 2053-2063.
52. De-Maat MPM, Pietersma A, Kofflard M, Sluiter W, Kluft C. Association of plasma fibrinogen levels with coronary artery

disease, smoking and inflammatory markers. Atherosclerosis.1996; 121: 185-191.

53. Silva MP, Ferhndez RJM, Luque CMD. Light scattering-based determination of fibrinogen in human plasma using an automated continuous system. Analytica Chimica Acta. 1996; 327: 101-106.
54. Bach J, Haubelt H, Hellstern P. Sources of variation in factor VIII, von Willebrand factor and fibrinogen measurements: Implications for detecting deficiencies and increased plasma levels. Thrombosis Res. 2010; 126: e188–e195.
55. Miesbach, W., Schenk, J., Alesci, S., and Lindhoff-Last, E. Comparison of the fibrinogen Clauss assay and the fibrinogen PT derived method in patients with dysfibrinogenemia. Thrombos. Res. 2010; 126: e428–e433.
56. Seppanen-Laakso T, Laakso I, Lehtimak T, Rontu R, Moilanen E, Solakivi T, Seppo L, Vanhanen H, Kiviranta K, Hiltunen R. Elevated plasma fibrinogen caused by inadequate α-linolenic acid intake can be reduced by replacing fat with canola-type rapeseedoil. Prostagland. Leukotr. and Essent. 2010; Fat. Acids. 83: 45–54.
57. Chandler LW, Valen T. Estimating the rate of thrombin and fibrin generation in vivo during cardiopulmonary bypass. Blood. 2003; 101: 4355-4362.
58. Dickneite G, Leithäuser B. Influence of antithrombin III on coagulation and inflammation in porcine septic shock. Arterioscler Thromb Vasc Biol. 1999; 19(6):1566-72.
59. Marlar, RA. Protein C in thromboembolic disease. Semin Thromb Hemost. 1985; 11(4):387-93.
60. Jamison C, Scott, McDowell A, Susan, Marl A, Richard D J. Sandra Friezner. Developmental expression of protein C and protein S in the rats. Thrombosis Research. 1995; 78 (5): 407-419.
61. 5. Arid CW. Natural anticoagulant inhibitors: activated Protein C. Best Practice & Res. Clinical Haematol. 2004; 17 (1): 161-182
62. Lipe B, Ornstein LD. Deficiencies of natural anticoagulants, protein C, protein S, and ntithrombin. Circulation. 2011; 124: e365-e368.
63. Moriau M, Lavenne-Pardonge E, Crasborn L, Frenckell RV. The treatment of severe recurrent deep venous thrombosis. Beneficial effects of the co-administration of antiplatelet agents with or without rheological effects and anticoagulants. Thrombosis Res. 1995; 78: 469-482.
64. Lane AD., Philippou H, Huntington AJ. Directing thrombin. Blood. 2005; 106: 2605-2612.
65. Wolberg, SA. Thrombin generation and fibrin clot structure. Blood. 2007; 21: 131-142.
66. Doormaal FF, Buller HR, Middeldorp S. Development in anticoagulant therapy. Critical Review I Oncol/Hematol. 2008; 66: 145-154.
67. Aarts PA, Bolhuis PA, Sakariassen KS, Heethaar RM, Sixma JJ. Red blood cell size is important for adherence of blood platelets to artery subendothelium. Blood. 1983; 62, 214-217.
68. Harrison P, Frelinger AL, Furman IM, Michelson DA. Measuring antiplatelet drug effects in the laboratory. Thromb. Res. 2007; 120: 323-336.
69. Wilcox, J. Lisa, Borradaile, M., Nica, Huff, W. M. Antiatherogenic properties of naringenin, a citrus flavonoid. Cardiovascular drug reviews. 1999; 17 (2): 160–178.
70. Hamilton JR. Protease-activated receptors as targets for antiplatelet therapy. Blood Reviews. 2008; 06: 1-5.