In vitro/in vivo effect of *Citrus limon* (L. Burm. f.) juice on blood parameters, coagulation and anticoagulation factors in rabbits

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Abstract: The genus Citrus of the family Rutaceae includes many species e.g. *Citrus indica, Citrus aurantifolia* and *Citrus limon*, among which *Citrus limon* L. Burm. f. has been reported to have highest antimicrobial activity. It is used as antidote against certain venom, due to its platelet inhibitory effect and also reported to have hypocholesterolemic effect. However its anticoagulant and thrombolytic effect were not been investigated, hence a prospective in-vitro/in-vivo study was designed to determine the effect of *Citrus limon* on blood parameters, coagulation and anticoagulation factors. In-vitro tests revealed highly significant increase in thrombin time and activated partial thromboplastin time by *Citrus limon*, whereas fibrinogen concentration was significantly reduced in comparison to control, however prothrombin time was not affected significantly. In-vivo testing of *Citrus limon* was done at three different doses i.e. 0.2ml/kg, 0.4ml/kg and 0.6ml/kg in healthy rabbits. Significant changes were observed in hematological parameters such as erythrocytes, hemoglobin and mean corpuscular hemoglobin concentration. Bleeding time and thrombin time was significantly prolonged and there was increase in protein C and thrombin antithrombin complex levels. These results may be due to inactivation of thrombin because it significantly decreases fibrinogen concentration and inhibit platelet aggregation. *Citrus limon* showed maximal anticoagulant effect at 0.4ml/kg, which suggest that *Citrus limon* possesses an anti-thrombin component and could prevent thrombosis playing a cardio protective role.

Keywords: Thrombin time, activated partial thrombin time, Fibrinogen concentration, mean corpuscular hemoglobin concentration

INTRODUCTION

Defects in coagulation and thrombosis are one of the important causes of atherosclerosis and cardiovascular diseases (Little *et al*., 2002; Wang *et al*., 2007). Studies regarding coagulation, anticoagulant agents have been done for the prevention and treatment of thrombogenic state (Ahmed *et al*., 2008; Riaz *et al*., 2009). Many factors of blood coagulation like increased level of factor VIII, von Wille brand factor and platelet activation are associated with cardiovascular disease as well as hemo stasis (Abdullah *et al*., 2010). Few studies indicates the role of flavonoids and polyphenol compounds in prevention of cardiovascular diseases either by reversing endothelial dysfunction (Heiss *et al*., 2005; McCullough *et al*., 2012) or increasing nitric oxide bioavailability or acting as antioxidant and anti-inflammatory (Kim *et al*., 2004; Chun *et al*., 2008; Grassi *et al*., 2010).

Fruits and their juices are the major dietary sources of polyphenol compounds, flavonols, flavanones, and anthocyanidins. It has been exhibited that Citrus fruit have highest antioxidant activity (Chun *et al*., 2008), due to the presence of abundant flavonoids, vitamin C and carotenoids (Xu *et al*., 2008). Common species of the genus Citrus are *Citrus indica, Citrus aurantifolia,* and

Citrus limon, in which *Citrus limon* (L. Burm. f.) is available in Pakistan and commonly known as *Limo*. It has significant economic value for its essential oil and is reported to be the source of magnesium, potassium, vitamin C, folic acid, limonoids and flavonoids (Deyhim *et al*., 2006). *Citrus limon* has shown usefulness as antidote against certain venom, due to its platelet inhibitory effect (Arias *et al*., 2005), however it needs further confirmation. More attention has also been paid on antioxidant capacity of *Citrus limon* (Berhow *et al*., 1995; Xu *et al*., 2008), since increase dietary antioxidant constituents could help to prevent athero-sclerosis (Hernandez *et al*., 2009; Gonzalez *et al*., 2010).

Number of studies has suggested the possibility of *Citrus limon* in preventing cardiovascular diseases due to its hypocholesterolemic activity (Gonzalez *et al*. Khan *et al*., 2010). *Citrus limon* was thought to produce anti-thrombotic effect, since hypercholesterolemia and thrombosis are interrelated (Vazquez *et al*., 2004; Pfister, 2006; Son *et al*., 2008). Beneficial effects of *Citrus limon* are due to its wide range of bioflavonoids, including rutin, hesperidin, quercitrin, eriocitrin, narirutin, didymin and naringin (Nijveldt *et al*., 2001; Tripoli *et al*., 2007). Two more isomers of hesperidin, neohesperidin and homoeriodictyol rutinoside have also been identified in *Citrus limon* (Gonzalez *et al*., 2010). Other micronutrient

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includes magnesium, potassium, vitamin C, folic acid, limonoids and xanthoxyletin. Systemic studies on anticoagulant effects are scare, however there are studies on Rutin and Hesperidin (Kuntic *et al*., 2011), xanthoxyletin (Teng *et al*., 1992), antifungal activity (Viuda *et al*., 2008) and anti-cancer activity of *Citrus limon* (Arias *et al*., 2005).

Present study was designed as a part of the therapeutic approach to evaluate the anticoagulant and anti-thrombotic effects of *Citrus limon* both *in-vitro* and *in-vivo*. The effect on coagulation and anticoagulation factors was assessed by determining thrombin time (TT), prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration (Fb), bleeding time (BT), platelet function and thrombin antithrobin (TAT) complex and protein C (PC) levels in rabbit blood.

MATERIALS AND METHODS

Citrus limon L. was purchased from local market, identified by center of plant conservation, University of Karachi and voucher specimen no C.L 11-11 was deposited in Department of Pharmacognosy, University of Karachi. Fruit were cut and squeezed by hand to yield fresh juice which was filtered immediately before use.

In-vitro Study
Blood Samples
In-vitro study was carried out on blood samples drawn from marginal vein of nine white healthy rabbits. 5 ml sample from each rabbit was collected in coagulation tubes containing 3.8% tri sodium citrate solution (9:1 v/v).

Design of experiment
Thrombin time (TT), Prothrombin time (PT), activated partial thromboplastin time (aPTT) and Fibrinogen concentration (Fb) were estimated using standard kits of Merck (Germany) by coagulation analyzer, Humaclot Duo (Kung-chi *et al*., 2007). Determination of TT, PT, aPTT and Fb was done by taking equal volume of plasma with fresh *Citrus limon* juice or water for injection or heparin sodium, 25,000IU (Huons Co. Ltd) to measure test, control and standard reading respectively.

Measurement of TT, PT, aPTT and Fb
Sample collection and animal handling was in accordance with the NCCLS approved guideline H21-A3 (Wayne, 1998). Plasma from each sample was immediately separated at 1500 rpm for 15 minutes by centrifugation in 14 K Humax centrifuge machine. The separated plasma samples were stored at -20ºC for determination. The principle used in Humaclot duo coagulometer (Human, Germany) was turbidimetric clot detection to assess coagulation endpoint. While TT was measured by mixing 100ul *Citrus limon* juice or water for injection or heparin

and 100ul plasma incubated with 100ul thrombin reagent. PT was measured by mixing 50ul *Citrus limon* juice or water for injection or heparin and 50ul plasma incubated with 200ul pre-warmed thromboplastin reagent. aPTT was measured by mixing 50ul *Citrus limon* juice or water for injection or heparin and 50ul plasma incubated for 1-2 min at 37˚C, followed by addition of 100ul aPTT-EL, then incubated with 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 was recorded required for clot formation. Fb concentration was measured as described by McNerlan *et al*. (1997) using Clauss, (1957) method.

In-vivo Study
Animal Selection
Sixty healthy white rabbits of either sex were selected for in-vivo study. All animals had mean body weight of 1300 ± 50 grams. Body weights of the animals were measured weekly during 60 day study. Rabbits were housed individually in steel rod bottom cages, under controlled condition of temperature 23±2°C, humidity 50-60%. Diet and water was provided *ad libitum*.

Design of experiment
Animals were divided into six groups with ten rabbits in each group. Three groups were given *Citrus limon* juice, once daily in three doses i.e. 0.2ml/kg, low *Citrus limon* dose (LCLD), 0.4ml/kg, moderate *Citrus limon* dose (MCLD) and 0.6ml/kg, high *Citrus limon* dose (HCLD). Fourth group was given saline in same dose equivalent to their body weights and considered as control group. Fifth and sixth groups were given aspirin and warfarin as standard drugs. Aspirin was suspended in normal saline and administered in the dose of 150 mg/kg once daily for 6 days a week (Merchant *et al*., 2004). Warfarin was suspended in distilled water and dose was scheduled for 6 days only, 5mg/kg for first 3 days and 10mg/kg next three days (Warfarin dosing guideline, 2009). All drugs were given by gastric intubation for 60 days. Blood samples were collected from ear vein in the EDTA containing tubes, trisodium citrate (3.8%) tubes in the ratio of 9:1 and gel tubes at 30 and 60 day at the end of dosing period.

Measurement of TT, PT, aPTT and Fb
Blood samples collected were centrifuged in Humax 14 K (Human, Germany) at 2000 xg for 10 min to separate plasma. Tests were performed on Humaclot duo (Human, Germany) coagulometer using the principle of turbidimetric clot detection to assess coagulation endpoint by measuring change in optical density in plasma samples. To measure TT 200ul plasma was incubated with 100ul thrombin reagent, while PT was measured by incubating 100ul plasma with 200ul pre-warmed

thromboplastin reagent. While aPTT was measured by incubating 100ul plasma for 1-2 min at 37˚C, with 100ul aPTT-EL reagent, followed by adding 100ul CaCl2. Incubation time was for 3 min at 37˚C; Fb concentration was measured as described by McNerlan *et al.*, (1997) using Clauss, 1957 method. All parameters were determined using standard kits by Human, Germany.

Measurement of bleeding time (BT)

Bleeding time was measured by cutting the ear tip as describe by (Johnstone *et al.*, 1990; Garcia *et al.*, 2001; Li *et al.*, 2005). First the ear was shaved then small incision of 5mm long and 1mm deep was made to the central ear artery using a template bleeding device. The incision sites were carefully blotted at 30 sec intervals with filter paper until bleeding has ceased.

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. Platelet rich plasma (PRP) was obtained as a supernatant fluid after centrifuging blood at 100 g for 10-15 minutes in centrifuge machine (Humax 14 K, Human, Germany). The remaining blood was further centrifuged at 1600-2000 g for 10-15 minutes to prepare platelet-poor plasma (PPP). Each PRP sample was standardized (approx. 250 000/mm^3) with autologous PPP as needed (Son *et al.*, 2008). Platelet reactivity was traced for 10 minutes at 37°C as previously described by Jeong *et al.*, (2010). 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), Ristocetin (1500 μg/ml) and Arachidonic acid (500μg/ml). Platelet aggregation assay was performed with turbidimetric monitoring device, Helena Agg RAM aggregometer (Helena Laboratories Corp, Beaumont, TX, USA), according to manufacturer's instructions. Briefly 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 Thrombin-antithrombin (TAT) complex Elisa kit (Cusabio Biotech Co. LTD). Standard curve was prepared for these two parameters taking absorbance of standard plasma 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 (Wayne, 1998).

Hematological 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).

Liver Function Tests (LFTs)

Blood collected in gel tubes, was allowed to clot at room temperature. Serum was separated by centrifugation at 2500 rpm for 10 minutes and used for estimation of liver function by measuring SGPT (serum glutamic-pyruvic transaminase), γGT (Gamma glutamyltransferase) and total bilirubin concentration (Fischbach *et al.*, 1992) by using standard kits of Human, Germany.

Histopathological Examinations

Microscopic changes were observed through random selection of liver samples from each test and control animals. Tissues were preserved in 10% formalin followed by dehydration in ascending grades of alcohol. Clearing by xylene and embedding in paraffin wax. Paraffin sections (5μm thickness) were stained with hematoxylin and eosin (H & E) for histological examination (Diab *et al.*, 2012).

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 $p < 0.05$ were considered significant and $p < 0.005$ as highly significant.

RESULTS

Table 1 shows *in vitro* comparison between water for injection, *Citrus limon* and heparin sodium. There was highly significant increase in TT and aPTT in animals of *Citrus limon* group as compare to control (water for injection), aPTT values were very near to the value of heparin sodium. Whereas Fb concentration was significantly reduced by *Citrus limon* as compare to control, however PT was not affected significantly.

Table 2 shows the effect of *Citrus limon* juice on BT, TT, PT, aPTT and Fb. There was highly significant increase in BT and TT in animals at moderate dose and significant decrease in Fb level as compared to control at 30 and 60 days. However at 60 days only BT was increased significantly in animals at low dose, whereas other changes were insignificant both at LCLD and HCLD.

Table 1: *In vitro* comparison of *Citrus limon*, heparin and control on coagulation parameters

Parameters	Groups		
	Water for injection	*Citrus limon*	Heparin sodium
Thrombin time (Sec)	5.8±0.2	422.2±49.4**	600.0±0.0**
Prothrombin time (Sec)	8.4±0.2	5.3±0.1	600±0.01**
Activated partial thromboplastin time (Sec)	193.0±29.8	509.7±35.5**	569.9±20.7**
Fibrinogen concentration (mg/dl)	102.03±18.18	11.26±2.05**	7.21±2.51**

n=10, Values are means ± S.E.M., **p<0.005 highly significant as compared to control

Table 2: *In vivo* effect of *Citrus limon* juice and warfarin on coagulation parameters

Parameters (Sec)	30 DAY				60 DAY				
	Control	LCLD	MCLD	HCLD	Control	LCLD	MCLD	HCLD	Warfarin
BT	99.60 ±5.35	103.8 ±5.25	152.9± 8.77**	107.7 ±5.54	101.66 ±5.8	128.10 ±10.3*	168.50± 8.32**	109.8± 5.28	133.9± 12.7*
TT	9.36± 0.92	9.2± 0.13	12.4± 0.59**	8.8± 0.08	9.41± 1.02	9.21± 0.41	12.86± 0.54**	9.17± 0.09	16.58± 1.74**
PT	5.2± 0.12	5.12± 0.02	6.34± 0.73	5.84± 0.04	5.32± 0.12	5.22± 0.02	6.73± 0.89	6.57± 0.14	13.59± 1.73**
Aptt	8.25± 0.62	12.52± 2.08	12.1± 1.55	8.67± 0.11	8.33± 0.61	12.51 ±1.82	12.22± 1.30	9.13± 0.07	15.73± 1.77**
Fb(mg/dl)	439.47 ±43.70	412.17 ±28.71	333.11± 29.55*	380.23± 32.62	437.50± 43.14	407.08 ±28.65	336.33± 26.48*	339.58± 24.35*	315.40± 25.54*

n=10, Values are means ± S.E.M
*P ≤0.05 significantly different as compared to control, **P ≤0.005 highly significant as compared to control
LCLD: Low *Citrus limon* dose 0.2ml/kg/day; MCLD: Moderate *Citrus limon* dose 0.4ml/kg/day; HCLD: High *Citrus limon* dose 0.6ml/kg/day.

Table 3 shows the effect of *Citrus limon* on hematological parameters. There was significant increase at MCLD in RBC, hemoglobin concentrations, MCHC and highly significant decrease in red cell distribution width (RDw) at 30 and 60 day, while animals received high dose of *Citrus limon* showed significant increase in RBC both at 30 and 60 day. Whereas at low dose there was no significant affect on any hematological parameters. However hematocrit, MCV, WBC and platelets count were not affected by any dose of *Citrus limon*.

Table 4 shows the influence of three doses of *Citrus limon* on platelet aggregation. There was significant inhibition in platelet aggregation at moderate dose induced by adenosine diphosphate (ADP), collagen (Col), epinephrine (Epi) and arachidonic acid (AA) both at 30 and 60 days. However significant inhibition in platelet aggregation induced by ristocetin (Risto) was only observed at high dose both at 30 and 60 days, whereas reduction in platelet aggregation was not affected at low dose.

Fig. 1 shows the effect of *Citrus limon* on Protein C (PC) at different doses. There was significant increase in PC level at moderate dose both after 30 and 60 days. Whereas PC level was not changed significantly at low and high dose.

Fig. 2 shows the effect of *Citrus limon* on thrombin antithrombin (TAT) complex. There was significant increase in TAT complex at moderate dose of *Citrus limon*, after 30 days and highly significant increase after 60 days. While significant increase was observed at high dose both after 30 and 60 days. However there was no significant change in TAT complex level at low dose of *Citrus limon*.

Liver function as determined by measuring SGPT, γGT and total bilirubin was not changed significantly as compared to control (data not shown). No histological changes were also observed in hepatic tissues of treated groups at any dose (data not shown).

DISCUSSION

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 and/ or prevention of chronic diseases. However little work has been done to observe the effect of *Citrus limon* on coagulation and hematological parameters.

In present study no significant effect of *Citrus limon* on PT was observed both *in-vivo* and in-vitro. Hence it

shows that *Citrus limon* has no effect on extrinsic coagulation factors, since prolong PT is due to deficiency of extrinsic coagulation factors, I, II, V, VII, and X (Rao *et al.*, 2000).

Similarly there was no significant change in aPTT *in-vivo*. It means that *Citrus limon* has no effect on intrinsic coagulation factors *in vivo*. Since prolong aPTT is due to deficiency of intrinsic coagulation factors I, II, V, VIII, IX, X, XI and XII (Khanin *et al.*, 1998; Mann *et al.*, 2003;

Kanahara *et al.*, 2008), but there was significant prolongation in aPTT *in-vitro*, Kuntic *et al.*, (2011) showed that Citrus flavonoids-Rutin and Hesperidin could prolong aPTT *in vitro*. However, in-vitro results alone not sufficient to draw definite conclusion about the usefulness of flavonoids in the diet. Vitamin C has role in prevention of blood protein as reported by Gonzalez *et al.*, (2010). Hence it could be suggested that *Citrus limon* may play a crucial role in preventing the change in PT, APTT or coagulation factors due to essential content, vitamin C.

Table 3: Effect of *Citrus limon* and aspirin on Hematological parameters of rabbits

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	61.00 ±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	62.30 ±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*	63.60 ±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*	65.10 ±0.83*	14.22± 0.08**	22.90± 0.67*	30.47 ±0.75	324.60 ±15.27
LCLD	30	4.34± 0.26	3.77± 0.28	9.78± 0.22	26.56 ±2.85	60.40 ±0.68	15.75± 0.19	20.10 ±0.79	28.88 ±0.43	296.50 ±33.37
	60	4.69± 0.43	3.84± 0.28	9.90± 0.21	25.63 ±2.78	61.70 ±0.77	15.91± 0.22	20.61 ±0.66	29.77 ±0.50	283.10 ±36.78
MCLD	30	4.81± 0.321*	4.14± 0.36	10.98± 0.55*	30.23 ±2.67	61.40 ±0.56	14.16± 0.06**	19.73 ±0.62	31.72 ±0.70*	287.10 ±37.29
	60	4.94± 0.30*	4.38± 0.412	10.95± 0.55*	32.37 ±2.37	62.30 ±0.44	14.33± 0.06**	20.53 ±0.53	33.29 ±0.59*	298.80 ±29.90
HCLD	30	4.67± 0.31*	4.17± 0.308	10.02± 0.20	31.63 ±7.96	61.50 ±1.00	15.85± 0.21	19.17 ±0.68	30.67 ±0.64	294.10 ±27.68
	60	4.81± 0.288*	4.27± 0.285	10.31± 0.37	33.94 ±2.64	61.90 ±0.75	15.81± 0.20	20.17 ±0.68	31.07 ±0.56	285.60 ±15.27

n=10, Values are means ± S.E.M.
*P≤0.05 significantly different as compared to control. **P≤0.005 highly significant as compared to control

Table 4: Effect of *Citrus limon* juice and aspirin on inhibition of Platelet aggregation

Groups	Days	% Inhibition of Platelet Aggregation				
		ADP (20µM)	Col (10µg/ml)	Epi (300 µM)	Risto (1500 µg/ml)	AA (500µg/ml)
Control	30	42.20±2.13	18.15±0.92	13.88±0.98	17.48±2.29	69.81±8.91
	60	42.30±4.12	18.21±1.14	13.41±0.73	21.82±2.61	75.77±6.66
Aspirin	30	29.90±0.85*	10.43±0.77*	12.85±1.54	9.98±0.93*	44.57±8.95*
	60	24.84±0.70**	10.72±0.93*	13.0±2.62	12.76±1.76*	46.14±8.88*
LCLD	30	32.01±0.75	22.53±1.95	11.77±1.30	13.15±2.40	65.87±5.72
	60	31.28±0.69	22.53±1.95	11.77±1.30	21.67±2.79	61.68±8.51
MCLD	30	31.43±0.85*	10.34±0.75*	9.37±0.48*	12.73±2.35	43.99±8.73*
	60	26.09±0.77*	10.25±0.72*	10.77±0.80*	21.40±2.58	48.19±7.29*
HCLD	30	50.50±7.62	22.78±2.03	11.98±1.08	10.67±0.97*	65.30±5.62
	60	47.66±8.09	21.76±1.94	13.0±2.62	13.89±2.86*	49.70±5.87*

n=10, Values are means ± S.E.M.
*P≤0.05 significantly different as compared to control. **P≤0.005 highly significant as compared to control
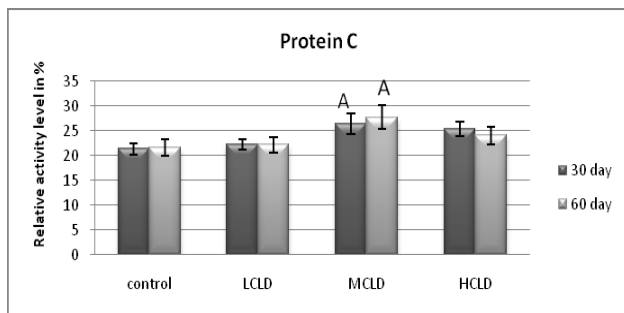
Fig. 1: Effect of *Citrus limon* on plasma activity of Protein C
n=10; Values are means ± S.E.M. Columns with (A) are significantly different, P≤0.05

Results of present study showed significant inhibition of platelet aggregation by *Citrus limon* at MCLD as well as HCLD in presence of ristocetin and arachidonic acid a response identical to aspirin (Walter *et al.*, 1969). This might be due to flavonoids, vitamin C and coumarin compound-xanthoxyletin which are reported to inhibit the activity of cyclooxygenase and lipoxygenase pathways (Nijveldt *et al.*, 2001) or modify membrane fluidity of platelets (Furusawa *et al.*, 2003). There are several studies which show that these compounds have anti-atherosclerotic and anti-platelet aggregation effects (Teng *et al.*, 1992; Heiss *et al.*, 2005; Kim *et al.*, 2008; Violi *et al.*, 2010; Bojic *et al.*, 2011). Hence it may be proposed that inhibition of platelet aggregation by *Citrus limon* is due to these essential components.
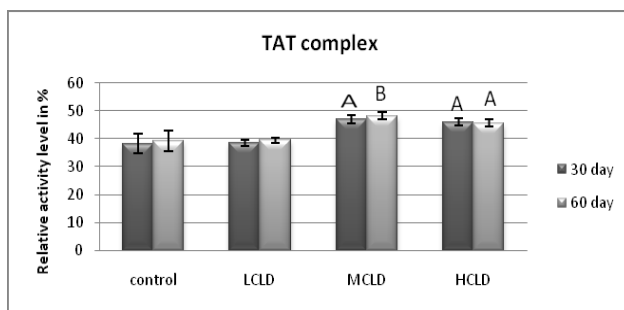

Fig. 2: Effect of *Citrus limon* on plasma activity of Thrombin antithrombin (TAT) complex
n=10; Values are means ± S.E.M; Columns with (A) are significantly different, P≤0.05; Columns with (B) are highly significant, P≤0.005.

Results of present study also reveals marked decrease in Fb, hence it may be concluded that inhibition of platelet aggregation might be due to this effect of *Citrus limon*, since platelets need fibrinogen to activate aggregation of platelets (Denninger *et al.*, 1987; Peterson *et al.*, 1989). More over there are several studies which show that platelet hyperaggregability and hyper-fibrinogenaemia are associated risk factors for coronary heart disease (De-Maat *et al.*, Silva *et al.*, 1996; Bach *et al.*, Miesbach *et al.*, Seppanen-Laakso *et al.*, 2010).

In present study *Citrus limon* have shown identical results both *in-vitro* and *in-vivo* i.e. prolonged TT, BT and decrease Fb, which might be due to impaired activity of thrombin, since thrombin plays an important role in platelet aggregation and conversion of fibrinogen to fibrin, during coagulation (Lane *et al.*, 2005; Wolberg, 2007; Doormaal *et al.*, 2008). These results suggest that *Citrus limon* have anticoagulant effects due to inhibition of thrombin, rather than altered activity of coagulation factors.

Present study shows increase in PC and TAT complex which suggest that anticoagulant and antiplatelet action of *Citrus limon* may be caused by impaired activity of thrombin. Since PC and TAT complex are important measures of inhibited thrombin (Arid, 2004; Chandler *et al.*, 2003; Lipe *et al.*, 2011). Several studies have shown that the level of PC and/or TAT complex, were decreased in thrombotic disease patients and cardio-vascular events (Moriau *et al.*, 1995; Little *et al.*, 2002). Hence *Citrus limon* by elevating PC and TAT complex, may provide beneficial effects in patients of thrombotic diseases.

Result of the present study showed significant changes in hematological parameters by *Citrus limon*. There was significant increase in RBC and Hb as compare to control. While aspirin showed significant increase in WBC and significant decrease in Hb, however there was no change in RBC which is in consistency to the result of Merchant *et al.*, (2004). According to this report aspirin causes chronic blood loss due to alteration of iron uptake. It could be suggested that beneficial hematological effects of *Citrus limon* are due to vitamin C, flavonoids, iron and pyridoxine as their essential components. Hence several studies showed their role in iron absorption due to antioxidant action or nitric oxide synthesis their consumption reduces the risk of death from CVD (Gonzalez *et al.*, 2010; Grassi *et al.*, 2010; McCullough *et al.*, 2012).

Results of present study showed no change in PLT, Hct and WBC by *Citrus limon*. Since normal platelet count is essential for normal blood coagulation that might not leads to bleeding problem in normal healthy subjects (Harrison *et al.*, 2007). No significant change in Hct is beneficial effect with respect to platelet aggregation. Since increase Hct enhance platelet adherence and aggregation (Aarts *et al.*, 1983). WBC's were not significantly changed because present study is on healthy rabbits and *Citrus limon* might affect WBC in disease conditions. Since Immuno-modulating and antibacterial effects of *Citrus limon* and vitamin C has been reported in several studies (Prabuseenivasan *et al.*, 2006; Sun *et al.*, 2009).

Contrary to this expectation, all observed beneficial effects in this study at MCLD seem to be depressed at

HCLD group, which might be due to high contents of vitamin C. Since high dose of vitamin C cause no change as compare to low dose (Antunes *et al*., 1998). Hence dosage adjustment is necessary to maintain drug concentrations within their therapeutic windows (Tripoli *et al*., 2007). It is suggested that different duration and dosage of *Citrus limon* juice may play important role in all their observed effects.

In recent years a dual role of thrombin has been revealed. It is not only involved in blood coagulation, but also associated with inflammatory response, cell-mediated immunity and cell death (Di-Cera, 2008; Jenkins *et al*., 2006; Krupiczojc *et al*., 2008). Anti-inflammatory response of the flavonoids of *Citrus limon* is already being documented (Chun *et al*., 2008). Hence it may be proposed that *Citrus limon* may retard progression of atherosclerosis and prevent cardiovascular diseases due to its anti-inflammatory affect. Since coagulation and inflammation has been reported as biological mediators of cardiovascular disease (Arid, 2004; Rallidis *et al*., 2004; Hamer *et al*., 2008).

On the basis of present data it may be concluded that the *Citrus limon* have maximum anticoagulant and anti-platelet effects in rabbits at moderate dose i.e 0.4ml/kg, which opens the door for further investigation on different doses of *Citrus limon*, since it is a food rich in flavonoids and vitamin C which may play vital role in the reduction of CVD risks.

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