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Tomatoes have natural anti-thrombotic effects

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The prevention of arterial thrombotic diseases has a high priority in developed countries. An inappropriate diet may be an important risk factor for thrombotic events. The daily intake of an anti-thrombotic diet may offer a convenient and effective way of prevention. The aim of the present study was to test tomato extracts for anti-thrombotic effects and to identify those varieties that have such an effect. A shear-induced platelet-function test (haemostatometry) was used to test anti-thrombotic potential *in vitro*. Extracts from those tomato varieties that showed a significant anti-thrombotic activity *in vitro* were further assessed *in vivo*, using a laser-induced thrombosis test in mice. One tomato variety (KG99-4) showed significant anti-thrombotic activity both *in vitro* and *in vivo*. KG99-4 inhibited not only platelet-rich thrombus formation but also had a thrombolytic effect. It is concluded that haemostatometry can detect and classify the anti-thrombotic potential of fruits and vegetables and offers a simple way of screening for such effects.

Tomatoes: Platelet aggregation: Thrombosis: Cardiovascular disease

The prevention of 'lifestyle-related atherothrombotic diseases' such as myocardial infarction and stroke is an important and urgent social task in many developed countries. Epidemiological studies have provided irrefutable evidence for the causative role of inappropriate diet in the development and clinical outcome of thrombotic diseases.

Mortality from cardiovascular diseases has been found to be significantly lower in the French population, who otherwise consume a high-fat diet, than those in other countries who eat similar high-fat diets (Ulbricht & Southgate, 1991). As an explanation, it was suggested that the French's habitual and large amount of red wine consumption was responsible for the lower cardiovascular mortality (Renaud & de Lorgeril, 1992). This so-called 'French paradox' accelerated laboratory studies for finding anti-thrombotic fruits and vegetables. Epidemiological studies provided evidence for foods with experimentally proven anti-thrombotic effects that can prevent cardiovascular diseases and stroke (Gillman *et al.* 1995; Joshipura *et al.* 1999, 2001; Liu *et al.* 2000; Bazzano *et al.* 2002).

Animal studies have suggested an anti-thrombotic effect of red wine. A special red wine (1987 Chateauneuf-du-Pape), which was brewed from a specified variety of grapes and a specified grape juice (Welch's natural purple grape juice), showed a significant anti-thrombotic effect in an animal model of thrombosis (Demrow *et al.* 1995). Juice made from a specified variety of raw onion also showed anti-thrombotic activity (Briggs *et al.* 2001). In contrast to grape juice, orange juice purchased from the market and grapefruit juice, prepared without special attention to variety, had no significant anti-thrombotic effect (Keevil *et al.* 2000). In an epidemiological study, wine (regardless of red or white), but not beer or spirits, had a preventive effect against stroke (Truelsen *et al.* 1998). In contrast, another study showed no difference in the cardiovascular mortality-preventive effect of beer, white or red wine and concluded that, in addition to the alcohol, the frequency of drinking was the decisive factor (Mukamal *et al.* 2003). Without adequate experimental studies, these contradictions cannot be resolved.

In arterial thrombotic diseases such as cardiovascular disease and stroke, platelets play a pivotal role. It is therefore the possible anti-platelet effects of fruits and vegetables that have to be tested. The conventional *in vitro* platelet-function test is platelet aggregometry, which measures platelet aggregation induced by various chemical agonists. However, platelet aggregation induced by shear

Abbreviations: CT1, coagulation time 1; CT2, coagulation time 2; H1, area of the pressure-recording curve showing 30 % pressure recovery; H2, area of the pressure-recording curve showing 90 % pressure recovery.

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forces is more similar to the pathological process of arterial thrombosis than agonist-induced platelet-aggregation tests (Fuster *et al.* 1992; Sixma, 1994). Furthermore, an *in vitro* test performed from native, non-anticoagulated blood has much more relevance to *in vivo* conditions than using anticoagulated blood (Kovacs *et al.* 1989; Gorog & Kovacs, 1990, 1995; Ratnatunga *et al.* 1992; Nakajima *et al.* 2000; Taka *et al.* 2002; Yamamoto *et al.* 2003).

The aim of the present study was to test different tomato varieties for anti-platelet and anti-thrombotic effects. As a first screening, a shear-induced *in vitro* platelet-function test was used, and then anti-thrombotic activities were studied *in vivo*, using a He–Ne laser-induced thrombosis test in mice.

Materials and methods

Animals

Male Wistar ST rats, 10–11 weeks old (SLC Co. Ltd., Hamamatsu, Japan) and male C57BL/6 mice, 10 weeks old (SLC Co. Ltd., Hamamatsu, Japan) were used. The animals were purchased 1 week before the experiment and given standard solid chow (CE-2; Japan Clea Co. Ltd., Tokyo, Japan) and tap water *ad libitum*. The animals were maintained in compliance with the Physiological Society of Japan's guiding principles for the care and use of animals in the field of physiological sciences.

Tomatoes

Tomato varieties were obtained from Kagome Co. Ltd. (Tochigi, Japan), the National Institute of Vegetable and Tea Science (Mie, Japan) and from the Japanese Agricultural Association Hyogo-Rokko (Kobe, Japan). All tomato varieties were cultivated in the same field and harvested in the same year.

Preparation of tomato filtrates. More than six tomatoes per variety were used to avoid individual variation among fruits. The fruits were cut into small pieces by knife and crushed by mortar at room temperature. The juice was centrifuged (1872 ***g***, 15 min, 4°C) and filtrated through a Millipore filter (pore size 0·2 μm; Millipore, Tokyo, Japan). Clear filtrates were stored at − 80°C until use.

In vitro *assessment of tomato filtrates on platelet reactivity and dynamic coagulation by haemostatometry*. Details of haemostatometry have been described elsewhere (Kovacs *et al.* 1989; Gorog & Kovacs, 1990; Ratnatunga *et al.* 1992; Taka *et al.* 2002). Blood was withdrawn from the abdominal aorta of anaesthetised rats 30 min after Nembutal administration (60 mg/kg intramuscularly). The non-anticoagulated blood was mixed with tomato filtrate or saline (9 g NaCl/l) (blood:filtrate, 9:1) in a syringe by inversion. Saline was used as the control in all *in vitro* experiments because it was practically impossible to choose something else as a control to various tomato extracts with probably different densities. The syringes with the blood samples were placed into a syringe holder (Fig. 1 (a), left) of a three-channel haemostatometer. This instrument, which is identical to the original haemostatometer used by Kovacs *et al.* (1989), was purpose-built in the Laboratory of Physiology, Faculty of Nutrition, Kobe Gakuin University, Japan. It should be emphasised that the haemostatometer is significantly different from the marketed instrument called the 'Clot Signature Analyzer'. The principle of haemostatometry is shown in Fig. 1. Briefly, liquid paraffin was pumped into the blood sample, thereby displacing blood through a plastic tube into a reservoir. The blood flow in the tubing was 0·057 ml/min. The perfusion of blood resulted in a steady 60 mmHg perfusion pressure. The tubing was punched with a fine needle (diameter 0·18 mm) at 150 s after blood withdrawal (Fig. 1 (a), middle), resulting in 'bleeding' into the surrounding warmed saline. The initial shear stress after punching was 375 dynes/mm^2. Eventually, platelet-rich haemostatic plugs occluded the punched holes, so that the 'bleeding' stopped. Subsequent to such haemostasis, coagulation led to the arrest of flow in the main tubing (dynamic clotting time). Pressure changes, which accompanied the above events, were monitored and analysed. The areas of the pressure recording curves (Fig. 1 (b)) showing 30 % (H1) and 90 % (H2) pressure recovery after the initial pressure drop due to punching were used as indices of platelet reactivity. The time from the start of the test until the first drop of perfusion pressure $\geq$ 10 mmHg (CT1) and to a level of $\leq$ 10 mmHg (CT2) reflected coagulation. H1 and H2 were used as indices of platelet reactivity (platelet adhesion plus platelet aggregation) and CT1 and CT2 of dynamic coagulation. The effect of the tomato filtrates on platelet reactivity was indicated by changes in H1 and H2. The effect on dynamic coagulation was shown by CT1 and CT2. An increase of H1 and H2 indicated inhibition, while a decrease of H1 and H2 showed enhanced platelet reactivity. Prolongation of CT1 and CT2 indicated the inhibition of dynamic coagulation while the shortening of CT1 and CT2 suggested hypercoagulation. Subsequent to clotting, the flow in the plastic tubing may re-start, causing an increase in pressure and indicating thrombolysis.

Assessment of anti-thrombotic effect by helium–neon laser-induced thrombosis in mouse carotid artery

Thrombus formation. Mice were anaesthetised with Nembutal (65 mg/kg, intramuscularly). A polyethylene tube (PE10; Becton Dickinson and Company, Spark, MD, USA) was inserted into the left femoral artery to inject the dye. The carotid artery (450–500 μm in diameter) was exposed by incision for laser irradiation. The mouse was placed on the stage of a microscope (Olympus Model CH-2; Olympus Co. Ltd., Tokyo, Japan) and the centre of the exposed carotid artery was irradiated with a laser (Model Neo-50MS, 30 mW power; Nihon Kagaku Engineering Co. Ltd., Osaka, Japan). Before irradiation, Evans blue dye (30 mg/kg) was injected intravenously. Thrombus formation at the site of irradiation was monitored under epi-illumination and simultaneously recorded on videotape using a CCD camera (Model TMC-7; Takenaka System Co. Ltd., Kyoto, Japan).

Calculation of thrombus size. Details of this technique have been described elsewhere (Ijiri *et al.* 2002). In every

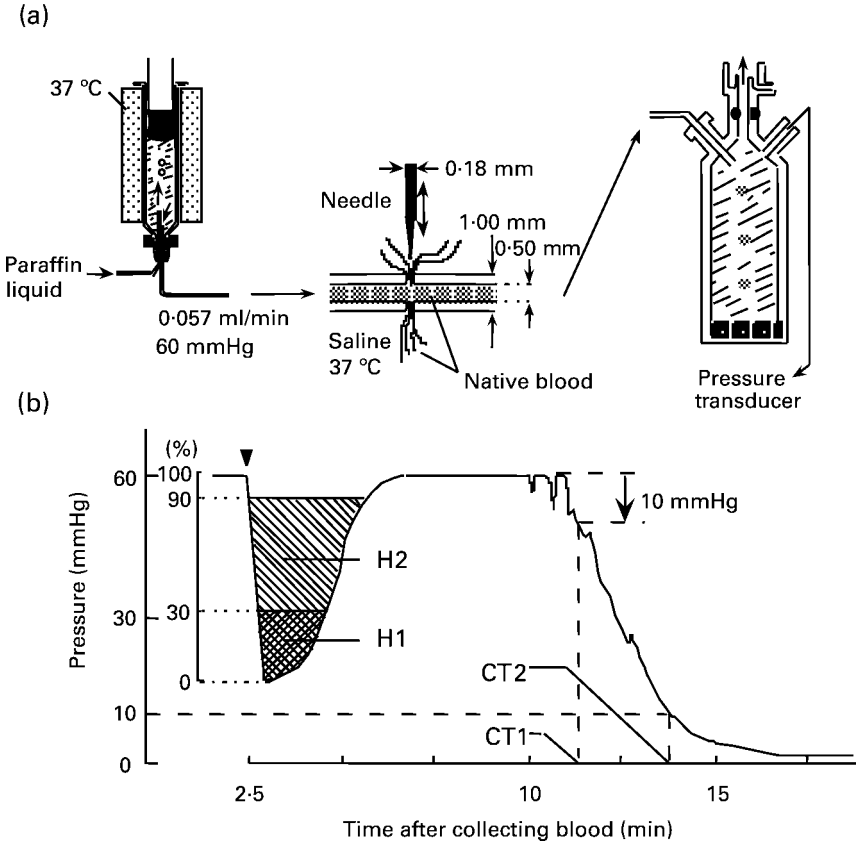

Fig. 1. The principle of haemostatometry (a) and a typical haemostatogram (b). CT1, coagulation time 1; CT2, coagulation time 2; H1, 30 % pressure recovery; H2, 90 % pressure recovery.

10 s, an image-frame of the thrombus was computer-analysed (Macintosh Power Mac G3; Apple, Cupertino, CA, USA). Thrombus mass was outlined by a defined threshold grey scale and the area of thrombus was calculated. Thrombus size was calculated by multiplication of the area and grey scale. Image analysis was performed by software (Automatix Inc., Billerica, MA, USA). During the growth of the thrombus, its size sometimes decreased due to embolisation. Thrombotic status was expressed by the total sum of sizes obtained in 10 min after irradiation.

Assessment of anti-thrombotic activity. The tomato filtrate was diluted with distilled water (the controls received distilled water) and was administered in a volume of 3·85 ml/kg by a gastric tube and the same volume of filtrate or water was given again 30 min after the first treatment. The mouse was then anaesthetised and the thrombosis experiment started 90 min after the second oral administration. Anti-thrombotic efficacy was assessed by the calculated total thrombus size (a smaller size indicated anti-thrombotic activity).

Molecular-size measurement of the components having anti-platelet (anti-thrombotic) activity

The tomato filtrate was ultrafiltrated through a molecular sieve membrane filter (NMWL 10 000; Millipore, Tokyo, Japan). The anti-platelet and anticoagulant activities of the filtrate and the non-ultrafiltrated samples were assessed by haemostatometry. Briefly, the non-ultrafiltrated sample of KG99-4 (starting filtrate) was ultrafiltrated until the volume of the starting non-ultrafiltrated sample had been reduced to 0·25 of the starting volume. Saline (9 g NaCl/l) was added to the remaining non-ultrafiltrate and ultrafiltration was performed. This procedure was further repeated twice. Subsequently, the anti-platelet activity of the first, second and third ultrafiltrates, finally the remaining non-ultrafiltrate and the starting samples were measured with haemostatometry.

Heat-stability measurement

The filtrates remained in boiling water for 5 min. When it cooled down to room temperature, the filtrates were tested with haemostatometry.

Statistical analysis

H1 and H2 were converted to logarithmic values and analysed by Student's paired *t* test. Raw CT1 and CT2 values were analysed by *t* tests. Thrombus size was analysed by unpaired *t* tests. Values were expressed as means and standard errors of the mean. $P<0{\cdot}05$ was considered as the limit of significance.

Table 1. Anti-platelet and anticoagulant activities of tomato varieties†

		Platelet reactivity		Coagulation	
Variety	Dilution	Inhibition	Enhancement	Inhibition	Enhancement
KG99-1	× 1	N	N	**	
	× 10	N	N		**
	× 100	N	N	N	N
KG99-2	× 1	N	N		**
	× 10	*			**
	× 100	N	N	N	N
KG99-3	× 1		*	N	N
	× 10	**			**
	× 100	**			*
KG99-4	× 1	**		N	N
	× 10	**			*
	× 100	**		N	N
	× 1000	N	N	N	N
KG99-5	× 1	N	N	**	
	× 10	N	N		**
	× 100	**		N	N
KG99-6	× 1	N	N	**	
	× 10	*			**
	× 100	N	N	N	N
KG99-7	× 1	**		N	N
	× 10	**		N	N
	× 100	N	N		**
KG99-8	× 1		**	**	
	× 10	N	N	N	N
	× 100	N	N	N	N
MA99-9	× 1		**	N	N
	× 10	N	N		**
	× 100	*		N	N
MIE01-1	× 1	N	N	N	N
	× 10	N	N	N	N
	× 100	N	N	N	N
MIE01-2	× 1	N	N	N	N
	× 10	N	N	N	N
	× 100	N	N	N	N
MIE01-3	× 1	N	N	N	N
	× 10	N	N	N	N
	× 100	*		N	N
MIE01-4	× 1	**		N	N
	× 10	**		N	N
	× 100	*		N	N
	× 1000	N	N	N	N
MIE01-5	× 1	N	N	N	N
	× 10	N	N	N	N
	× 100	N	N	N	N
MIE01-6	× 1	N	N	N	N
	× 10	*		N	N
	× 100	N	N	N	*
MIE01-7	× 1	N	N	*	
	× 10	N	N	N	N
	× 100	N	N	N	N
MIE01-8	× 1	**		ND	ND
	× 10	N	N		*
	× 100	*		N	N
JA00-1	× 1	*		N	N
	× 10	N	N	N	N
	× 100	N	N	N	N
JA00-2	× 1	N	N	N	N
	× 10	N	N		**
	× 100	N	N	*	
JA00-3	× 1	N	N	N	N
	× 10	N	N	N	N
	× 100	N	N	N	N
JA01-1	× 1	N	N	N	N
	× 10	N	N	N	N
	× 100	N	N	N	N

N, no effect, ND, not determined.
*$P<0{\cdot}05$, **$P<0{\cdot}01$.
† The activities of filtrates were measured by haemostatometry in undiluted (× 1) filtrates and in filtrates diluted from 10 times to 1000 times with saline (six measurements per sample).

Results

Anti-platelet and anticoagulant activities of tomato varieties

The results are shown in Table 1. Tomato variety KG99-1 had a variable effect on dynamic coagulation. The undiluted filtrate inhibited, the $\times 10$ dilution enhanced, and the $\times 100$ dilution did not affect dynamic coagulation. Undiluted KG99-3 enhanced platelet reactivity, but in the $\times 10$ and $\times 100$ dilutions inhibited platelets. KG99-4 inhibited platelet reactivity regardless of dilution. Of the tested varieties, KG99-4 showed the strongest and constant anti-platelet effects. The effect on platelet reactivity was independent of the effect on dynamic coagulation. Only KG99-4 had thrombolytic activity.

Anti-thrombotic effects of KG99-4 tested by helium–neon laser-induced mouse carotid artery thrombosis test

The effect of the orally administered KG99-4 filtrate is shown in Fig. 2. A significant inhibition of thrombus growth was observed after two oral treatments of mice with KG99-4, but not after only a single oral treatment.

Difference in anti-platelet activity of KG99-4 at harvest time

The tomatoes were harvested at various stages of maturation and anti-platelet and anticoagulant activities were examined by haemostatometry. The H2 values of the results are shown in Fig. 3. Anti-platelet activity was observed at all stages but the earlier the harvest stage, the stronger the platelet inhibition was. A weak thrombolytic activity was observed at the mature and over-mature phases.

Heat stability of the active component(s)

The undiluted filtrate of KG99-4 was treated in boiling water for 5 min and the activity was measured at room temperature by haemostatometry. The activity was heat stable.

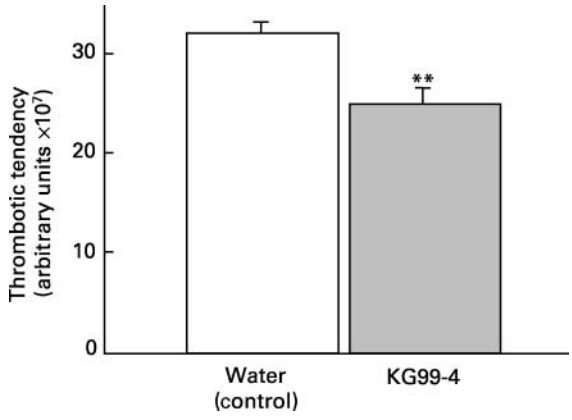

Fig. 2. The anti-thrombotic effect of tomato variety KG99-4 *in vivo*. Six measurements were performed in each sample. The values are means, with the standard errors of the means represented by vertical bars. **Mean value was significantly less than that for the water control ($P<0{\cdot}01$).

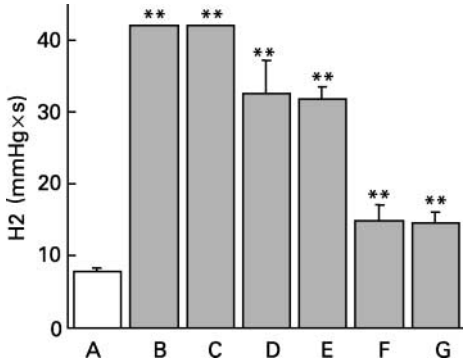

Fig. 3. Anti-platelet activity in tomatoes at different harvest stages according to the area of the pressure-recording curve showing 90 % pressure recovery (H2). Six measurements were performed in each sample. A, saline (control); B, filtrate from tomatoes at mature green phase; C, filtrate at turning phase I; D, filtrate at turning phase II; E, filtrate at pink phase; F, filtrate at mature phase; G, filtrate at over-mature phase. **Mean values were significantly greater than that for the saline control ($P<0{\cdot}01$).

Estimation of the molecular weight of the active component(s)

The results are shown in Fig. 4. An anti-platelet effect was observed in the starting filtrate, the first, the second and the third ultrafiltrates, but not in the finally remaining non-ultrafiltrate. Because only component(s) with a molecular weight of less than 10 000 Da could pass this particular sieve membrane filter, the present results indicate the molecular weight of the active component(s) to be less than 10 000 Da.

Discussion

The 'French paradox' is a great inspiration for finding natural anti-thrombotic effects not only in wines but also in various fruits and vegetables, in juices and in fermented products. The primary objective of the present study was to

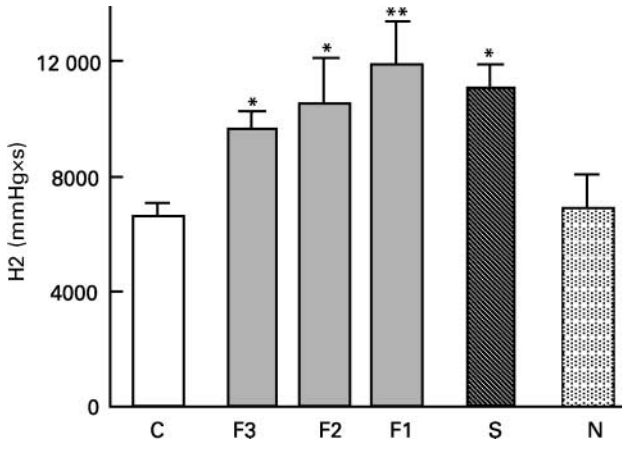

Fig. 4. Molecular weight of component(s) with anti-platelet activity. Six measurements were performed in each sample. C, saline (control); F3, ultrafiltrated three times ($\times 16$ dilution); F2, ultrafiltrated twice ($\times 4$ dilution); F1, ultrafiltrated once (non-dilution); S, starting sample; N, remaining non-ultrafiltrated fraction. Mean values were significantly greater than that for the saline control: *$P<0{\cdot}05$, **$P<0{\cdot}01$.

find antioxidant activity, which might be beneficial in atherothrombotic conditions. As fruits and vegetables are rich in polyphenolics, they have natural antioxidant activity. However, epidemiology has raised doubts about the effectiveness of antioxidant vitamins in the prevention of cardiovascular diseases (Brown *et al.* 2001). While it is easy to assess the overall antioxidant capacity of fruits and vegetables, the assessment of anti-thrombotic potential has been hampered by methodological difficulties. Even if a promising anti-thrombotic effect is observed *in vitro*, such an effect should be demonstrated also *in vivo*, after oral administration of the extract. This is extremely difficult in clinical trials, but can be done by using physiologically relevant animal models of thrombosis. This approach has already been followed (Demrow *et al.* 1995; Osman *et al.* 1998; Briggs *et al.* 2001; Shanmuganayagam *et al.* 2002).

The most difficult challenge is to find an animal model of thrombosis that closely resembles the pathological process of arterial thrombosis in man. In the present study the He–Ne laser-induced thrombosis test was employed. This test is based on the principle that subsequent to the administration of Evans blue dye, laser-irradiation of a targeted artery induces platelet-rich thrombus formation in the lumen of the vessel. The role of Evans blue dye is to absorb the otherwise harmless laser energy, convert it to heat, thereby causing heat injury of the endothelium (Kovacs *et al.* 1975; Yamamoto *et al.* 1989, 1997; Ijiri *et al.* 2002). This test, however, is complicated and is not suitable for screening large numbers of species or extracts. This is why it has to be preceded by an *in vitro* test; the *in vitro* test selects those species or extracts with significant effects, which are worthwhile for further studies. A novel platelet-function-test haemostatometry was used, to select specimens with definite anti-thrombotic effects. Haemostatometry uses shear stresses to induce platelet activation and aggregation. As the test is performed on native blood samples, in addition to platelet reactivity, this test allows the simultaneous measurement of dynamic coagulation and thrombolysis. The present findings revealed that the anti-platelet activity of tomatoes differed from variety to variety and that one variety, KG99-4, had potent anti-thrombotic activity.

Based on the present findings, it is suggested that haemostatometry can be useful in classifying fruits and vegetables on the basis of their effect on platelets. The criteria that are used for classification are shown in Table 2 and the classifications of the tested tomato varieties are shown in Table 3.

The active component(s) having anti-platelet effects were heat stable and have a molecular weight less than 10 000 Da. The chemical characteristics of the component(s) will be explored in forthcoming studies. Tomatoes are rich in the antioxidant lycopene and this was thought to be responsible for the beneficial effect in cardiovascular diseases (Arab & Steck, 2000). The present study does not support this assumption. Anti-platelet activity decreased in the process of maturation while, at the same time, the lycopene concentration in the tomatoes increased. It has been suggested that heat-stable and low-molecular-weight components such as adenosine and others might be responsible for the anti-platelet effect (Dutta-Roy *et al.* 2001). However, the great difference in anti-thrombotic potential between the various tomato varieties questions a single active component. Rather like in grapes, where a combination of active components have been found and thought to be responsible for anti-platelet activity (Shanmuganayagam *et al.* 2002), tomatoes may have several active components.

The KG99-4 tomato filtrate had a direct inhibitory effect on platelets. However, as the mechanism of the

Table 2. The criteria for classification of anti-platelet activity of fruits and vegetables*

Intensity index	Activity on platelet reactivity	Definition
+4	Very strong inhibition	Inhibition in undiluted filtrate ($P<0{\cdot}05$) and inhibition in filtrate diluted 10 times or more ($P<0{\cdot}05$) or the ratio of H2 of undiluted filtrate *v.* H2 of control $\geq 2{\cdot}5$ in all six measurements
+3	Strong inhibition	Inhibition in undiluted filtrate ($P<0{\cdot}05$) and the ratio of H2 of undiluted filtrate *v.* H2 of control $\geq 2{\cdot}5$ in three to five of six measurements
+2	Inhibition	Inhibition in undiluted filtrate ($P<0{\cdot}05$) and the ratio of H2 of undiluted filtrate *v.* H2 of control $\geq 2{\cdot}5$ in zero to two in six measurements
+1	Trend of inhibition	No inhibition in undiluted filtrate ($P>0{\cdot}05$) but enhancement in filtrate diluted 10 times or more ($P<0{\cdot}05$)
0	No effect	No inhibition in filtrate diluted no, 10 and 100 times ($P>0{\cdot}05$)
−1	Trend of enhancement	No enhancement in undiluted filtrate ($P>0{\cdot}05$) but inhibition in filtrate diluted 10 times or more ($P<0{\cdot}05$)
−2	Enhancement	Enhancement in undiluted filtrate ($P<0{\cdot}05$) and the ratio of H2 of undiluted filtrate *v.* H2 of control $\leq 0{\cdot}25$ in zero to two of six measurements
−3	Strong enhancement	Enhancement in undiluted filtrate ($P<0{\cdot}05$) and the ratio of H2 of undiluted filtrate *v.* H2 of control $\leq 0{\cdot}25$ in three to five of six measurements
−4	Very strong enhancement	Enhancement in undiluted filtrate ($P<0{\cdot}05$) and enhancement in filtrate diluted 10 times or more ($P<0{\cdot}05$) or the ratio of H2 of undiluted filtrate *v.* H2 of control $\leq 0{\cdot}25$ in all six measurements
+/−	Dilution-dependent inhibition and enhancement	Inhibition and enhancement in all dilutions ($P<0{\cdot}05$)

H2, area of pressure-recording curve showing 90 % pressure recovery.
* This was determined by haemostatometry (*in vitro*). Six measurements were performed in each sample and the intensity of activity to platelet reactivity was grouped on the basis of the activity to H2.

Table 3. Activities of tomato varieties on platelet reactivity

Inhibition or enhancement*	Tomato varieties
+4	KG99-4, KG99-7, MIE01-4, MIE01-8
+3	
+2	JA00-1
+1	KG99-2, KG99-5, KG99-6, MIE01-3, MIE01-6
0	KG99-1, KG00-2, JA00-2, JA00-3, JA01-1, MIE01-1, MIE01-2, MIE01-5, MIE01-7
−1	
−2	
−3	
−4	KG99-8, KG00-1
+/−	KG99-3, MA99-9, KG00-3, KG00-4

+/−, Activities on platelet reactivity are changed by dilution.
* Positive figures show inhibition, negative ones enhancement.

anti-thrombotic effect *in vivo*, a protective effect on vascular endothelium, similar to the one found in grapes, cannot be ruled out (Stein *et al.* 1999). Identification of the anti-thrombotic component(s) in tomatoes and the mechanism of the effect is the subject of future investigations.

In conclusion, a significant anti-thrombotic effect in tomatoes was demonstrated and it was shown that this effect varies from variety to variety. A particular variety (KG99-4) showed potent anti-thrombotic effects both *in vitro*, and also after oral intake, *in vivo*.

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