

Anticoagulant, Antiplatelet and Antianemic Effects of *Citrus paradisi* (Grape Fruit) Juice in Rabbits

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Abstract

Medicinal plants are under continuous analysis worldwide to explore new medication to treat atherosclerosis and cardiovascular diseases. Citrus fruits have displayed utmost antioxidant activity, particularly flavonoids in *Citrus paradisi* have exhibited strong antioxidant and anti-inflammatory characteristics. This study was intended to examine effects of *C. paradisi* on blood coagulation and anticoagulation at doses of 0.1, 0.3 and 0.5 ml/kg. Highly significant increase in thrombin time (TT) and substantial decline in fibrinogen (Fb) level was noticed as related to control. There was substantial increase in TT, prothrombin time (PT) and activated partial thromboplastin time (aPTT) and noteworthy decrease in Fb level by warfarin. *C. paradisi* at 0.3 ml/kg considerably inhibited aggregation of platelet by collagen, adenosine phosphate, arachidonic acid and epinephrine, while there was also substantial rise in Protein C, TAT complex, RBC and hemoglobin concentration. In conclusion, these results suggest that the addition of *C. paradisi* to the diet of patients at risk of developing cardiovascular events could significantly decrease the morbidity or mortality in these patients due to high contents of phytochemicals like naringin, hesperidin, limonene and other flavonoids.

Keywords

Anticoagulant, Antiplatelet, Antianemic, Grape Fruit

1. Introduction

It has been documented that individuals consuming citrus fruits regularly have decreased risk of cardiovascular disease and ischemic stroke [1] [2]. Grapefruit is considered among the most widely consumed citrus fruits in the form of juice. The nutrients and bioactive phytochemicals present in grapefruit juice include sugars, organic acids,

flavonoids and other phenolic compounds [3] [4].

Flavonoids are widely dispersed group of polyphenol compounds with health-related characteristics that are based on their antioxidant activity, e.g. inhibition of platelet aggregation, anticancer, anti-inflammatory and antiviral activities. The flavonoids present in Citrus are Flavanones, flavones, and flavonols, although flavones and flavonols are found in low concentrations as compared to flavanones, but are potent antioxidants and free radical scavengers [5].

Numerous unique threats have been proposed as probable measures for better revealing of subclinical atherosclerosis; however clinically attention has been paid to parameters like lipoprotein (a), apolipoprotein (apo) A-I, apo B-100, C-reactive protein (CRP) and fibrinogen to reveal risk factors for systemic atherosclerosis [6]. Moreover, it has been indefinite whether screening for some of these unique biomarkers significantly expands risk estimation over that related to standard lipid screening [7]-[9].

Most significant reasons for atherosclerosis and cardiovascular diseases are flaws in coagulation and thrombosis [10] [11]. Prevention and treatment of thrombotic state have been investigated through coagulation and anticoagulant agents [12] [13]. Various factors of blood coagulation have been found to be associated with cardiovascular disease as well as hemostasis e.g. augmented level of factor VIII, von Willebr and factor and platelet activation [14].

The role of flavonoids and polyphenols has been studied to prevent and treat cardiovascular diseases due to several reasons [15] [16] including antioxidant and anti-inflammatory activity [17]-[19], as increase dietary intake of antioxidants may prevent atherosclerosis [20] [21], since increased cholesterol and occlusion is correlated [22]-[24].

Grape fruits (*C. paradisi*) have high vitamin C and potassium and are worthy source of calcium, folate, iron and other minerals. They also hold protective plant chemicals like phenolic acid, limonoids, monoterpenes, terpenes and bioflavonoids that are prevents cancer and heart diseases. Naringin is the chief bioflavonoid in grapefruit having diverse pharmacological affects e.g. antioxidant, hypolipidemic [25] [26] and anticarcinogenic activity [5].

Recently, thrombin has been found to have dual role and all the anti-inflammatory effects of grape fruits contribute towards anti-coagulant state in the body [27] [28]. Thus current study was aimed to measure anticoagulant, hematological and antithrombotic effects of *C. paradisi* to endorse its preventive and therapeutic role in various situations; hence thrombin and prothrombin time, activated partial thromboplastin time (aPTT), fibrinogen level (Fb), platelet function, thrombin antithrombin (TAT) complex and protein C (PC) were determined to evaluate the effects of these parameters on blood in rabbits.

2. Materials and Methods

2.1. *Citrus paradisi* Juice

Grapefruit (*C. paradisi*) was bought from the local vendors. The fruit was recognized by

Prof. Anjum Parveen (Director, Plant Conservation Center; University of Karachi) and voucher sample no C.P 09-10 was deposited in the Department of Pharmacognosy, University of Karachi. The fruit was peeled and pressed to obtain fresh juice which was used promptly after filtration. The fresh juice was then given by mouth in three doses *i.e.* 0.1, 0.3 and 0.5 ml/kg as per body weight of the animals.

2.2. Animals

Study was conducted on 60, healthy white rabbits of either sex with average body weight 1300 ± 50 grams after agreement from board of advance studies and research, University of Karachi. Steel rod bottom cages were used for individual housing of the rabbits with unrestricted contact to diet and water at a temperature $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$, humidity 50% - 60% with 12 hour light/dark cycle.

2.3. Dosing

Animals were allocated in 6 groups having 10 rabbit's each. *C. paradisi* was administered to three groups in the doses of 0.1, 0.3 and 0.5 ml/kg respectively for 30 days. Animal group served as normal control received saline in doses equivalent to their body weights, while remaining two groups received standard drugs aspirin and warfarin. Aspirin was administered on once daily basis (150 mg/kg) suspended in normal saline for 6 days per week [29], warfarin was given for 6 days only in distilled water *i.e.* 5 mg/kg first 3 days and 10 mg/kg next 3 days [30]. Drugs and juices were administered via gastric intubation.

2.4. Sample Collection

The National Committee for Clinical Laboratory Standards (NCCLS) approved guidelines H21-A3 were followed for Sample collection and handling [31]. Blood samples were gathered in EDTA and 3.8% tri sodium citrate containing tubes in the ratio of 9:1 and gel tubes at the end of dosing period from ear vein. Centrifugation of Blood samples was done to separate plasma at $2000 \times g$ for 10 min in Humax 14 K (Human, Germany).

2.5. Coagulation and Hematological Testing

Coagulation parameters were determined by Huma clot duo (Human, Germany) using standard reagent kits provided by Human, Germany. Turbidimetric clot were detected and coagulation endpoint was assessed in plasma. 100 ul thrombin reagent was used to incubate 200 ul plasma for TT; 100 ul plasma with 200 ul pre-warmed thromboplastin reagent was incubated for PT. While 100 ul plasma was incubated for 1 to 2 min at 37°C with 100 ul aPTT-EL reagent then 100 ul CaCl_2 was added for activated partial thromboplastin time. All plasma samples were incubated at 37°C for 3 min. Clauss method [32] was used to evaluate Fb concentration as defined by McNerlan *et al.* [33].

Fully automated hematology analyzer, Huma Count (Human, Germany) was employed to study hematological parameters *i.e.* red blood cell count (RBC), white blood

cell count (WBC), platelet count (PLT), Hematocrit (Ht), hemoglobin (Hb), mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH).

Helena Agg RAM aggregometer (Helena Laboratories Corp, Beaumont, TX, USA) was used for the assay of platelet aggregation. Plasma separated from blood was considered as platelet rich plasma (PRP), how-ever further centrifugation of blood for 10 - 15 minutes at 1600 - 2000 rpm yielded platelet-poor plasma (PPP). Standardization of PRP (approx. 250,000/mm³) with autologous PPP was done as required [34].

Platelet reactivity was found at 37°C for 10 min [35], for untreated PRP mixed with the aggregation reagent, the absorbance represents 0% aggregation; the absorbance denotes 100% of PPP control. Epinephrine (300 µM), Arachidonic acid (500 µg/ml), Adenosine diphosphate (20 µM) and Collagen (10 µg/ml), were used as platelet aggregation inducers. Platelet aggregation in samples was measured as described by Riaz *et al.* [36]. Pipette was used to measure and pour 450 µl PRP into cuvettes which were then incubated at 37°C. PPP cuvette was inserted into suitable channel and set to 100% aggregation; 50 µl of aggregation inducing substance was then added. Change in light transmission was considered as resulted aggregation and expressed as % PPP transmission.

The activity of PC and TAT complex in plasma was assessed by commercial Protein C and TAT complex Elisa kit (Cusabio Biotech Co. LTD). Standard plasma was used for the preparation of standard curves and absorbance was observed at 450 nm. The activity of TAT complex and Protein C in the samples was stated as percentage related to the level of activity of standard plasma. All the tests were performed following NCCL guidelines.

2.6. Statistical Analysis

Software package for statistical analysis (SPSS) version 17 was employed for Data entry and analysis presenting the data as mean ± S. E. M with 95% confidence interval. ANOVA followed by post hoc was executed for the comparisons of values with control. The Values of $p \leq 0.05$ denote significant and $p \leq 0.005$ denote highly significant results.

3. Results

The results of *C. paradisi* juice and warfarin on TT, PT, aPTT and Fb are shown in **Table 1**. There was substantially high increase in TT and significant increase in aPTT, however there was noteworthy decrease in Fb level at 0.3 ml/kg of *C. paradisi* compared to control. However warfarin caused substantially high increase in TT, PT and aPTT and significantly reduced Fb level.

Table 2 displays result of *C. paradisi* on aggregation of platelet. There was considerable inhibition in aggregation of platelet by collagen (Col), adenosine phosphate (ADP), arachidonic acid (AA) and epinephrine (Epi) at 0.3 ml/kg dose of *C. paradisi*. However at 0.5 ml/kg only AA revealed significant inhibition in platelet aggregation.

Table 3 shows results of *C. paradisi* in different doses on Protein C and TAT complex. There was considerable rise in Protein C and TAT complex at 0.3 ml/kg, while

Table 1. Effects of warfarin and *C. paradisi* on coagulation factors.

Group/Dose ml/kg	Parameters			
	TT (Sec)	PT (Sec)	aPTT (Sec)	Fb (mg/dl)
Control	9.55 ± 0.42	5.11 ± 0.06	8.18 ± 0.05	438.75 ± 42.38
<i>C. paradisi</i> (0.1)	9.20 ± 0.13	5.22 ± 0.02	12.51 ± 1.82	407.07 ± 28.65
<i>C. paradisi</i> (0.3)	12.41 ± 0.58**	6.35 ± 0.73	11.46 ± 0.31*	333.10 ± 29.55*
<i>C. paradisi</i> (0.5)	8.81 ± 0.08	5.85 ± 0.04	8.68 ± 0.11	380.24 ± 32.61
Warfarin	16.57 ± 1.74**	13.60 ± 1.73**	15.74 ± 1.77**	315.39 ± 25.54*

Values, mean ± S.E.M. *P ≤ 0.05 significantly unlike as compared to control **P ≤ 0.005 unlike extremely significant as compared to control Warfarin: 5 ml/kg (first three days) and 10 mg/kg (another 3 days).

Table 2. Effects of *C. paradisi* on aggregation of platelet.

Group/Dose ml/kg	parameters			
	ADP (20 µM)	Col (10 µg/ml)	Epi (300 µM)	AA (500 µg/ml)
Control	53.85 ± 5.36	18.20 ± 1.15	13.89 ± 0.97	60.84 ± 8.38
<i>C. paradisi</i> (0.1)	44.45 ± 0.97	22.52 ± 1.95	11.78 ± 1.31	61.69 ± 8.51
<i>C. paradisi</i> (0.3)	32.40 ± 0.58*	10.35 ± 0.75*	9.38 ± 0.49*	48.20 ± 7.31*
<i>C. paradisi</i> (0.5)	42.96 ± 0.75	21.75 ± 1.94	13.01 ± 2.63	49.71 ± 5.87*
Aspirin	32.29 ± 0.71*	10.44 ± 0.78*	12.84 ± 1.53	24.51 ± 1.18**

Values, mean ± S.E.M. *P ≤ 0.05 significantly unlike as compared to control **P ≤ 0.005 unlike extremely significant as compared to control ADP = Adenosine diphosphate; Col = Collagen; Epi = Epinephrine; AA = Arachidonic acid.

Table 3. Impact of *C. paradisi* on protein c and TAT complex.

Group/Dose ml/kg	parameters	
	Protein C	TAT complex
Control	32.08 ± 0.81	2.36 ± 0.15
<i>C. paradisi</i> (0.1)	36.25 ± 1.59	2.71 ± 0.14*
<i>C. paradisi</i> (0.3)	38.96 ± 1.48*	3.25 ± 0.07*
<i>C. paradisi</i> (0.5)	33.35 ± 2.23	2.71 ± 0.14

Values, mean ± S.E.M. *P ≤ 0.05 significantly dissimilar as compared to control.

there was no significant change in Protein C and TAT complex at 0.1 ml/kg and 0.5 ml/kg.

Table 4 shows results of *C. paradisi* on hematology; at 0.3 ml/kg considerable rise in RBC and hemoglobin was observed, while at 0.5 ml/kg only significant rise in RBC was seen whereas at 0.1 ml/kg no significant change in any hematological factors was observed. However WBC, PLT, hematocrit and mean corpuscle volume were not influenced by any dose of *C. paradisi*.

Table 4. Effect of *C. paradisi* and aspirin on hematological parameters.

Group/Dose ml/kg	parameters						
	RBC ($\times 10^5/\text{cm}$)	WBC ($\times 10^3/\text{cm}$)	PLT ($\times 10^3/\text{cm}$)	Hb (g/dl)	Ht (%)	MCV (%)	MCH (Pg/cell)
Control	3.84 \pm 0.3	3.32 \pm 0.4	291.04 \pm 27.6	9.77 \pm 0.4	28.23 \pm 4	62.4 \pm 0.7	19.5 \pm 0.6
<i>C. paradisi</i> (0.1)	4.68 \pm 0.4	4.14 \pm 0.4	283.10 \pm 36.7	9.90 \pm 0.2	30.21 \pm 3	61.6 \pm 0.7	20.6 \pm 0.6
<i>C. paradisi</i> (0.3)	4.95 \pm 0.3*	5.90 \pm 0.4	298.70 \pm 29.9	11 \pm 0.5*	32.38 \pm 2	62.3 \pm 0.5	19.7 \pm 0.6
<i>C. paradisi</i> (0.5)	4.82 \pm 0.3*	4.26 \pm 0.3	294.10 \pm 27.7	10.01 \pm 0.2	33.95 \pm 3	61.9 \pm 0.8	20.2 \pm 0.7
<i>Aspirin</i>	3.64 \pm 0.4	3.77 \pm 0.3	281.6 \pm 32.4	5.28 \pm 0.2	20.6 \pm 0.5	63.5 \pm 0.7	21.7 \pm 0.8*

Values, mean \pm S.E.M. *P \leq 0.05 significantly unlike as compared to control Aspirin: 150 mg/kg once daily for 6 days a week.

4. Discussion

This research was planned particularly to study the effectiveness of *C. paradisi* on coagulation factors. Dietary flavonoid is one of the herbal components which may alter coagulation and fibrinolysis. It is known that dietary lipids may alter factor VII [37], fibrinogen, plasminogen activator and tissue plasminogen [38].

The risk of bleeding may better be defined by coagulation assay like PT, TT, aPTT and Fb [39]. The effect of *C. paradisi* juice was thus determined at different doses on platelet count, activated partial thromboplastin time, thrombin time, prothrombin time, and fibrinogen concentration, since these parameters can better define the risk of bleeding [40], hence were utilized to observe the efficacy of *C. paradisi* on coagulation process as well as their efficacy in CVD.

Hepatic synthesis of clotting factors stimulated by increased catabolic rate of prothrombin, results in increased plasma concentration of clotting factors [41]. Therefore it may be concluded that increased concentration of cholesterol may lead to increased plasma concentration of coagulation factors or vice versa.

One possible explanation of prolonged aPTT could be cholesterol lowering effect of flavonoids and other components. Moreover current investigation revealed remarkable inhibition of platelet aggregation by *C. paradisi* stimulated by collagen, adenosine diphosphate, arachidonic acid and epinephrine, hence *C. paradisi* seems to be more effective against thrombosis since existing antiplatelet agents only inhibit thromboxane A2 or ADP platelet activation pathways without interfering number of other platelet activation pathways that may contribute to thrombotic event [42].

C. paradisi may have anticoagulant effect due to inhibition of intrinsic coagulation factor and thrombin, since factor IX and X are essentially required for thrombin generation [28]. There is evidence that deficiency of PC and TAT complex leads to cardiovascular events [10] [42] [43]. While present outcomes reveals raise in PC and TAT complex, which may lead to inhibition of thrombin activity, prolongation of TT and decrease Fb. hence it may be concluded that *C. paradisi* may have a beneficial effect in thrombotic disease patients.

Our result also revealed significant rise in hemoglobin and RBC as compared to control,

these beneficial effects of *C. paradisi* may be due to the presence of flavonoids (hesperidin hesperetin and nobiletin), vitamin C, pyridoxine and iron in citrus fruits reported in several studies [15] [17] [44]-[48].

5. Conclusions

Normal blood flow depends on the balance between pro-coagulant and anti-coagulant factors, unless this balance is shifted towards pro-coagulant mechanisms due to injury or increased vascular sclerosis. Thus present results show that grape fruit juice can act as an effective anticoagulant as it keeps the balance shifted towards anti-coagulant mechanisms.

These results suggest that addition of *C. paradisi* juice may be tested by adding to the diet of patients at risk of developing cardiovascular or neurovascular events and may also be helpful in improving the general health of these patients due to high contents of vitamin C, B and phytochemicals such as carotenoids, flavonoids and limonoids.

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Conflict of Interest Statement

None of the author involved in the study has received any financial assistance from any organization and there is no conflict of interest to declare.

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