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Agnese Barin

Ranjit K. Das

Nasser E. Bastani

Per Ole Iversen

Asim K. Duttaroy

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Agnese Barin

University of Insubria

Ranjit K. Das

University of Texas Rio Grande Valley

Nasser E. Bastani

University of Oslo

Per Ole Iversen

University of Oslo

Asim K. Duttaroy (✉ a.k.duttaroy@medisin.uio.no)

University of Oslo

Research Article

Abstract

Fruit extracts may be cardioprotective via favorable modulation of platelet-blood vessel interaction. We here show that sugar-free extracts of tamarillo, horned melon (kiwano), and raspberry in a dose-dependent manner inhibited ADP-induced platelet aggregation in platelet-rich plasma. In contrast, pear extract had no such effect. Furthermore, analysisof untargeted metabolites revealed the presence of platelet inhibitory components such as benzoic acid, caffeic acid, and gallic acid in the sugar-free extracts of tamarillo, raspberry, and kiwano, but not in pear extract. All these three fruit extracts inhibited the platelet production of TxB2 and the release of platelet factor 4. In conclusion, our work suggests that tamarillo, raspberry, and kiwano inhibit platelet aggregation partly due to the high levels of anti-platelet compounds such as benzoic, caffeic, and gallic acids.

Introduction

Human blood platelets play a crucial role in hemostasis and thrombosis and are also involved in other biological processes, including inflammation, immunity, wound healing, cancer, and angiogenesis [2–5]. Platelets mediate complex vascular homeostasis via specific receptors, granule release, RNA transfer, and mitochondrial secretion, subsequently regulating hemostasis, infection, and innate and adaptive immunity. Activated platelets release mediators, such as 5-hydroxytryptamine (serotonin), ADP, ATP, and lysophosphatidic acid, and their hyperactivation is the causal factor for the initiation and progression of atherosclerosis and in the cardiovascular disease (CVD) [1, 2]. Atherosclerosis is the leading cause of CVD – a chronic inflammatory state in the arterial blood vessel walls with subsequent thrombus formation [3, 4]. Factors contributing to the development of atherosclerotic include increased lifespan, genetic susceptibility, adverse environment, and personal lifestyle choices such as smoking, a high-fat diet, and lack of exercise, all of which ultimately lead to plaque formation [4–6]. Regular blood platelet activity maintains hemostasis and adequate blood flow [3]. However, hyperactive platelets interact with vessel walls by shedding macro-particles, secreting adhesive growth factors, and inflammatory cytokines, interrupting blood flow and promoting a pro-thrombotic state. Activated platelets release mediators, such as 5-hydroxytryptamine (serotonin), ADP, ATP, and lysophosphatidic acid [7]. This is particularly evident in individuals with obesity, diabetes, a sedentary lifestyle, hypertension, and smokers [8–11]. Therefore, platelet activation is causal in triggering and maintaining the pro-inflammatory and pro-thrombotic state of obesity, creating a feedback loop involving adipose tissue, activated platelets, and vascular endothelium that culminates in an environment favorable for atherothrombotic vascular events [12, 13]. Hence, hyperactive platelets contribute to plaque formation and have thus been proposed as a CVD risk factor [14, 15].

Aspirin (acetylsalicylic acid) remains a cornerstone in anti-platelet treatment to prevent vascular thrombosis. However, resistance to aspirin treatment occurs in 25–30% of patients, and aspirin can also lead to several serious side effects, particularly bleedings, rendering it often unsuitable for primary CVD prevention [16–18]. In addition, other platelet inhibitors such as dipyridamole, ADP-receptor-, and glycoprotein antagonists also confer side effects, making it challenging to use them to prevent CVD. In

line with this, aspirin and other anti-platelet drugs are not recommended for primary CVD prevention or in subjects with low CVD risk. Therefore, finding alternative safe anti-platelet inhibitors for those with hyperactive platelets is essential to reduce CVD risk.

There is growing interest in naturally occurring compounds with potential anti-platelet effects that lack the side effects of current anti-platelet inhibitors [18]. In line with this, nutraceuticals with anti-platelet compounds are suggested to prevent CVD [19]. Various fruits are being investigated for their cardioprotective effects due to their high polyphenol content and bioactive component profile [20]. To this end, emerging data indicate that fruit extracts may be cardioprotective via favorable modulation of the platelet-blood vessel interaction [21].

Therefore, we now systematically investigate bioactive compounds' impact in high polyphenol-content fruits on human blood platelet aggregation. In addition, we aim to utilize the findings to characterize the mechanisms involved in this process to identify potential dietary anti-platelet components.

Dietary polyphenols have been shown to reduce platelet activation and aggregation via multiple pathways [18, 22, 23]. Plant polyphenols, such as flavonols, phenolic acids, anthocyanins, and procyanidins, are found in exceptionally high concentrations in various berries, e.g. tamarillo, and kiwano (horned melon) [24–27]. Tamarillo (*Solanum betaceum Cav.*) is a sub-tropical fruit known for its high nutritional value and beneficial effect on CVD, attributed to numerous phytochemicals [28]. Kiwano (*Cucumis metuliferus*), a fruit from the *Cucurbitaceae* family, also contains several polyphenols [29]. Raspberry (*Rubus idaeus*) is another fruit that is an excellent source of polyphenols [30]. Although these fruits contain extensive high polyphenols, their effects on platelet aggregation have not been studied. Thus, our first objective was to investigate the effects of extracts prepared from these fruits on ADP-induced platelet aggregation. Secondly, we aimed to unravel their mechanism of action on platelet activation. In this paper, we report that these fruits exhibited potent anti-platelet activity, and their anti-platelet effects can be due to benzoic acid, caffeic acid, and gallic acid. These fruits can be exploited to develop cardioprotective functional food.

Materials and Methods

Tamarillo (*Solanum betaceum*), Kiwano or horned melon (*Cucumis metulifer*), Raspberry (*Rubus idaeus*), and pears (*Pyrus communis*) were obtained from local grocery shops in Oslo. Hydro-benzoic acid, caffeic acid, and gallic acid were obtained from Sigma (Oslo, Norway). TxB_2 ELISA kit was obtained from Cayman Chemical (Ann Arbor, Michigan, USA), whereas a human platelet factor 4 (PF4) ELISA kit was obtained from Thermo Fisher (Waltham, USA). Fruitflow® (sugar-free tomato aqueous extract) was kindly provided by DSM Nutritional Products (Basel, Switzerland). ADP was obtained from Helena (New York, USA). All other reagents used were of analytical-grade quality.

Study participants and Ethical approval

For the study, healthy volunteers (n = 18) of both genders, aged 20–39 years, were recruited after assessing their dietary history. All volunteers abstained from platelet-interfering medications for at least 14 days before the study. Exclusion criteria were the presence of overt vascular, hematological, or respiratory disease, hypertension, infection, frequent consumption of drugs that affect platelet function (e.g., aspirin, paracetamol, non-steroid anti-inflammatory drugs, steroids, habitual consumption of n-3 fatty acid supplements), previous or current cardiovascular, neoplastic, infectious, or immune disease, those on specific dietary restrictions (e.g., vegetarians and vegans), and failure to comply with oral/written instructions. Ethical approval for this project was obtained from the Regional Committee for Medical and Health Research Ethics (no. 2015/396/REK Sør-Øst C), Norway.

Preparation of fruit extracts and the removal of sugars

To prepare 100% fruit juice, peeled fruits were homogenized with a Brown Turbo Mixer for 90–200 seconds at the highest speed, and the homogenate was centrifuged at 2500 x g for 15 min at 22 ℃. The first centrifugation removed most seeds and fruit pulp and was excluded from the experimental workflow. Then, the supernatant was centrifuged at 3000 x g for 15 min at 22 ℃ to obtain a more clarified fruit extract to be stored at -20°C. The supernatant was then concentrated, dried, and reconstituted in PBS, and the pH was adjusted to 7.4 for further studies. The fruit extracts, as prepared above, usually contain soluble sugars and polyphenols. Therefore, 1 ml of the fruit extracts was freeze-dried for 72 h after being stored at -80° overnight. These dried extracts were dissolved in double-distilled water and filtered before solid-phase extraction column chromatography was used to remove soluble sugars. The sugar-free extracts were dried and reconstituted in water before further experimental use[1, 23, 31].

When prepared as above, the fruit extracts contained more than 50–60% water-soluble sugars. Solid phase extraction column chromatography was used to remove soluble sugars using a J.T.Baker® Bakerbond SPE 1ml disposable extraction columns. Typically, 1 ml fruit extract (as prepared above) was loaded onto the cartridge after conditioning the column with 1 ml methanol and 1 ml distilled water. The cartridge was thoroughly dried before eluting the non-sugar components with 1 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. [1, 23, 31].

The freeze-dried and sugar-free materials from each extraction step were tested for anti-platelet factors. In addition, platelet-rich plasma was incubated with fruit extracts to measure its effects on the ADP-induced platelet aggregation [32]. As previously described, inhibition of platelet aggregation was expressed as a decrease in the area under the curve compared with the control [33].

Platelet-rich plasma preparation and platelet aggregation study

Venous blood (20–30 ml) was collected from volunteers who had not taken any medications for at least 14 days before donation. Blood coagulation was prevented by mixing the blood samples with acid citrate buffer (135 mM) in a ratio of nine parts by blood volume with one part by volume of acid citrate [32]. The

blood was collected using a butterfly needle and stored in B.D. Vacutainer containing sodium citrate (ratio 9:1) to prevent blood coagulation. The platelet-rich plasma (PRP) was prepared as described earlier [34]. Due to probable artificial activation, samples with fibrinopeptide A > 6 ng/ml were excluded [35]. The anti-platelet activity of the fruit extracts prepared at different steps was investigated for anti-platelet factors. Fruit extracts at different concentrations (0–1.50 mg/ml) were incubated with 0.45 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 ADP (10 µM). Controls were run parallel using 10 µl of phosphate buffer (pH 7.4) instead of the fruit extract. As described before, platelet aggregation in PRP was monitored using Aggram, Aggregometer (Helena, USA) at a constant stirring speed of 1000 rpm at 37°C [32]. The extent of ADP-induced platelet aggregation in PRP was measured 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 was expressed as the decrease in the area under the curve compared with the control. Each sample was measured in triplicate.

Untargeted sample analysis

Further chromatographic analyses were conducted to identify the unknown compounds in the sugar-free fruit extracts derived from tamarillo, kiwano, raspberry, and pear.

Chromatographic separation was performed with a Dionex Ultimate 3000 UHPLC system, coupled with a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer, in turn, equipped with a heat electrospray ionization (HESI) source (Bremen, Germany). The UPLC column used was HSS T3 C18 (Waters, Milford). The flow rate was 600 l min^{-1}, and the injection volume was 3 µl. Separations were performed using a binary gradient. The mobile phase was 0.1% formic acid in reagent water (A) and 0.1% formic acid in 70% acetonitrile and 30% methanol (B). A gradient of both mobile phases (running time 11 min) was used: 1–98% A at 0–8 min, 1% A for 9–10 min, 1% A for 10–11 min. The mass spectrometer was operated both in positive and negative ion mode, with the ion source parameters optimized, and the operating conditions were as follows: 3.0 kV (positive mode) and 2.5 kV (negative mode); sheath gas flow-rate 50 arbitrary unit; auxiliary gas flow-rate 13 arbitrary unit; heater temperature 425 °C; capillary temperature 263 °C; capillary voltage 45 V and lens voltage 60 V. Both ionization in positive (ESI+) and negative (ESI−) mode was used. The system operated in full-scan mode (70–1050 m/z) at a resolving power of 70,000 (200 ms). The resolution of the entire M.S. scan was 70,000, and the range was 700–1050 m/z. In MS2 mode at 35,500, samples were analyzed at 10, 30, and 60 normalized collision energy (NCE).

Inhibition of platelet factor 4 release by sugar-free fruit extracts in platelet-rich plasma

To measure the inhibitory effect of different fruit extracts on PF4 release, PRP (450 µl) was incubated in the presence of these extracts at different concentrations and followed by 10 µM ADP treatment, as described [36]. After 5 min of incubation, PRP was centrifuged to prepare platelet-poor plasma, and PF4 was measured using the PF4 assay kit. Human Platelet Factor 4 was assayed using a HuPF-4 ELISA kit (ThermoFisher), as described earlier [32].

Thromboxane B$_2$ assay

Thromboxane (Tx) A$_2$ is produced from arachidonic acid by many cells and causes irreversible platelet aggregation and contraction of vascular and bronchial smooth muscle. Thromboxane (Tx)B$_2$, the breakdown product of TxA$_2$, was estimated using a TxB$_2$ assay kit (Cayman Chemical, Ann Arbor, USA), as described before [32]. PRP (450 µl) was incubated with 10 µl of sugar-free fruit extracts. At the end of the platelet aggregation experiment, plasma was centrifuged at 3000 x g for 5 min at 22C. The supernatants were collected and stored at -80°C and subsequently used for TxB$_2$ assay using the ELISA Kit according to the manufacturer's instructions.

Data processing

For data acquisition, Thermo Xcalibur 3.0 (Thermo Scientific, San Jose, CA, USA) and Compound Discover (CD) software 3.1 (Thermo Scientific) were used. Natural product analysis and untargeted metabolomics workflow were selected, and the databases included the MZcloud database. Principal component analysis (PCA), a method for reducing the dimensionality of such datasets and increasing interpretability while minimizing information loss, was used to detect variation between data groups. Partial least squares discriminant analysis (PLS-DA), a method that reduces the variables to predict to a smaller set of predictors, was used to distinguish the compounds between two data groups. Differential peak area analysis was performed to normalize and compare the relative intensity of chromatographic peaks. This analysis allowed for the estimation of p-values through data transformation, specifically using log-10 areas. Peaks with larger differences in intensity and smaller p-values are considered more significant.

Statistics

The Results are presented as the mean ± S.D. Results were analyzed by the Student's t-test. Other statistical analyses were performed using ANOVA where appropriate; values were considered significantly different when $p < 0.05$. A two-tailed student t-test calculated statistical test, and the p-value was adjusted using Benjamin-Hochberg correction for a false discovery rate.

Results

Isolation of water-soluble extracts of fruits

Samples of different fruit extracts were evaluated regarding the anti-aggregation effect on platelet-rich plasma. 100% juice (w/v) was initially used. All the fruit extracts had a dose-dependent inhibition of platelet aggregation, the maximum inhibition being 70 to 80% (Table 1). Results were expressed as the percent inhibition of aggregation in response to ADP compared with control. The maximum inhibitory effect (70−80%) was with tamarillo, raspberry, and kiwano extracts, whereas pear had little or no activity (2−5%).

Table 1
The inhibitory effect of different fruit extracts on platelet aggregation in PRP.

Fruit extraect	Inhibition of platelet aggregation (n = 9)
Raspberry	77 ± 8%
Tamarillo	81 ± 11%
Kiwano	75 ± 12%
Pear	3 ± 2%
Values are means ± SD.	

Soluble sugars were removed by using SPE column chromatography. Under the experimental conditions, polar compounds were eluted earlier than nonpolar compounds. The de-sugared extracts contained less than 0.2 mg/ml of glucose, fructose, and no detectable sucrose. The soluble sugars showed no activity toward platelet aggregation (data not shown). One hundred grams of tamarillo, kiwano, or raspberry produced 6.57 mg, 1.59 mg, and 9.6 mg of sugar-free extract containing anti-platelet factors. Figure 1 shows the effect of different sugar-free fruit extracts on ADP-induced platelet aggregation in PRP. The dose-dependent inhibition of ADP-induced aggregation was also demonstrated. The IC_{50} (minimum concentration required for 50% inhibition of platelet aggregation induced by ADP in 450 μl of PRP of various fruit extracts was around 5–7 μl (100% juice). Because the juice prepared from these three fruits (kiwano, raspberry, tamarillo) had the maximum anti-platelet activity, we compared the yield of anti-platelet factors and their potency against platelet aggregation. The sugar-free extracts containing cardioprotective compounds yielded 6.57 mg, 1.59 mg, and 9.6 mg per 100 g of tamarillo, kiwano, and raspberry, respectively. The IC_{50} was 2.2 ± 0.2 mg/ml, 2.2 ± 0.2 mg/ml, and 2.6 ± 0.11 mg/ml for tamarillo, kiwano, and raspberry, respectively ($p < 0.05$). Because, among all fruits, tamarillo, kiwano, and raspberry have a considerable amount of anti-platelet factors, we isolated and compared their yields and potencies. Pear extract had no detectable effect. As expected, aspirin and Fruitflow® [37] markedly inhibited platelet aggregation (95%). The (IC_{50} in PRP induced by ADP was determined. Kiwano, raspberry, and tamarillo showed their anti-platelet effect., however, Tamarillo extract was the most potent platelet aggregation inhibitor.

Effect of fruit extracts on TxA_2 synthesis

To determine whether the inhibitory effect of different fruit extracts (sugar-free) on platelet aggregation was due to the reduced synthesis of TxA_2, the levels of TxB_2, the stable breakdown product of TxA_2, were measured in PRP in the presence and absence of these fruit extracts (500 μg) and ADP. Incubation of PRP with fruit extracts inhibited TxB_2 production. The inhibition of platelet aggregation by sugar-free fruit extracts was concomitantly associated with the inhibition of TxB_2 synthesis. Fruit extract inhibited TxB_2

synthesis in the platelets. All three sugar-free extracts inhibited ADP-induced TxB_2 synthesis by $80-90\%$ compared with their respective controls ($p < 0.05$).

Effects of fruit extracts on platelet PF4 release

To examine the effect of these fruit extracts on the extracellular release of granule contents, we measured PF4 (a constituent of platelet-derived α granules) in the supernatants from ADP-stimulated platelets in the presence and absence of different fruit extracts. The level of PF4 was determined in PRP in duplicates. The concentration of HuPF-4 in control was around a maximum of ≈ 3100 pg/ml. All three sugar-free extracts lowered the concentration of HuPF-4 by around $45-50\%$ compared with the control ($p < 0.05$).

Chemical analysis of sugar-free fruit extracts

Chemical analysis showed that benzoic acid, caffeic acid, and gallic acid were present significantly in all the fruit extracts (tamarillo, raspberry, and kiwano) that had anti-platelet activity (Fig. 2A-C). In contrast, the pear extracts did not contain detectable amounts of these compounds. Benzoic acid, caffeic acid, and gallic acid were then further evaluated on their platelet anti-aggregatory effect to assess whether they had a role in the inhibitory activity of these fruits. Benzoic, caffeic, and gallic acids showed potent anti-platelet activity (Fig. 3).

Discussion

The potent anti-platelet factors were identified in water-soluble extracts of tamarillo, kiwano, and strawberry, which significantly inhibited platelet aggregation, In contrast, the extract of pear had no such effect. Untargeted metabolite analysis suggested that benzoic acid, caffeic acid, and gallic acid were significant in all the fruit extracts (tamarillo, raspberry, and kiwano) but not in pear extracts.

We also found a decrease in TBx_2 and PF-4 levels in response to all three fruits, indicating that these molecular mechanisms may contribute to their anti-platelet effects. These effects are already impressive in their pure form but could be improved with synthetic or recombinant alternatives.

Nutraceuticals are bioactive substances found in everyday food or botanical-based sources. They can be delivered as dietary supplements or functional food, providing beneficial effects and essential nutritional components. Several studies have demonstrated the significant beneficial effects of nutraceuticals on immune system functions, such as boosting immunomodulatory activity and reducing the impacts of autoimmune disorders and hypersensitivity [38, 39].

Maintaining regular platelet activity is critical to overall hemostasis. However, due to several side effects, anti-platelet drugs (aspirin, etc.) cannot be used as primary prevention. Therefore, alternative safe anti-platelet inhibitors for the population with hyperactive platelets are being sought to reduce the risk of developing CVD. This paper demonstrates that extracts of three fruits, tamarillo, raspberry, and kiwano (horned melon), inhibited ADP-induced platelet aggregation.

In conclusion, the study's findings indicate the promising anti-aggregation potential of these fruit extracts, but further understanding of their mechanisms of action and evaluation for possible clinical use is needed. Human trials should also be conducted to assess the in vitro effects of these fruits. Nevertheless, our data suggest that these fruits have the potential therapeutic and/or preventive application against CVDs and potentially a broader range of platelet-related acquired disorders.

Declarations

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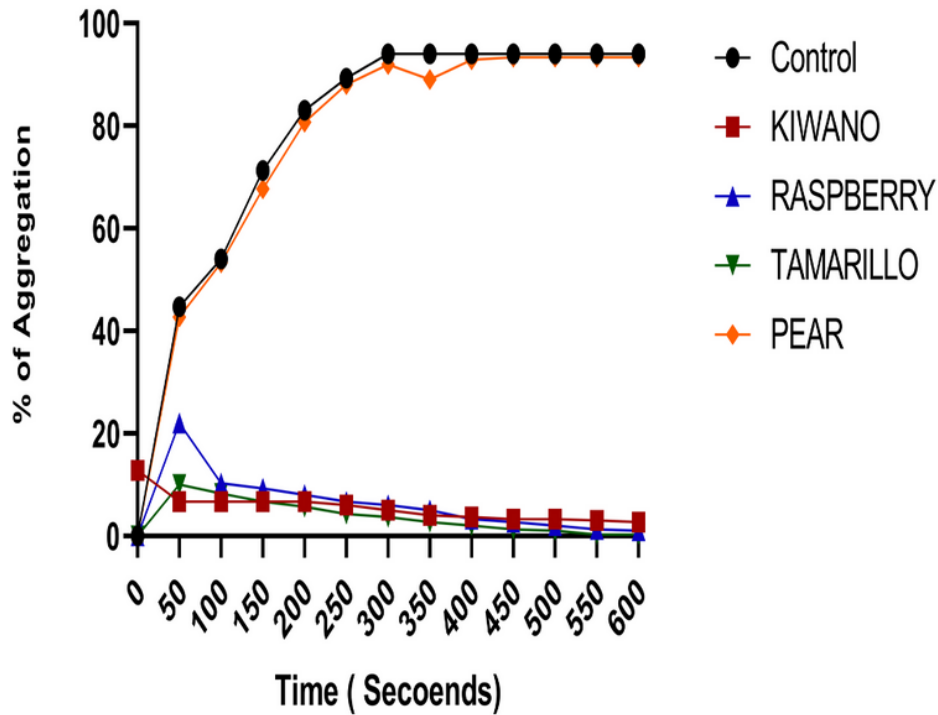
Figures

Figure 1

Inhibition of platelet aggregation by different fruit extracts and Fruitflow

PRP was prepared as described in the "Methods" section. PRP (final volume, 0.225 ml) was then incubated with different amounts of sugar-free fruit extracts 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 Fruits (a), b), (c), and (d) on ADP-induced aggregation of platelets is shown. For details, see the section "Methods."
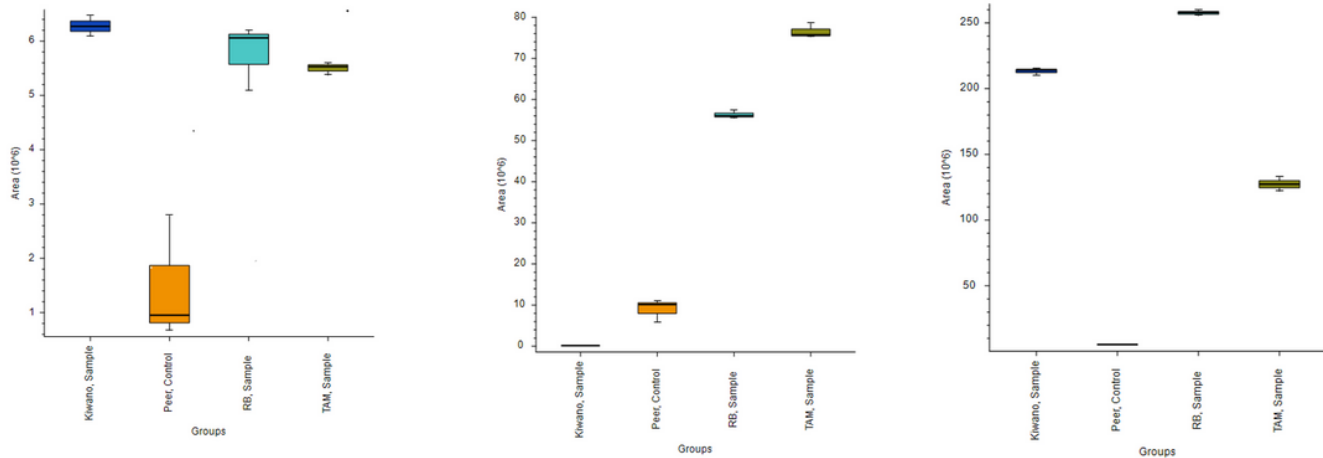
Figure 2

The presence of benzoic acid, caffeic acid, and gallic acid in the sugar-free extract of raspberry, kiwano, and tamarillo was determined by the untargeted analysis.

Further chromatographic analyses were conducted to identify the unknown compounds in the sugar-free fruit extracts derived from tamarillo, kiwano, raspberry, and pear. Pear was used again as a negative control to underline differences in components in other fruit extracts.
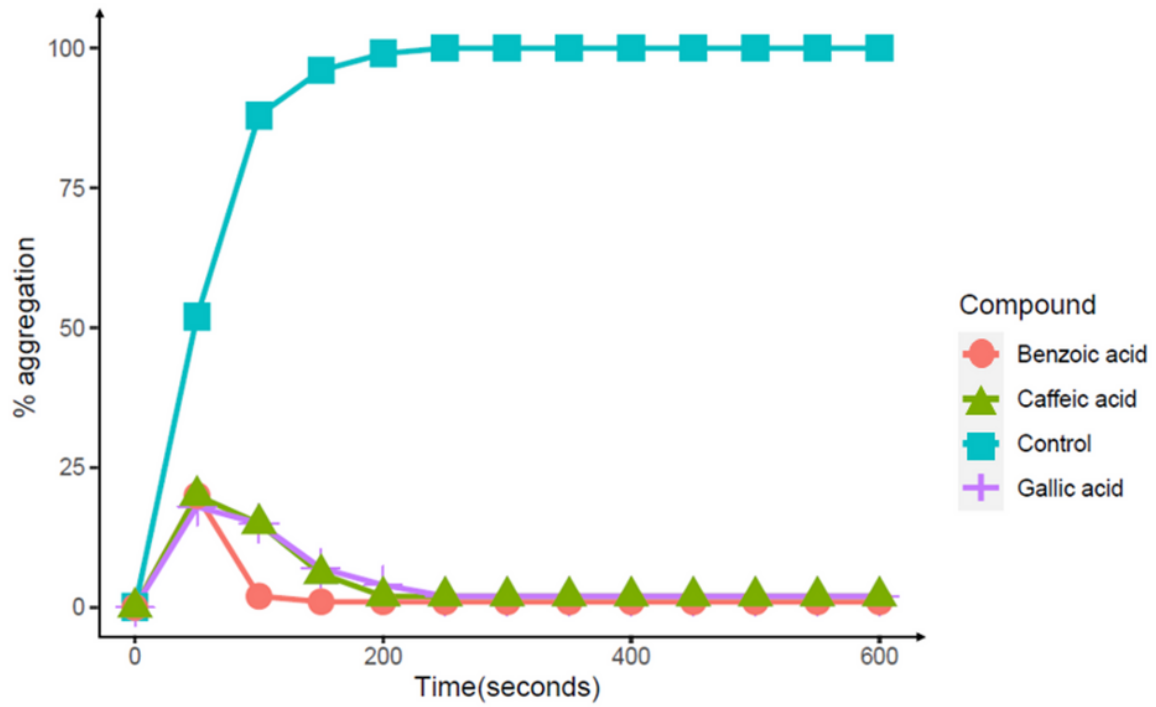
Figure 3

Inhibition of ADP-induced platelet aggregation in PRP by gallic, benzoic, and caffeic acids

PRP was incubated with 10 ml of gallic acid, benzoic acid, and caffeic acid (2%) for 15 min before adding the ADP (10mM). The aggregation was measured as described in Figure 1.