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ORIGINAL ARTICLE

Inhibitory effects of kiwifruit extract on human platelet aggregation and plasma angiotensin-converting enzyme activity

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Abstract

Previous human studies suggest that supplementation with kiwifruits lowers several cardiovascular risk factors such as platelet hyperactivity, blood pressure and plasma lipids. The cardiovascular health benefit of fruit and vegetables is usually attributed to the complex mixture of phytochemicals therein; however, kiwifruit's cardioprotective factors are not well studied. In this study, we investigated the effects of kiwifruit extract on human blood platelet aggregation and plasma angiotensin-converting enzyme (ACE) activity. A sugar-free, heat-stable aqueous extract with molecular mass less than 1000 Da was prepared from kiwifruits. Typically, 100 g kiwifruits produced 66.3 ± 5.8 mg (1.2 ± 0.1 mg CE) of sugar-free kiwifruit extract (KFE). KFE inhibited both human platelet aggregation and plasma ACE activity in a dose-dependent manner. KFE inhibited platelet aggregation in response to ADP, collagen and arachidonic acid, and inhibitory action was mediated in part by reducing TxA_2 synthesis. The IC_{50} for ADP-induced platelet aggregation was 1.6 ± 0.2 mg/ml (29.0 ± 3.0 μg CE/ml), whereas IC_{50} for serum ACE was 0.6 ± 0.1 mg/ml (11.0 ± 1.2 μg CE/ml). Consuming 500 mg of KFE (9.0 mg CE) in 10 g margarine inhibited *ex vivo* platelet aggregation by 12.7%, 2 h after consumption by healthy volunteers ($n = 9$). All these data indicate that kiwifruit contains very potent antiplatelet and anti-ACE components. Consuming kiwifruits might be beneficial as both preventive and therapeutic regime in cardiovascular disease.

Keywords

Angiotensin-converting enzyme (ACE), cAMP, kiwifruit, kiwifruit extract, platelet, platelet aggregation, tomato, TxB2

History

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Introduction

Consumption of fruits and vegetables protects against the development of cardiovascular disease (CVD) [1–3]. The bioactive compounds present in fruits and vegetables, although not yet well characterized, individually or in concert may protect the cardiovascular system by favorably modulating oxidative stress, plasma lipid levels, hypertension, platelet hyperactivity and other CVD risk factors [4, 5]. Hyperlipidemia, hypertension and hyperactivity of blood platelets are the critical contributors to pathogenesis of CVD [6]. Human blood platelets are not only involved in the thrombotic events but are also involved in the initiation and progression of atherosclerotic plaque. Consequently, platelets act as a bridge between the processes characteristic of atherosclerosis and thrombosis [7, 8]. Hyperactive platelets as observed in diabetes mellitus, insulin resistance, obesity and smoking contribute to the development of CVD [9–14]. There is also a positive linear relationship between blood pressure and predisposition to platelet hyperactivity. In fact, hypertension is another very important risk factor for CVD. In hypertensive patients with elevated plasma rennin–angiotensin activity, a five-fold increased incidence of myocardial infarction was demonstrated [15]. Angiotensin-converting enzyme (ACE) (EC 3.4.15.1, dipeptidyl carboxypeptidase) is a glycoprotein peptidyldipeptide hydrolase that cleaves histidyl leucine dipeptide from angiotensin I forming the potent vasoconstrictor angiotensin II. Studies demonstrated that ACE inhibitors significantly reduced the morbidity and mortality in patients with myocardial infarction and the incidence of recurrent myocardial infarction and ischemic events in patients with CVD, even in the absence of blood pressure-lowering effects [16–18]. The mechanism by which ACE inhibitors affect atherosclerosis is not well understood, but it has been postulated that these inhibitors may have multiple effects, including blood pressure lowering, antiproliferative effect on vascular cells, inhibitory effect on platelet aggregation and inhibition of lipid peroxidation [19–23]. The main ACE inhibitors in foods are ACE inhibitory peptides, flavonoids and other polyphenols. Flavonoids and flavonoid-rich extracts have been demonstrated as natural competitive ACE inhibitors [24], where the ACE activity is identified as a critical factor in regulating high blood pressure. Flavonoids are also inhibitors of cyclic nucleotide phosphodiesterase and TxA_2 synthesis, two of the main mechanisms responsible for the inhibition of platelets aggregation. Consequently, these bioactive components in fruits may reduce more than one CVD risk factors, such as platelet hyperactivity and hypertension [25, 26].

Kiwifruit is a best-known crop in the genus *Actinidia*. Kiwifruit contains very significant amounts of various phytochemicals, such as anthocyanidins and flavonols [27]. The common green kiwifruit, *Actinidia deliciosa*, has been used in several trials to examine effects on biomarkers relevant to CVD [4, 28–30]. Daily consumption of two or three kiwifruits reduced platelet aggregation response, blood pressure and plasma

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lipids [4, 28–30]. The presence of these diverse activities such as antiplatelet and anti-ACE was also demonstrated in differently processed extracts of kiwifruits [4, 31]. Therefore, it was reasonable to investigate whether a standardized kiwifruit extract contained both the antiplatelet and anti-ACE activities.

The aim of this study was therefore to isolate these bioactive components in a standardized extract of kiwifruits. We here describe that an aqueous kiwifruit extract contained compounds (Mw <1000 Da) that strongly inhibited both platelet aggregation and plasma ACE activity. The molecular mechanisms of their actions are not yet known; however, the platelet inhibitory action of the extract may be mediated in part by reducing TxA_2 synthesis.

Materials and methods

Materials

Green kiwifruits (1.class, New Zealand) and tomatoes were obtained from a local grocery shop in Oslo. Collagen was obtained from Chrono-Log (Havertown, PA), while ADP and arachidonic acid were obtained from Helena, Beaumont, TX. Bond Elut ENV cartridges were obtained from Agilent, Santa Clara, CA. The angiotensin-converting enzyme assay REA kit was from BÜHLMANN Laboratories AG, Switzerland, and theTxB_2 EIA assay kit and IBMX were obtained from Cayman, Ann Arbor, MI. Platelet factor 4 human ELISA kit was obtained from Abcam, UK. ACE of rabbit lung (EC 3.4.15.1), prostaglandin E_1 and captopril were obtained from Sigma, St Louis, MO. Lipidex-1000 column was obtained from Packard, Downers Grove, IL. All other reagents used were of analytical grade quality.

Preparation of sugar-free kiwifruit extract

To prepare the 100% fruit juice, the peeled kiwifruits were homogenized with a Brown Turbo Mixer 20–30 s at highest speed, the whole homogenate was boiled at 90 °C for 20 min, and the homogenate was then centrifuged at 22 000*g* for 15 min at 4 °C. The supernatant was then freeze-dried overnight. The dried extract was dissolved in double distilled water, and subjected to ultrafiltration with molecular weight cut-off of 1000 kD using Microsep™ Centrifugal Devices (Pall Corporation, NY). The ultrafiltrate was freeze-dried and reconstituted in water, and pH was adjusted to 7.4 for further studies. The kiwifruit extract (KFE) as prepared above contained more than 50% water-soluble sugars. Solid phase extraction column chromatography was used for the removal of sugars using a Bond Elut ENV cartridge (Agilent) [32]. Typically, 1 g dried extract (as prepared above) was dissolved in 4 ml MilliQ water and loaded onto the cartridge. The water-soluble components were eluted 3–4 times with 4 ml of MilliQ water. The cartridge was then dried out completely before elution of the non-sugar components with 3×2 ml of 100% methanol. The eluted fractions were dried under N_2 at 45 °C, and then re-dissolved in phosphate buffered saline (PBS) for further use. The freeze-dried materials from each of the extraction steps were tested for the presence of antiplatelet and ACE inhibitory factors. In some cases, sugar-free extract of tomatoes was also prepared following the same procedure as described.

Delipidation of KFE

To determine whether the cardioprotective activity (antiplatelet and anti-ACE factors) in KFE were a lipid-like compounds, the extract was delipidated using Lipidex-1000 column chromatography, as described [33]. Elution of polar compounds was performed with 0.5 mM K-phosphate buffer pH 7.4 (5–$10 \times$ column volume). The lipids retained in the column were eluted with methanol. Because only the aqueous fraction of the freeze-dried sample had both significant antiplatelet and anti-ACE properties further studies were carried out with the aqueous fraction.

Determination of sugar content in the KFE

Sugar contents of KFE extracts (before and after removal of sugars) were analyzed by HPAEC-PAD using ICS3000 software (Dionex Corp., Sunnyvale, CA). Trehalose was used as an internal standard. All samples were analyzed in duplicate.

Determination of total phenolic contents in the extracts

The concentration of phenolics in kiwifruit juice or extracts was determined using a spectrophotometric method as described before [34]. Typically, the reaction mixture was prepared by mixing 0.5 ml of methanolic solution of extract (1 mg/ml), 2.5 ml of 10% Folin–Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% $NaHCO_3$. The same procedure was repeated for the standard solution of catechin and the calibration curve was constructed. Total polyphenol content was calculated from absorption values and linear regression equation using catechin as standard. Results were shown as mg or μg CE (Catechin Equivalent).

LC-DAD/ESI-MS/MS of the purified sugar-free factors of kiwi fruits

Liquid chromatography/diode-array detection tandem mass spectrometry (LC-DAD/ESI-MS/MS) with electrospray ionization (ESI) was used (4000 Q TRAP linear MS/MS spectrometer, Applied Biosystems, Foster City, CA) for separation of components in KFE. The sugar-free KFE was subjected to an Agilent ZORBAX SB-C18 column (4.6 mm × 50 mm, 1.8 μm). Elution was accomplished by going from 100% mobile phase water-formic acid (100:0.1, v/v/v) to 100% acetonitrile-formic acid (100:0.1, v/v/v) over 35 min. MS scan 100–1000 Mw in negative mode and UV spectral scan (200–400 nm) were performed. Applied Biosystems software, Analyst version 1.6.1, was used for data acquisition and processing.

Platelet aggregation study

Antiplatelet activity of the kiwifruit juice or extract prepared from fruits at different steps of the preparation was investigated. The pH of all samples was adjusted to 7.4 with KOH solution prior to testing their effect on platelet aggregation. Venous blood was collected from volunteers who had not taken any medications for at least 14 days before donation. Blood (20–30 ml) was collected using a 21 G butterfly needle. Blood coagulation was prevented by mixing the blood samples with acid citrate buffer (135 mM) in the ratio of nine parts by volume of blood with one part by volume of acid citrate. Platelet-rich plasma (PRP) was prepared from the samples by centrifuging the blood at 180 *g* for 15 min at room temperature. The pH of KFE was adjusted to 7.4 with sodium hydroxide. KFE at different concentrations (0–1.44 mg KFE/ml) was incubated with 0.225 ml of PRP at 37 °C for 15 min after which the effect of the extract on agonist-induced platelet aggregation was monitored with the addition of either ADP (1–5 μM), collagen (1–5 μg/ml) and arachidonic acid (500 μg/ml). Controls were run in parallel using 10–30 μl of phosphate buffer, pH 7.4 instead of the fruit extract. Platelet aggregation in PRP was monitored using Aggram, Helena, Beaumont, TX, at a constant stirring speed of 1000 rpm at 37 °C [5]. Measurement of the extent of ADP-induced platelet aggregation in PRP was carried out at each time point.

The maximal aggregation (100%) was defined as the maximum change in light transmission observed over 15 min without extracts.

Inhibition of platelet aggregation is expressed as the decrease in the area under the curve compared with the control. In some cases, PGE_1 and aspirin were used as controls. Each sample was measured in triplicate. The IC_{50} values (the concentration necessary to reduce the induced platelet aggregation by 50% with respect to control) were obtained from concentration-effect curves.

For *ex vivo* studies, effects on platelet aggregation observed after treatment or control interventions are expressed as the percentage change in area under the aggregation curve after consumption of extract or placebo, as compared with baseline values.

Thromboxane B_2 assay

Thromboxane B_2 (TxB_2), the breakdown product of TxA_2, was estimated using a TxB_2 assay kit, as described before. Briefly, at the end of the platelet aggregation experiment, plasma was centrifuged at 22 000*g* for 10 s. The centrifuged plasma was diluted to 1:50 with assay buffer and was assayed according to the manufacturer's instruction.

Cyclic AMP assay

Cyclic AMP was determined in PRP as previously described [35]. PRP aliquots (700 µl) were incubated with KFE at different concentrations 0, 0.18 mg/ml (0.003 mg CE/ml), 0.36 mg/ml (0.006 mg CE/ml) and 0.72 mg/ml (0.012 mg CE/ml) for 12 min in the absence and presence of 10 µM IBMX (a phosphodiesterase inhibitor). In some cases, PGE_1 (10 µM) was used to compare its stimulation of cAMP with those of KFE in PRP. After 12 min of incubation, ethanol was added to the plasma at a ratio of 2:1, and vortexed for 10 s. The mixture was kept at 4 °C for 15 min. Subsequently, the samples were centrifuged at 1500 *g* for 15 min at 4 °C, and the resultant supernatant was dried under N_2 at 55 °C. The dried material was then reconstituted in assay buffer and cAMP was measured using the cAMP assay kit.

Inhibition of platelet factor 4 (PF4) release by KFE in platelet rich plasma

To measure the inhibitory effect of KFE on PF4 release, PRP (130 µl) was incubated in the presence of KFE at different concentrations 0, 0.5 mg/ml (0.009 mg CE/ml) and 1.5 mg/ml (0.027 mg CE/ml) and then followed by 3 µM ADP treatment, as described [36]. After 5 min of incubation, PRP was centrifuged to prepare platelet poor plasma and PF4 was the measured using the PF4 assay kit.

Stability of the antiplatelet factors in kiwifruit extract

Kiwifruit juice (KFJ) was prepared after homogenization of the peeled fruits, followed by centrifugation at 22 000 *g* for 15 min. The other fraction of supernatant was boiled at 90 °C for 20 min and centrifuged again. The supernatants as prepared above were kept at 4 °C for determining their antiplatelet activity at different time periods (0, 4, 8, 15 and 18 days). Stability of the supernatant was determined by incubating PRP with an equal amount of supernatant (after adjusting the pH to 7.4) for 15 min, and the inhibition was compared with control (in the absence of juice) using 3 µM ADP as an aggregating agent.

Effect of sugar-free KFE on serum ACE

The effect of KFE on the serum ACE activity was measured using the angiotensin-converting enzyme assay REA Direct kit. Typically, the serum (100 µl) was incubated with different concentrations of KFE for 15 min at 37 °C. After the incubation the ACE activity of the serum was measured. ACE inhibitory activity (%) = (A − B)/(A − C) × 100, where A represents cpm in the presence of ACE and sample, B is absorbance of control and C is absorbance of the reaction blank.

IC_{50} values (the amounts in mg per ml required to produce 50% ACE inhibition) were calculated using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA). Nonlinear regression was performed on the dose–response data and a sigmoidal curve with variable slope was fitted to each of the data sets. The equation used for the sigmoidal curve with variable slope was:

$$Y = \text{bottom} + (\text{top} - \text{bottom})/\left(1 + 10^{(\log IC50 - X)\text{Hillslope}}\right)$$

where bottom is the *Y*-value at the bottom plateau; top is the *Y*-value at the top plateau; log IC_{50} is the *X*-value of response halfway between top and bottom. Hill slope is the Hill coefficient or slope factor (controls slope or curve). For curve fitting, only the mean value of each data point, without weighting, was considered.

The minimum inhibitory concentration and the maximal inhibitory concentration were calculated from the *X*-values at the intercept between the slope and the bottom plateau and top plateau, respectively. The IC_{50} was calculated from the *X*-value of the response halfway between top and bottom plateau.

Effect of sugar-free KFE on rabbit lung ACE enzyme

To investigate whether the observed inhibitory effect of KFE on serum ACE was mediated through direct interaction with enzyme, we investigated the effect of sugar-free KFE on the activity of rabbit lung ACE. Rabbit lung ACE enzyme (6.6 mU) was incubated with increasing concentrations of sugar-free KFE (0–0.65 mg/ml) (0–0.012 mg CE/ml) for 30 min at 37 °C. After the incubation, residual ACE activity was measured.

Comparative effects of extracts of kiwifruits and tomatoes

Because, among all fruits, tomatoes and kiwifruits have the considerable amount of antiplatelet factors [4, 5], we therefore isolated and compared the yields and potencies of these factors in these two fruits. Effects of sugar-free extracts prepared from tomato and kiwifruit were then tested for their inhibitory effects on platelet aggregation and TxB_2 synthesis.

Human volunteers

Subjects were aged 25–60 years and had no history of serious disease or hemostatic disorders. Written informed consent was obtained from all subjects. The study was approved by the local ethical committee, Oslo university hospital. Suitability for inclusion into the study was assessed by using diet and lifestyle questionnaires and by medical screening, during which platelet function was assessed. Subjects were selected on the basis of high platelet function, as determined by the platelet aggregation response to ADP. Subjects habitually consuming dietary supplements were asked to suspend these supplements for a minimum of 1 month before participating in the study. Subjects were instructed to abstain from consuming drugs known to affect platelet function for a 10-day period before participation. Volunteers were overnight fasted. It was a single blind cross-over study. Venous blood samples of ~20 ml were drawn at each sampling time point (time 0) and then the volunteers consumed 10 g of margarine with or without 500 mg sugar-free KFE (9.04 mg CE) under the supervision of a lab personnel. Blood was then again drawn after 2 h after consuming KFE in margarine. For measurements of platelet function, blood was collected into plastic syringes and transferred into citrated blood collection tubes (final sodium

citrate concentration, 13 mM). Platelet aggregation was then measured as described above.

Statistical analysis

All variables were tested for normal distribution by use of the Kolmogorov–Smirnov test. A one-way repeated-measures ANOVA was used to compare the effects of different volumes or concentrations of kiwifruit juice, KFE and captopril on platelet aggregation and ACE activity, followed by Post hoc Bonferroni's t tests for comparison with controls. Mauchly's test was used to confirm the sphericity of the data. Results are presented as the mean ± SD. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed with IBM Statistics for Windows (20.0; SPSS Inc., Chicago, IL).

Results

Preparation of Kiwifruit extract

Table I summarizes the effects of KFJ (100% fruit juice after adjusting their pH to 7.4) on the platelet aggregation and plasma ACE activity. KFJ inhibited the platelet aggregation and ACE activity in a dose-dependent manner. KFJ at 30 µl (0.006 mg CE/ml PRP) inhibited 71% platelet aggregation and 80% of plasma ACE activity. The sugar-free KFE extract was then purified following the methods as outlined in Figure 1. Delipidation followed by ultrafiltration of the KFE indicated that the active factors in kiwifruit were water-soluble, heat-stable and molecular mass was >1000 Da. Kiwifruit extract had glucose (8.9 ± 0.4 mg/ml), fructose (9.9 ± 0.5 mg/ml) and sucrose (2.3 ± 0.2 mg/ml). Soluble sugars were removed by using SPE column chromatography. The de-sugarized KFE contained less than 0.2 ± 0.0 mg/ml of glucose and fructose and no detectable sucrose. The soluble sugars did not show any activity towards platelet aggregation and plasma ACE activity (data not shown). Typically, 100 g of kiwifruits produced 66.3 ± 5.8 mg (1.2 mg± 0.1 CE) of sugar-free KFE containing both antiplatelet and anti-ACE activities. Figure 2(A) and (B) show the UV spectrum and MS scan of KFE before and after sugar removal, respectively. The polar compounds were eluted earlier than the nonpolar compounds under the experimental conditions. After removal of sugars, the peaks eluted between 6 min to 16 min disappeared.

Effect of boiling on the activity of kiwifruit juice (KFJ)

Boiling of KFJ at 90 °C for 20 min did not destroy antiplatelet or anti-ACE activities. Rather boiling of the KFJ imparted stability to the inhibitory activity of the juice, as the non-boiled juice lost 75% of its activity within a week of preparation whereas the antiplatelet activity of the boiled juice remained the same till the day 18.

Inhibition of platelet aggregation by the sugar-free KFE

Sugar-free KFE inhibited platelet aggregation induced by different aggregating agents, ADP, collagen and arachidonic acid (data not shown). Figure 3 shows the dose-dependent

Table I. Effects of 100% freshly prepared kiwifruit juice on the platelet aggregation and serum ACE activity.

		Inhibition of platelet aggregation (%)	Inhibition of ACE activity (%)
Control		0	0
Kiwifruit juice (10 µl)	(0.002 mg CE/ml PRP)	23 ± 11*	61.43 ± 3.41*
Kiwifruit juice (20 µl)	(0.004 mg CE/ml PRP)	38 ± 13*	83.23 ± 4.32*
Kiwifruit juice (30 µl)	(0.006 mg CE/ml PRP)	71 ± 16*	86.36 ± 4.67*

Results are expressed as mean ± SD. Kiwifruit juice contained 0.050 mg CE per ml. Experiments were performed as described in the ''Materials and methods'' section. TxB_2 was measured after platelet aggregation was induced by ADP as described in the ''Materials and methods'' section. Each experiment was done in triplicate ($n = 6$). ACE activity was measured in serum as described in the ''Materials and methods'' section. Serum was incubated with different volumes of kiwifruit juice for 15 min. The ACE activity of serum was then measured using the ACE kit as described in the ''Materials and methods'' section. For details, please see the Statistical analysis section.

*$p < 0.05$ statistically significant different from controls (incubated in the absence of extract).

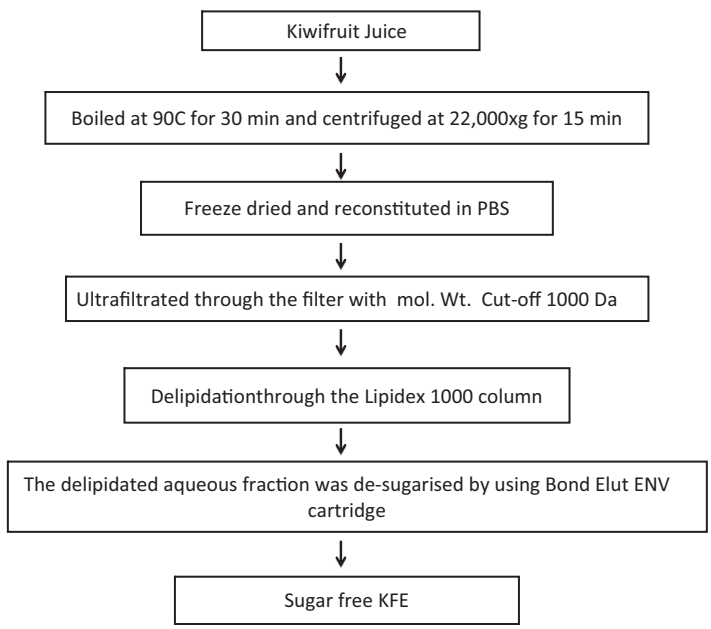

Figure 1. Outline of kiwifruit extract preparation. Kiwifruits were homogenized and the juice was boiled at 90 °C for 15 min and was centrifuged at 22 000*g* for 15 min. The supernatant was then delipidated using Lipidex-1000 column and followed by filtration using filter with molecular cut-off less than 1000 Da. The filtrated fraction was de-sugarized by using hydrophobic column, as described in the ''Materials and methods'' section.

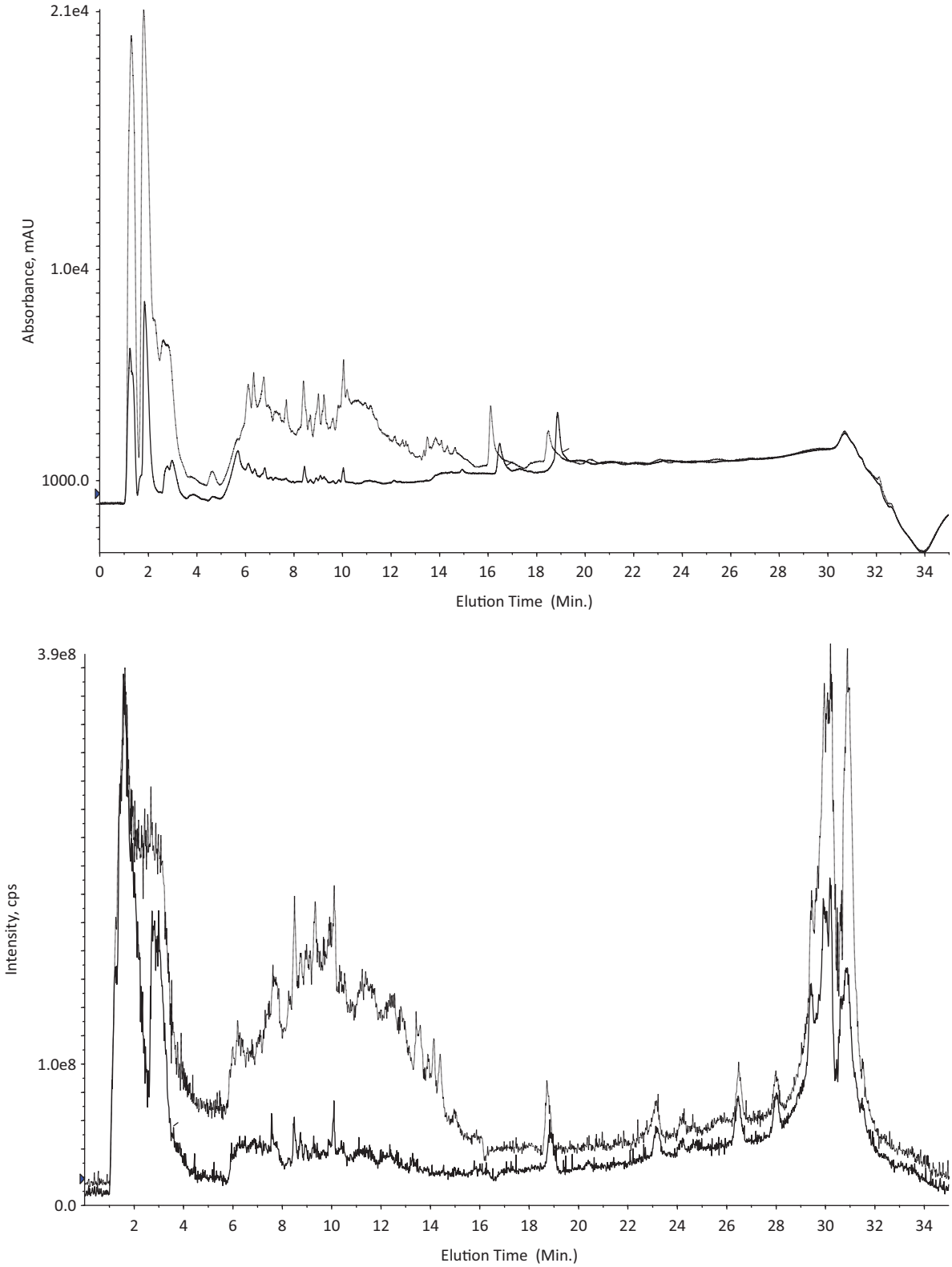

Figure 2. UV scanning and MS spectra of the delipidated, ultrafiltrated kiwifruit extract. The UV spectra (A) and MS (B) of the KFE before and after removal of soluble sugars. For details, please see the ''Materials and methods'' section. Sugar-free KFE (—); KFE (- - -).

inhibition of ADP-induced platelet aggregation by sugar-free KFE. ADP-induced aggregation was inhibited by 11% with 0.3 mg/ml (0.005 mg CE/ml), 71% inhibition with 0.9 mg/ ml (0.01 mg CE/ ml) and 80% with 1.44 mg/ml (0.03 mg CE/ml) of KFE, compared with control. KFE also inhibited collagen-induced platelet aggregation; however, the level of inhibition was lower with 1.47 mg/ml (0.03 mg CE/ml) and 2.0 mg/ml (0.04 mg CE/ml) incubations. Inhibition of arachidonic acid–induced platelet aggregation exhibited a very different profile with only 58% inhibition at the highest KFE amount 2.4 mg/ml (0.04 mg CE/ml tested and very little inhibition at lower amounts of KFE. The fraction isolated from kiwifruit was effective against all three platelet aggregating agents, collagen, ADP and arachidonic acid. The concentrations of KFE required to inhibit platelet aggregation by 50% (IC_{50}) induced by collagen and ADP were determined. The IC_{50} for ADP-induced platelet aggregation was 1.5 ± 0.3 mg/ml of KFE (0.03 ± 0.006 mg

CE/ml) and for collagen–induced aggregation, the value was 1.8 ± 0.2 mg/ml (0.03 ± 0.004 mg CE/ml). Under the similar conditions, PGE_1 (10 μM) and aspirin (5 μM) inhibited ADP-induced platelet aggregation by 90% and 73%, respectively.

Effects of KFE on PF4 release

To examine the effect of KFE on the extracellular release of granule contents, we quantitated PF4 (a constituent of α granules) in the supernatants from ADP-stimulated platelets in the presence and absence of KFE. The level of PF4 was determined in PRP in duplicate ($n = 2$). The presence of increasing amounts of KFE inhibited ADP-induced PF4 release in a dose-dependent manner. The release of PF4 was inhibited by 30% and 50% by KFE at different concentrations of 0.5 *mg/ml*, (0.009 *mg CE/ml*) and 1.5 mg/*ml* (0.03 *mg CE/ml*), respectively, compared with control ($p < 0.05$).

Effect of KFE on TxA_2 synthesis

To determine whether the inhibitory effect of KFE on platelet aggregation was due to the reduced synthesis of TxA_2, levels of TxB_2, the stable breakdown product of TxA_2, were measured in PRP in the presence and absence of KFE and aggregating agents ADP and collagen. Incubation of PRP with KFE inhibited TxB_2 production as shown in Table II. The inhibition of platelet aggregation by sugar-free KFE was concomitantly associated with inhibition of TxB_2 synthesis. KFE dose-dependently inhibited TxB_2 synthesis in the platelets. KFE at 1.68 mg/ml (0.30 mg CE/ml) inhibited ADP- and collagen-induced TxB_2 synthesis by 91% and 81%, respectively, compared with their respective controls ($p < 0.05$). KFE at this concentration inhibited 54% and 45% platelet aggregation induced by ADP and collagen, respectively.

Effect of KFE on cAMP synthesis

cAMP levels in PRP were determined after treating these cells with different concentrations of KFE 0, 0.2 mg/ml (0.003 mg CE/ml), 0.4 mg/ml (0.006 mg CE/ml), 0.7 mg/ml (0.01 mg CE/ml) in the absence and the presence of IBMX for 12 min. IBMX at 10 mM concentration increased cAMP level from basal level 54 pmol/ml to 72 pmol/ml ($p < 0.05$). The presence of 0.7 mg/ml (0.01 mg CE/ml) of KFE increased cAMP to only 89 pmol/ml ($p > 0.05$) in the presence of IBMX. KFE at 0.7 mg/ml (0.01 mg CE/ml) on its own increased cAMP levels to 58 pmol/ml ($p > 0.05$) from basal levels (54 pmol/ml) ($p > 0.05$). PGE1 at 10 μM increased cAMP level by fourfold compared with the basal levels ($p < 0.05$)

Effects of sugar-free KFE on activity in human serum ACE and pure rabbit lung ACE

KFE extract inhibited serum ACE activity in a dose-dependent manner (Table III). The IC_{50} of KFE for serum ACE was 0.6 ± 0.08 mg/ml (11.0 ± 1.2 μg CE/ml) whereas the IC_{50} of captopril was a 1000-fold less (0.6 ± 0.08 μg/ml). The orange extract prepared under similar conditions did not show any inhibition of ACE activity (data not shown). KFE also inhibited

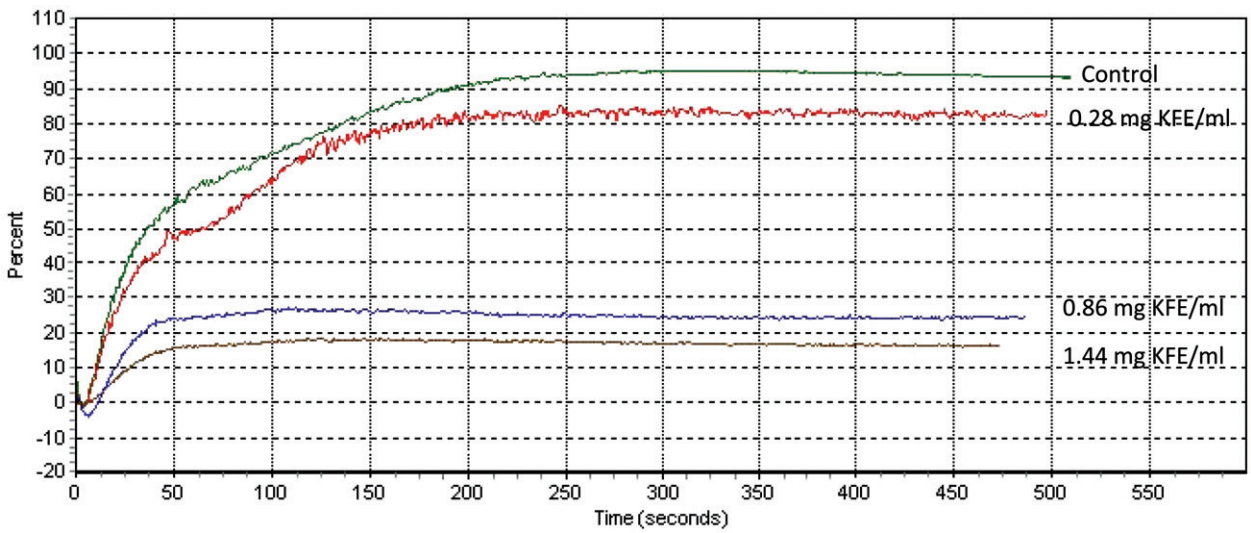

Figure-3. Dose-dependent inhibition of platelet aggregation by sugar-free kiwifruit extract. PRP was prepared as described in the ''Material and Methods'' section. PRP (final volume, 0.225 ml) was then incubated with different concentrations of sugar-free KFE for 15 min at 37 °C before ADP-induced aggregation was initiated. Aggregation was followed at 37 °C with stirring. A representative inhibition profile of KFE on ADP-induced aggregation of platelets was shown.

Table II. Effects of sugar-free kiwifruit extract on platelet aggregation and TxB_2 synthesis.

	ADP-induced aggregation		Collagen-induced aggregation	
Sugar-free KFE extract	% inhibition	TxB_2 (pg/ml)	% inhibition	TxB_2 (pg/ml)
0	0	125 ± 17	0	121 ± 14
0.42 mg/ml (0.008 mg CE/ml)	16 ± 5*	110 ± 10	8 ± 4*	83 ± 19*
0.84mg/ml (0.016 mg CE/ml)	25 ± 8*	25 ± 4*	13 ± 5*	35 ± 8*
1.68 mg/ml (0.032 mg CE/ml)	54 ± 7*	12 ± 3*	45 ± 11*	23 ± 7*

Results are expressed as mean ± SD. Experiments were performed as described in the ''Materials and methods'' section. TxB_2 was measured after platelet aggregation was induced by agonists as described. Each experiment was done in triplicate.
*$p < 0.05$ statistically significant different from controls (incubated in the absence of extracts) ($n = 6$).

Table III. Effects of sugar-free KFE and captopril on plasma ACE activity.

Kiwifruit extract (mg/ml)	μg CE/ ml	ACE activity (%)	Captopril (μg/ml)	ACE activity (%)
0	0	100 ± 0	0	100 ± 0
0.12	2.25	90.0 ± 12.0	1.2	40 ± 5*
0.34	6.12	68.0 ± 8.0*	2.5	19 ± 3*
0.63	11.34	57.0 ± 7.2*	5.0	11 ± 3*
1.25	22.50	38.0 ± 8.4*	7.5	5 ± 2*
2.06	37.08	20.0 ± 6.2*	10.0	4 ± 2*

Results are expressed as mean ± SD. ACE activity was measured in serum as described in the ''Materials and methods'' section. Serum was incubated with different amounts of sugar-free KFE for 15 min. The ACE activity of serum was then measured using the ACE kit as described in the ''Materials and methods'' section ($n = 6$). For details, please see the Statistical analysis section.

*$p < 0.05$ statistically significant different from controls (incubated in the absence of extracts).

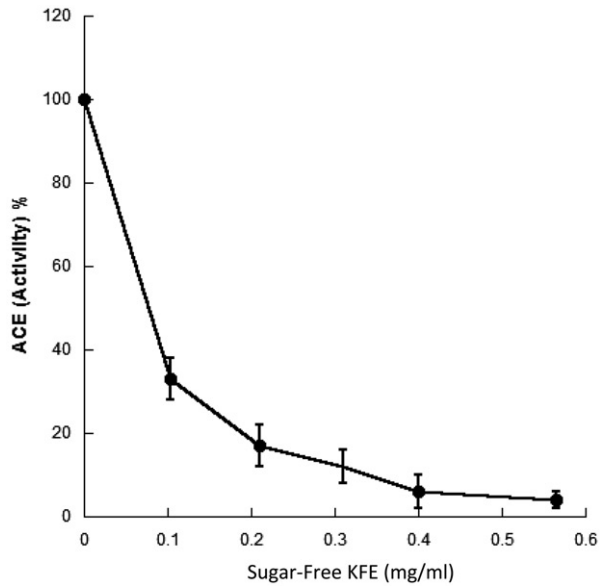

Figure 4. Inhibitory effects of sugar-free KFE on ACE activity. ACE enzyme was incubated with different concentrations of sugar-free KFE extract for 30 min at 37 °C. The residual activity of ACE was then measured as described in the ''Materials and methods'' section. $p < 0.01$ statistically significant different from controls (incubated in the absence of extracts). For details, please see the ''Materials and methods'' section.

rabbit lung ACE activity in a dose-dependent manner (Figure 4). KFE at 0.10 mg/ml (0.0018 mg CE/ml) inhibited 67% ACE activity whilst 100% inhibition of ACE activity was observed at 0.4–0.5 mg/ml of KFE (0.007–0.009 mg CE/ml) of KFE ($n = 3$).

Comparison of the antiplatelet factors in tomatoes and kiwifruit

Because the juice prepared from these two fruits (tomato and Kiwifruit) had the maximum antiplatelet activity [4, 5], we therefore compared both the yield of antiplatelet factors and their potency against platelet aggregation. The yields of the sugar-free extracts containing cardioprotective compounds were 66.1 ± 5.8 mg (1.2 ± 0.10 mg CE) and 72.6 ± 8.8 mg (1.4 ± 0.2 mg CE) per 100 g of kiwifruits and tomatoes, respectively. The IC_{50} (the amount required to inhibit 50% inhibition of platelet aggregation) for KFE and tomato extract were 1.6 ± 0.2 mg/ml (0.03 ± 002 g CE/ml) and 1.6 ± 0.11 mg/ml (0.030 ± 002 g CE/ml), respectively, $p < 0.05$. Both the extracts inhibited platelet aggregation and TxB_2 synthesis in a dose-dependent manner.

Acute study using crude kiwifruit extract

Baseline platelet function, as measured by the platelet aggregation response to ADP at 0 h, was initially determined for individual subjects. Supplementation with KFE in margarine resulted in a significant decrease in the platelet aggregation response at 2 h, whereas the control supplement resulted in no change. Platelet aggregation responses to ADP were significantly lower than baseline values by 12.9% (8.1–15.4%) $n = 9$, compared with control.

Discussion

This article reports that specially prepared sugar-free aqueous KFE exhibits an ability to inhibit both platelet aggregation and serum ACE activity. The water-soluble, colorless components in KFE have molecular weight less than 1000 Da, and do not loose bioactivity when boiled. The active components were found to be primarily associated with, or extractable from, the juice, the flesh surrounding the pips of the kiwifruit. Although there is no information on the nature of compounds presents in the sugar-free KFE, the possible presence of several compounds was indicated by UV and MS spectra. Total phenolic content extractable from kiwifruits by simple water treatment was reported around 0.6 ± 0.08 mg CE per g of kiwifruit pulp as measured by Folin–Ciocalteu assay [34]. In our case, the amount of total phenolic content in sugar-free KFE was 1.2 ± 0.10 mg CE per 100 g of kiwifruits. This difference was probably due to the fact that we followed the bioassay guided methods (such as centrifugation, boiling, ultrafiltration, delipidation and solid phase extraction column chromatography) to maximize the isolation of bioactivities (rather than polyphenols) from the fruits. Moreover, the bioactive compounds are not only polyphenols but could be of diverse nature as adenosine was shown to be an important antiplatelet compound in tomato extract [4, 5, 37, 38]. KFE had significant inhibitory effect on the multiple aggregating agents such as ADP, arachidonic acid and collagen. The mechanism of inhibitory action of KFE is not yet fully known. Reduction of TxA_2 synthesis in platelets may be in part involved in the KFE-mediated inhibition of platelet aggregation. Basal cAMP levels were not significantly different in the presence of the KFE as compared with control levels, and so cAMP may not be involved in the inhibition process [39]. Platelets are involved in the atherosclerosis process and therefore reduction of platelet hyperactivity decreases the incidence of CVD in diabetes, smokers and in metabolic syndromes [12, 26, 40]. Human trials demonstrated that both green and golden kiwifruits have cardioprotective effects [4, 28, 29]. Consumption of kiwifruits (green and golden) lowered the cardiovascular risk factors platelet aggregation, hypertension and blood lipids in both healthy human volunteers and smokers [4, 28, 29]. Our data presented here indicate that aqueous extract isolated from kiwifruits contains both antiplatelet and anti-ACE activities.

In addition to its antiplatelet activity, KFE inhibited ACE and thus may contribute to some extent to the reduction in blood pressure. KFE directly inhibited the ACE catalytic activity suggesting a possible interaction between the ACE protein and the components present in KFE as observed in case of ramipril-induced inhibition of ACE activity. As the IC_{50} value of components present in KFE was 1000-fold higher than that of the prescribed drugs for hypertension (e.g. captopril), KFE may be used as a preventative nutraceutical against especially for pre-hypertensive people, whose blood pressure is marginally or mildly high.

Our previous human trial data indicate that anti-ACE factors present in kiwifruits are absorbed in humans after consuming kiwifruits as reflected in reduction of both serum ACE activity and blood pressure compared with controls [28]. However, bioavailability of these factors in isolated extract may be different than from whole fruit. Further studies are required on their absorption, metabolism and bioavailability for a proper evaluation of their potential health effects. Future research should also be focused on further isolation and characterization of antihypertensive and antiplatelet properties of KFE. Increased serum ACE activity is associated with enhanced susceptibility to lipid peroxidation [21], and hence, the inhibitory effect of KFE on ACE activity can further contribute to an antioxidant environment and attenuated atherosclerotic risk. Reduction in serum ACE activity with the potent ACE inhibitor ramipril, even with no reduction in blood pressure, produces antiatherogenic effect in mice [41], and it is also associated with reduced mortality in cardiovascular patients [42].

Modulation of platelet reactivity towards collagen, ADP and ACE activity by KFE could be of potentially prophylactic and therapeutic benefit in preventing and halting pathologic processes that lead to CVD. The reduction in platelet function that can be achieved with this supplementation (up to 12.7% reduction of ADP aggregation) represents a significant reduction, which seems appropriate for the general healthy population at low risk of CVD-related events. It is now recognized that 20–30% of persons experience the so-called aspirin-resistance syndrome, in which the expected antiplatelet effects are not observed [43]. This finding indicates an advantage of the KFE's broad antiplatelet activity profile over single-target drugs such as aspirin. The greater benefits of combined antiplatelet therapies that target more than one mode of platelet aggregation, as compared with single-drug therapeutic strategies have been shown in clinical trials. Although the structure of individual compounds present in the extract is not known at present, the UV and MS spectra of the extract can be used as proof of reproducibility of sugar-free KFE preparation. The many different components in KFE contribute their cardioprotective effects via different mechanisms at different cellular signalling steps. Some of the compounds may have synergistic and protective effects during absorption of the extract. A thorough knowledge of the bioavailability of these bioactive compounds will help us to identify those that are most likely to exert protective health effects.

In conclusion, an extract of kiwifruit contained water-soluble and heat stable cardioprotective factors (Mw<1000 Da) that inhibited both platelet aggregation and serum ACE activity. Naturally occurring flavonoids have a potential to be used as mild or moderate ACE inhibitors. As the IC_{50} values of flavonoids were higher than those of the prescribed drugs for hypertension, flavonoids could be used as preventative nutraceuticals against hypertension rather than as being used as therapeutic drug for hypertension. KFE extract can be exploited for development of cardioprotective functional food. These claims still require further studies to elucidate their effects *in vivo*, including bioavailability, stability and metabolism of these compounds.

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Declaration of interest

The authors report no declarations of interest. This work was supported in part by Mills, DA Norway, and Throne Holst Foundation, Norway.

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