<Original Article>

Water soluble tomato concentrate regulates platelet function via the mitogen-activated protein kinase pathway

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Abstract: Tomato extract has been shown to exert antiplatelet activity in vitro and to change platelet function ex vivo, but with limitations. In this study, antiplatelet activity of water soluble tomato concentrate (Fruitflow I) and dry water soluble tomato concentrate (Fruitflow II) was investigated using rat platelets. Aggregation was induced by collagen and adenosine diphosphate and granule-secretion, [Ca2+]i, thromboxane B2, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels were examined. The activation of integrin αIIbβ3 and phosphorylation of signaling molecules, including mitogen-activated protein kinase (MAPK) and PI3K/Akt, were investigated by flow cytometry and immunoblotting, respectively. Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were examined. Moreover, in vivo thrombus weight was tested by an arteriovenous shunt model. Fruitflow I and Fruitflow II significantly inhibited agonist induced platelet aggregation, adenosine triphosphate and serotonin release, [Ca2+]i, and thromboxane B2 concentration, while having no effect on cAMP and cGMP levels. Integrin αIIbβ3 activation was also significantly decreased. Moreover, both concentrates reduced phosphorylation of MAPK pathway factors such as ERK, JNK, P38, and PI3K/Akt. In vivo thrombus formation was also inhibited. Taken together, these concentrates have the potential for ethnomedicinal applications to prevent cardiovascular ailments and can be used as functional foods.

Keywords: blood platelet, cardiovascular disease, natural cardioprotective agent, thrombosis, tomato

Introduction

The prevalence of cardiovascular diseases in the developed countries is increasing at a high pace [7]. Platelet hyperactivation is the causal factor for the growth and advancement of atherosclerosis and an important contributor in cardiovascular pathology [14]. Rupture of atherosclerotic plaque can lead to death by development of cardiovascular disease (CVD) [4]. Therapeutic antiplatelet agents have been proved to reduce the incidence CVD [15]. There is need to slow down the progression of these diseases along with focused attention on the influence of dietary compounds on the cardiovascular system [6]. Recently, increase in the popularity of ethnomedicine and natural products has strengthened interest in traditional remedies for CVD [10]. During the last few decades, consumption and popularity of tomatoes have been increased and previous literature suggests the overall health benefits of tomatoes and evidence to be considered as cardiovascular protective food [1, 5]. Nutrients available in the tomato are accompanying theoretical or proven effects on reduced risk of degenerative diseases [28] and cardiovascular system like cardioprotective effects of lycopene [1]. Previous studies have put forward a link between lower incidence of CVD and consumption of tomato in Mediterranean countries [5, 23]. Dutta-Roy et al. [2] and O’Kennedy et al. [16] proved that platelet activity can be influenced by water tomato concentrates (Fruitflow I and Fruitflow II) in vitro and ex vivo. But the available data is insufficient to recognize the underlying mechanism of action. In this study, we explored the effects of tomato ex vivo as well as in vivo in more detail using a rat model.

We hypothesize that observed cardioprotective benefits accredited to the tomato could be linked to reduce hyperactivity of platelets and the suppression of platelet function in vivo. This type of natural antithrombotic agent could have an application in the new era of ethnomedicine and prevention of CVD like atherosclerosis, myocardial infarction and coronary artery disease.

Materials and Methods

Chemicals preparation

Water soluble tomato concentrate (WSTC; Fruitflow I) and
dry water soluble tomato concentrate (DWSTC; Fruitflow II) were obtained from DSM Nutritional product (Switzerland). The Fruitflow II was diluted to the appropriate concentration immediately before all experiments were performed. Collagen and adenosine diphosphate (ADP) were purchased from Chronolog Corporation (USA). Fura-2/AM was obtained from Sigma Chemical (USA). ATP Assay kit was obtained from Biomedical Research Service & Clinical Application (USA). TXB2 EIA kit was purchased from Enzo Life Sciences (USA). Serotonin EIA kit was purchased from LDN Labor Diagnostika Nord (Germany). Fibrinogen Alexa Fluor 488 conjugate was obtained from Molecular Probes (USA). Antibodies against phospho-p44/42, p44/42, phospho-p38, p38, phospho-SAPK/JNK, SAPK/JNK, phospho-P13K (p85), P13K (p85), phospho-Akt and Akt were acquired from Cell Signaling Technology (USA). All chemicals were of reagent grade.

Animals and dosage
Male Sprague Dawley rats (6 weeks old) weighing from 240 to 250 g were obtained from Orient Bio (Korea). The animals were acclimated for 1 week before the experiments and maintained in an air-conditioned animal room with a 12/12 h light/dark cycle at a temperature of 23 ± 2°C and humidity of 50 ± 10%. The rats were randomly divided into 3 groups; one normal group, one Fruitflow I treated group and one Fruitflow II treated group (n = 10 for each group). As based on tomato feeding of 3 g/day for human consumption [20], dose normalization for rats was done to 900 mg/kg [11]. Aggregation was monitored by measuring light transmission using an aggregometer (Chrono-log Corporation, USA). Platelet aggregation assay was performed and the reactions were terminated by adding ice-cold 2.5 mM EDTA and 100 µM indomethacin. After centrifugation at 12,000 × g for 5 min at 4°C, the supernatant was collected and serotonin release was measured with Fura-2/AM as previously described [9] and Fura-2 fluorescence in the cytosol was measured with the spectrophotometer as previously described by Schaeffer and Blaustein [22] using following formula: [Ca$^{2+}$]i, in cytosol = 224 nM × (F−min)/(Fmax−F), in which 224 nM is the dissociation constant of the Fura-2-Ca$^{2+}$ complex, and min and max represent the fluorescence intensity levels at very low and very high Ca$^{2+}$ concentrations, respectively.

Adenosine triphosphate (ATP) release assay
ATP assay was performed as previously described [8]. After the aggregation reaction was terminated, the platelets centrifuged and the supernatants were used for the assay. ATP release was measured in a luminometer (GloMax 20/20; Promega, USA) using an ATP assay kit according to manufacturer’s instructions.

Serotonin release assay
After terminating the platelet aggregation reaction, the mixture was immediately centrifuged at 12,000 × g for 5 min at 4°C. The supernatant was collected and serotonin release was measured with a serotonin ELISA kit according to the manufacturer’s instructions.

Evaluation of thromboxane B2 (TXB2) generation
Platelet aggregation assay was performed and the reactions were terminated by adding ice-cold 2.5 mM EDTA and 100 µM indomethacin. After centrifugation at 12,000 × g for 5 min at 4°C, the supernatant was collected and the concentration of TXB2 was measured using a TXB2 EIA kit according to the manufacturer’s protocol.

Measurement of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels
After terminating the platelet aggregation reaction, cAMP or cGMP level was measured using a cAMP EIA kit or cGMP EIA kit according to the manufacturer’s protocol, respectively.

Assessment of fibrinogen binding to integrin αmβ3
Fibrinogen Alexa Fluor 488 conjugate binding to washed
platelets was quantified by flow cytometry as previously described [8]. The fluorescence of each platelet sample was analyzed using a FACS Caliber cytometer (BD Biosciences, USA), and the data were analyzed using CellQuest software (BD Biosciences).

**Prothrombin time (PT)/activated partial thromboplastin time (aPTT)**

aPTT and PT were measured using an Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory, Italy). Briefly, the platelet-poor plasma (PPP) from rats treated Fruitflow I and Fruitflow II was incubated at 37°C for 7 min. 100 µL of the incubated PPP was mixed with 50 mL of cephalin in the process plate, and coagulation started with the addition of 1 mM CaCl₂ and 100 mL thromboplastin to the PPP for the aPTT and PT assays, respectively.

**Ateriovenous shunt**

The *in vivo* antithrombotic activity of Fruitflow I and Fruitflow II was evaluated in a rat extracorporeal shunt model by the method of Umetsu and Sanai [26], with a little modification. Briefly, the ateriovenous shunt model was used and blood circulation in the cannula was carried out for 15 min, and thrombus weight was determined immediately.

**Immunoblotting**

Immunoblots were performed as described previously [3]. Briefly, after terminating the aggregation reaction, lysates were then prepared by solubilizing and centrifuging the platelets in sample lysis buffer. Protein concentration was determined using a bicinchoninic acid assay. Total cell proteins (35 µg) from the platelet lystate were separated using 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes in transfer buffer. The membranes were blocked in TBS-T containing 5% dry skim milk and incubated with primary antibody diluted in 5% bovine serum albumin solution. The blots were then incubated with horseradish peroxidase-conjugated secondary antibody that was visualized by binding an enhanced chemiluminescence (Advansta, USA).

**Statistical analysis**

Data were analyzed with a one-way analysis of variance followed by a *post hoc* Dunnett’s test in order to measure statistical significance of the differences observed (SAS Institute, USA). All data are presented as the mean ± SE. *P* values of 0.05 or less were considered to be statistically significant.

**Results**

**Effects on agonist-induced platelet aggregation**

The effect of WSTC (Fruitflow I) and DWSTC (Fruitflow II) on agonist-induced platelet aggregation was evaluated. Our result showed that Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) dramatically inhibited platelet aggregations induced with collagen (1.25 µg/mL) to 32 and 33% and with ADP 2.5 µM to 24 and 14%, and with ADP 1.25 µM to 27 and 15%, respectively compared to normal control group 93 ± 10% (Fig. 2).

**Effect on intracellular calcium concentration**

Intracellular calcium release acts as a key role in platelet degranulation and aggregation [17]. Therefore, we examined whether Fruitflow I and Fruitflow II inhibit intracellular calcium concentration. As shown in Figure 3, Fruitflow I and Fruitflow II significantly reduced intracellular calcium mobilization by 85 ± 10% and 84 ± 10% respectively vs. control group (Fig. 2).

**Effect on granule secretion (ATP and serotonin release)**

Activated platelets release the contents of granules such as alpha and dense-granules and these granules content secretion enhances platelet activation including intracellular signaling pathway. We checked the effect of ATP and serotonin release of dense granules. These findings suggests that the Fruitflow I and Fruitflow II inhibited ATP release by 45 ±
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5% and 46 ± 6% respectively (Fig. 4) as well as serotonin release by 75 ± 10% and 71 ± 10% (Fig. 5).

**Effect on TXB2 production**

Thromboxane A2 amplifies platelet activation during hemostasis as a mediator. As it is physiologically unstable, so we investigated its metabolite, TXB2 production effect. The result showed that Fruitflow I and Fruitflow II markedly decreased both ADP and collagen-induced TXB2 production by 82 ± 7% and 85 ± 6% respectively (Fig. 6).

**Fruitflow I and Fruitflow II inhibit fibrinogen binding to integrin αIIbβ3**

Binding of fibrinogen to integrin αIIbβ3 which take pivotal role in the platelet activation and aggregation, induce outside-in signaling leads to adhesion, spreading and complete aggregation [24]. So, we checked the effect of Fruitflow I and Fruitflow II on fibrinogen binding to integrin αIIbβ3. Washed platelets (3 × 10^8/mL) were pre-incubated for 2 min at room temperature in the presence of 0.1 mM CaCl₂ and then stimulated with collagen or ADP for 5 min and fibrinogen Alexa Fluor 488 (20 µg/mL), and then fixed with 0.5% paraformaldehyde at 4°C for 30 min. Representative FACS analysis results of four independent trials. (B) Bar graph summarizing the inhibitory effect of Fruitflow I and Fruitflow II on fibrinogen binding. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control.
and Fruitflow II on fibrinogen binding to integrin α\(_{\text{IIb}}\)β\(_{3}\) and the result showed that fibrinogen binding to integrin α\(_{\text{IIb}}\)β\(_{3}\) reduced by 45 ± 5% and 38 ± 2% respectively as the control group (Fig. 7).

**Arteriovenous shunt thrombosis model**

It is well established that the arteriovenous shunt thrombosis models have been used to evaluate in vivo antithrombotic effects [3, 26]. We therefore, investigated the effect of Fruitflow I and Fruitflow II on extracorporeal shunts model thrombus formation. As shown in Figure 8, Fruitflow I and Fruitflow II potently reduced the thrombus weight by 58 and 61% respectively.

**Fruitflow I and Fruitflow II attenuates agonist induced mitogen-activated protein kinase (MAPK) and PI3K/Akt phosphorylation**

It is well-known that the phosphorylation of MAPKs (ERK, JNK and P38-MAPK) mediate platelet activation pathway and PI3K/Akt signaling pathway is another critical step for platelet activation and aggregation. Fruitflow I and Fruitflow II markedly blocked the phosphorylation MAPK’s and PI3K/Akt indicating that Fruitflow I and Fruitflow II mediate the MAPK’s and PI3K/Akt signaling pathway (Fig. 9).

**Discussion**

Previous studies proposed that prothrombotic suppression of platelet activation can prevent prothrombotic state [25]. It slows down the progression of atherosclerosis and minimizes the risk of stroke and myocardial infarction, and side effects of prophylactic regimes outweigh their benefits [19]. A previous study demonstrated that Tomato extract inhibited collagen and ADP induced platelet aggregation ex vivo [16]. However, the underlying mechanism is not completely understood.

Therefore, in this study, we investigated the effect of WSTC (Fruitflow I) and DWSTC (Fruitflow II) on agonist-induced platelet activation ex vivo and thrombus formation in vivo. Fruitflow I and Fruitflow II significantly inhibited platelet aggregation induced by collagen and ADP. These data suggested antiplatelet activity of Fruitflow I and Fruitflow II involved in glycoprotein VI and P2Y\(_{12}\) receptor signaling pathway. In order to demonstrate the inhibitory mechanism, downstream signaling components including calcium mobilization, granule secretions, integrin α\(_{\text{IIb}}\)β\(_{3}\) activation were examined. Platelet granule molecules play key roles in hemostasis, thrombosis, including activating other cells or cellular adhesion [29]. We therefore studied the role of Fruitflow I and Fruitflow II in the secretion of dense granules in platelets, by measuring ATP and serotonin release assay. It reduced ATP and serotonin releases on collagen and ADP induced platelet activation. These results demonstrated that the antiplatelet effect of Fruitflow I and Fruitflow II can occur through its suppressive effect of platelet secretion.

It is well-known that cytosolic calcium play key role in platelet activation. Increasing calcium levels leads to several signaling pathways involved in actin-myosin interaction, protein kinase C [13]. As a result of our Ca\(^{2+}\) mobilization study, Fruitflow I and Fruitflow II significantly decreased the intracellular calcium concentration on collagen and ADP induced platelet aggregation. Thromboxane A2 (TXA2) production from arachidonic acid via the cyclooxygenase pathway is an important positive feedback loop mechanisms for platelet activation [12]. A significant reduction of its production was observed by Fruitflow I and Fruitflow II treatment. Cyclic nucleotides such as cAMP and cGMP generation lead to inhibition of platelet aggregation but Fruitflow I and Fruitflow II have no effect on cAMP and cGMP levels (data not shown).

Inhibitory potential of Fruitflow I and Fruitflow II was further confirmed by a clear suppression of granule secretion and integrin α\(_{\text{IIb}}\)β\(_{3}\) activation (conjoint congregating step of platelet activation). The above findings propose that impaired α\(_{\text{IIb}}\)β\(_{3}\) conformational changes may be induced by pretreatment with Fruitflow I and Fruitflow II on exposure to its high affinity fibrinogen binding site formerly called as inside-out signaling, followed by the platelet agonist interactions. Inversely, these findings also suggests that the outside-in signaling (the next step in fibrinogen binding followed by post-ligand occupancy proceedings) leading to platelet shape change and spreading may be weakened by given treatment. Number of evidences are available indicating that platelets are constantly exposed to a variety of activating factors, including collagen, fibrinogen, ADP, vWF, thrombin and thromboxane and inhibitory factors such as endothelial-derived NO, prostacyclin (PG\(_{1}\)) and ADPase [21, 27]. Thrombotic or bleeding disorders can be developed by impairment of this equilibrium. Thus, a durable equilibrium between two opposing processes of platelet activation and inhibition is thought to be critical for normal hemostasis. Our study suggests that pretreatment of activated...

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**Fig. 8. In vivo effects of Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) on thrombus formation.**

The arteriovenous shunt model was used and blood circulation in the cannula carried out for 15 mints, and thrombus weight determined immediately. Bar graph shows the mean ± SE of at least three independent experiments. ***p < 0.001 vs. control.
Fig. 9. Fruitflow I (900 mg/kg) and Fruitflow II (45 mg/kg) attenuated the phosphorylation of mitogen-activated protein kinase (i.e. ERK1/2, JNK, and p38) (A) and PI3K/Akt (B). Cell proteins were extracted after aggregation termination and proteins separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes which then probed to antibodies against total and phospho ERK1/2, JNK, and p38, and PI3K/Akt. All immunoblots were carried out in at least four independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. control.
platelets with Fruitflow I and Fruitflow II may contribute to the maintenance of this balance.

In the present study, observations indicate that Fruitflow I and Fruitflow II inhibits collagen and ADP-induced ERK1/2, JNK and p38-MAPK and potent inhibitory effect on PI3K/Akt signaling, indicating modulation of both pathways may be involved in tomato's anti-platelet activity. Our study indicate that tomato have GPVI and P2Y12 suppression potential, thus antagonism of these receptors may represents a novel therapeutic regime. PT and aPTT results did not show difference between normal and treatment group suggesting that Fruitflow I and Fruitflow II do not induce changes to the integrity of extrinsic and intrinsic cascade of coagulation system (data not shown).

In accordance with previous findings on platelet hemostasis and pathophysiology of coagulation cascades [21, 27] we conclude the ex vivo capability of Fruitflow I and Fruitflow II to inhibit agonist induced aggregation, TXA2 production, granule secretion, α<sub>IIb</sub>β<sub>3</sub> activation, [Ca<sup>2+</sup>] mobilization, via MAPK and PI3K/Akt phosphorylations and in-vivo thrombus formation inhibition without affecting coagulation time, which illustrates the potential use of given compound as a nominee to be considered as an effective ethnomedicinal antithrombotic agent.

WSTC (Fruitflow I) and DWSTC (Fruitflow II) are potent inhibitors of agonist induced ex vivo platelet aggregation and granule secretion. In addition, it also significantly inhibited in vivo thrombus formation, while it had no effect on coagulation. Our findings indicate that Fruitflow I and Fruitflow II inhibit collagen and ADP-stimulated platelet function through modulation of signaling downstream via MAPK pathway.

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