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Tomato pomace-derived nitrated fatty acids: Synthesis and antiplatelet activity

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ABSTRACT

This study investigates the antiplatelet properties of tomato pulp to combat cardiovascular diseases. Notably, it examines the formation of nitrated fatty acids (NO_2-FA) in tomato pomace, renowned for its potential antiplatelet effects. Through diverse assays, including tandem mass spectrometry, microplate-based platelet aggregation, and flow cytometry, the research identifies NO_2-OA, NO_2-LA, and NO_2-LnA as pivotal antiplatelet compounds. It demonstrates the concentration-dependent antiplatelet effects of nitrated tomato pomace against thrombin receptor activator peptide 6 (TRAP-6) and collagen-induced platelet activation, alongside the modulation of platelet activation markers. Additionally, synergistic effects were observed with nitrated tomato pomace extracts. The findings suggest therapeutic potential for NO_2-FA derived from tomato pomace in preventing blood clot formation, with nitrated extracts exhibiting superior efficacy compared to non-nitrated ones. This research highlights the promising role of natural products, such as tomato pomace, in mitigating cardiovascular risks and proposes novel strategies for population health enhancement and cardiovascular disease management.

1. Introduction

Cardiovascular diseases (CVD) constitute a substantial public health burden globally, standing as the principal cause of morbidity and mortality [2]. As our comprehension of these diseases' risk factors and underlying mechanisms advances, novel bioactive compounds with the potential to influence their development and progression have been identified [25]. Given the pivotal role of food in CVD, there is a growing demand for dietary sources that actively prevent CVD and enhance overall quality of life. Our research has unveiled the cardiovascular protective effects of a healthful diet, particularly one rich in fruits and vegetables, such as tomatoes. Tomatoes, in particular, make a noteworthy contribution to reducing CVD. This contribution is attributed to their ability to inhibit platelet aggregation and exhibit antithrombotic activity [15,16,33].

Tomato (*Solanum lycopersicum L.*) is a globally predominant vegetable crop consumed extensively. Large quantities of this versatile vegetable undergo annual processing to yield canned tomatoes, ketchup, tomato juice, sauce, and other products [49]. Tomatoes emerge as a rich dietary source of essential vitamins (C & E), carotenoids (e.g., β-carotene and lycopene), flavonoids (e.g., quercetin and kaempferol), and folates. In addition to enjoying fresh, tomatoes undergo industrial processing to generate various manufactured products, including sauces, purees, pastes, and juices. The commercial processing of tomatoes into these manufactured products involves a multi-step procedure, encompassing an initial heat treatment (either cold break at 60–65°C or hot break at 90–95°C), followed by pulping and homogenization techniques [27].

Given its seed's high fatty acid content exceeding 30 %, tomato

Abbreviations: ACD, Acid-citrate-dextrose; β−ME, β−mercaptoethanol; CVD, Cardiovascular diseases; DMSO, Dimethylsulfoxide; EPI, Enhanced Product Ion; FAO, Food and Agriculture Organization of the United Nations; IC_{50}, Half-maximal inhibitory concentration; HPLC−MS/MS, High-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry; LA, Linoleic acid; LnA, Linolenic acid; NO_2-LA, Nitrated linoleic acid; NO_2-LnA, Nitrated linolenic acid; NO_2-OA, Nitrated oleic acid; MRM, Multiple Reactions Monitoring; NO_2-FA, Nitrated fatty acids; NO_3^-, Nitrate; NO_2^-, Nitrite; OA, Oleic acid; PRP, Platelet-rich plasma; TRAP-6, Thrombin receptor activator peptide 6.

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pomace is a potentially rich source of healthy oils. Results indicate that tomato seed oil exhibits elevated thermal stability and an excellent physicochemical profile even after heating [46]. Consequently, from an annual production of 18 thousand tons of tomato pomace, approximately two thousand tons of cardioprotective healthy oil could be derived [1]. Studies reveal that more than 80 % of the fatty acids present in tomato pomace are unsaturated, encompassing health-promoting fats such as linoleic acid (cis, cis−9, 12 C18:2; LA) at 54 %, oleic acid (cis−9 C18:1; OA) at 22 %, linolenic acid (cis, cis, cis−9, 12, 15 C18:3; LnA) at 2 %, and palmitoleic acid (cis−9 C16:1, PnA) at 0.3 %. Consequently, the composition of fatty acids in tomato pomace oil, particularly in terms of LA-OA, closely resembles that of sunflower and soybean oils [56,57]. Despite being underutilized, tomato pomace holds promise as a valuable source of bioactive compounds, primarily enriched in healthy fatty acids.

We propose that fatty acids found in tomato pomace undergo nitration during digestion. Our hypothesis aims to explore the formation of nitrated fatty acids (NO_2-FA) and assess their potential antiplatelet properties. Nitro-fatty acids confer multiple health benefits, encompassing oxidative stress reduction, lipid profile improvement, enhanced insulin sensitivity, optimized endothelial function, and antithrombotic properties. These advantages are supported by the Mediterranean diet, renowned for its richness in vegetables containing NO_2^- and nitrates (NO_3^-). In this study, we have optimized conditions for synthesizing NO_2-FA from tomato pomace, a byproduct of tomato processing. This optimization can potentially confer added value to tomato pomace, which is currently considered agricultural waste. Furthermore, the NO_2-FA derived from tomato pomace may be utilized to develop novel foods and supplements offering cardiovascular health benefits.

2. Materials and methods

2.1. Chemicals

Thrombin receptor activator peptide 6 (TRAP-6) was obtained from Sigma-Aldrich (St. Louis, Missouri/MO, USA), and collagen was obtained from Farmalatina. LDH kit, Calcein Violet-AM, Na_3PO_4, and $NaNO_2$ also were obtained from Sigma-Aldrich (St. Louis, Missouri/MO, USA). All solvents for HPLC and HPLC-MS/MS were of HPLC grade. In all the experiments, the final concentration of dimethylsulfoxide (DMSO) did not exceed 0.2 % (v/v), which does not affect platelet function, and control conditions include DMSO as the appropriate vehicle.

2.2. Nitration of tomato pomace

Tomato pomace powder (100 mg) was dissolved under constant agitation in gastric acidic conditions [41]. Nitration was initiated by adding sodium nitrite ($NaNO_2$). The nitration process was carried out for 1 hour at 37°C [12,17,52]. The control condition involved incubating tomato pomace under the same conditions but without NO_2^- or defatted tomato pomace [45]. The overall procedure was repeated several times to have enough raw material, and each batch was analyzed before use with no observed changes in composition and effects between them.

The synthesis under gastric conditions allowed us to nitrate our vegetable matrix, which is rich in fatty acids, and subsequently identify the NO_2-FA, as we will describe below.

2.3. Removal of phenols from tomato pomace

To assess the influence of phenolic components present in tomato pomace on fatty acid nitration and the subsequent biological effects of nitrated pomace, we conducted the removal of phenolic compounds before and after nitration reactions [41]. One hundred milligrams of tomato pomace were combined with 1 mL of methanol, subjected to vigorous vortexing for 1 minute, and then centrifuged at 1800 rpm for 5 minutes. The upper phase, enriched in phenolic compounds, was subsequently separated. This procedure was performed three times to maximize the elimination of phenolic compounds, including phenolic acids and flavonoids [41].

2.4. Extraction, chemical characterization, and quantitation of nitro−fatty acids in tomato pomace

The fatty acid composition of tomato pomace was determined utilizing gas chromatography. A minimum sample quantity of 2 g was required for analysis. Lipid extraction followed the AOAC Official Method 996.06 for Fat (Total, Saturated, and Unsaturated) in Foods, with specific modifications [9]. Briefly, 2 g of the sample was weighed, and a solution containing 2 mL of acidified methanol and 1 mL of toluene was added. The samples were subjected to rotary tube shaking at 1700 rpm for 10 minutes and then placed in a water bath at 90°C for 2 hours, with temperature control during the initial 30 minutes. After this, the samples were cooled to room temperature, mixed with 5 mL of saturated NaCl, and centrifuged for 8 minutes at 4000 rpm. The toluene phase obtained was then injected into the gas chromatography system for further analysis.

The samples of nitrated and non-nitrated pomace were analyzed to determine the formation and identification of NO_2-FA after enzymatic hydrolysis with a triacylglycerol lipase and phospholipase followed by extraction of the reaction mixture using the n-hexane method [41,50,51]. Enzymatic hydrolysis and subsequent extraction with n-hexane allowed the release of the esterified fatty acids. The free fatty acid fraction, including nitrated fatty acids, was obtained before their analysis by mass spectrometry after solid-phase extraction (SPE), as previously reported by our group and others [37,41] and explained below. Additionally, the biological activity of nitrated tomato pomace was evaluated, as described later.

The identification of NO_2-FA in the experimental setup was carried out following previously established procedures by our research group utilizing High-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (HPLC−MS/MS). The characterization and quantification were accomplished by employing the Enhanced Product Ion (EPI) and Multiple Reactions Monitoring (MRM) scan modes following MRM transitions corresponding to potential nitrated isomers derived from OA, LA, and LnA [38,41]. For the qualitative and isomer identification analysis of NO_2−FA, we performed the experiments using a triple quadrupole−ion trap mass spectrometer (QTRAP4500, ABSciex, Framingham, MA) in the negative ion mode coupled to an Agilent 1260 HPLC.

The separation of NO_2-FA was conducted on a C18 reverse-phase column (150 × 2 mm, 3 μm, Phenomenex) with elution at a flow rate of 0.25 mL/min. The solvent system consisted of A (H_2O/0.1 % formic acid) and B (acetonitrile/0.1 % formic acid), with the following solvent gradient: 45 % B (0 min); 45–80 % % B (0–45 min); 80–100 % B (45–46 min); 100 % B (46–53.0 min); 100–45 % (53.0–53.1); 45 % (53.1–63.0), followed by column reequilibration to initial conditions [41]. In all instances, internal standards (e.g., NO_2-FA with ^{13}C or ^{15}N) were added before lipid extraction to facilitate the detection of NO_2-FA.

Fatty acids were released by treatment with triacylglycerol lipase and phospholipases to analyze esterified lipids. Free fatty acids originating from triacylglycerides were obtained after incubation with pancreatic lipase (5 mg protein/mL) and phospholipase (40 U) in 50 mM phosphate buffer, pH 7.4, for one hour at 37°C with stirring. The lipid fraction was extracted, dried under nitrogen, and purified from the lipid mixture through SPE using Strata NH2 cartridges (Phenomenex). Free fatty acids were eluted with diethyl ether/2 % acetic acid [39,51]. Samples were dried, and lipids were resuspended in 50 % methanol for HPLC−MS/MS analysis.

Furthermore, the presence of electrophilic NO_2-FA was validated by forming a covalent adduct with β−mercaptoethanol (β−ME) [43].

2.5. Preparation of human platelets

Blood samples were obtained from healthy donors who refrained from using anti-inflammatory drugs for two weeks. Prior informed consent was obtained from the donors, and the study was approved by the Ethics Committee at the University of Talca (Folio 03–2022). Blood was collected using acid-citrate-dextrose (ACD) at a ratio of 4:1 v/v and subsequently centrifuged at room temperature for 12 minutes (1200 rpm) to obtain platelet-rich plasma (PRP). The PRP was centrifuged at room temperature for 10 minutes at 3000 rpm. The resulting platelet pellet was resuspended in calcium-free Tyrode buffer with ACD (5:1 v/v). The platelets were washed by centrifugation at 3000 rpm for 10 minutes at the same temperature [30]. Platelet count was determined using a Hematology Counter (Mindray BC-3000 Plus Hematology Counter, Japan). Washed platelets were utilized to assess the antiplatelet potential of NO_2-FA derived from tomato pomace in platelet aggregation studies.

2.6. Cytotoxic activity

We incubated washed platelets (3×10^8 platelets/mL) with the highest tested concentration of nitrated tomato pomace (6 mg/mL) for 10 minutes at 37°C. Subsequently, we centrifuged the platelets at 3000 rpm for 8 minutes and analyzed the supernatant using the Lactate Dehydrogenase (LDH) Cytotoxicity Assay Kit (Cayman Chemical, USA). Following centrifugation, 50 μL of the resulting supernatant was subjected to the LDH assay at 490 nm. Triton X-100 at 0.3 % was a positive control [30].

2.7. Inhibition of platelet aggregation

The inhibition of platelet aggregation was assessed through a microplate-based assay with slight modifications [10]. Washed platelets were incubated with nitrated or non-nitrated tomato pomace (0.5, 1, 2, 4, and 6 mg/mL) or a vehicle (DMSO, 0.2 %) at 37°C for 6 minutes. Platelet aggregation was induced using TRAP-6 (5 μM) and collagen (2 μg/mL). The samples were loaded into 96-well plates (Greiner, Stonehouse, United Kingdom) and shaken at 1200 rpm for 5 minutes at 37°C using a plate shaker (Thermo Scientific). Washed platelets served as a control for maximum aggregation. Absorbance was measured at 450 nm using a spectrophotometer (Agilent BioTek).

The percentage of platelet aggregation was calculated as follows [34]:

Platelet Aggregation (%) = 100 x
([Control Abs (Washed Platelets - NO_2-FA Absorbance)] /
[Control Absorbance (Washed Platelets) - Blank Absorbance (TAF)])

The formula determined platelet inhibition:

Inhibition of Platelet Aggregation (%)=100- ([NO_2-FA Platelet Aggregation / Negative Control Platelet Aggregation] x 100)

The concentration required to achieve a 50 % reduction in platelet aggregation (IC_{50}) was derived from the concentration curves of nitrated tomato pomace (0.5, 1, 2, 4, and 6 mg/mL).

Various combinations of tomato pomace extracts were prepared to investigate the antiplatelet effects arising from interactions among the nitrated components of tomato pomace. The lipid fractions extracts that were evaluated included: i) E_1: Tomato pomace undergoes nitration after phenolic cleaning with methanol, followed by lipid extraction with n-hexane; ii) E_2: Pomace undergoes phenolic cleaning with methanol, and nitration conditions are simulated without adding $NaNO_2$; iii) E_3: Tomato pomace undergoes nitration, followed by phenolic cleaning and extraction with hexane to obtain the nitrated matrix free of phenols.The mixtures were evaluated at final concentrations of 1 mg/mL and 6 mg/mL and compared with the pure fractions of tomato pomace at concentrations of 0.5, 1, 3, and 6 mg/mL.

2.8. Inhibition of platelet activation and secretion

Platelets (200×10^9 platelets/L) were pre-incubated for 10 minutes at 37°C with fatty acids extracted from nitrated or non-nitrated tomato pomace (0.5, 1, 2, 4, and 6 mg/mL) or a vehicle (DMSO, 0.2 %). Subsequently, platelet aggregation was induced with various agonists (collagen 2 μg/mL or TRAP-6 5 μM). Platelet activation was then evaluated by incubating 50 μL of the previous sample for 30 minutes at room temperature in the dark with saturated concentrations of anti-CD61-FITC antibody (platelet marker), anti-CD63-PE antibody (CD63 secretion), anti-CD62P-PE antibody (P-selectin expression), or anti-Fibrinogen (Fibrinogen binding). Anti-CD61-FITC facilitated the precise selection of the platelet population. The samples were subsequently analyzed using a BD FACSLyric flow cytometer. Platelet populations were chosen based on cell size using forward scattering (FSC) versus side scattering (SSC) and CD61 positivity to distinguish them from electronic noise, as described by other researchers [14,34].

2.9. In vitro model of thrombosis

The thrombus formation experiments were conducted following the methodology outlined by Stainer et al., 2019 [48], with certain modifications. Whole blood was collected in citrate tubes from healthy volunteers. The sample was incubated with Calcein-AM (4 mM) for 1 hour at 30°C. The Vena8 BioChip microfluidic channels were coated with collagen (0.1 mg/mL) for 1 hour at room temperature. Subsequently, whole blood was incubated with nitrated and non-nitrated tomato pomace (IC_{50}=0.07 ± 0.02 mg/mL and IC_{50}=1.4 ± 0.05 mg/mL, respectively) or vehicle control (DMSO) for 10 minutes at 30°C before perfusion through the channels. Thrombus formation was quantified by perfusing citrated whole blood through the flow chamber for 6 minutes at a rate of 45 μL/mL. Blood was collected into a 5 mL BD syringe.

Thrombus formation was observed using fluorescence microscopy (AXIO Examiner Z.1, Zeiss, Oberkochen, Germany), and the same area was selected for each channel for all conditions evaluated. Platelet deposition was assessed with a 63x objective, utilizing an *in vivo* camera system equipped with the COLIBRI system filter at 470 nanometers (blue light) and 20 % intensity. Randomly selected images were analyzed for each condition using ImageJ software (version 1.26 t, NIH, USA) [29]. The area of thrombus formation was calculated and expressed in μm^2.

2.10. Statistical analysis

The data obtained are presented as the mean ± standard error of the mean (SEM) from individual experiments and were analyzed using Prism 6.0 software (GraphPad Inc., San Diego, CA, USA). All measurements were conducted using samples from six separate platelet donors. Results are expressed as a percentage of inhibition or a percentage of the vehicle (considered 100 %). The half-maximal inhibitory concentration (IC_{50}) of the obtained NO_2-FA was calculated from the dose-response curves. Differences were assessed using ANOVA with post-hoc Tukey. P values <0.05 were considered significant [29].

3. RESULTS

3.1. Determination of the fatty acid profile and polyphenolic quantitation of tomato pomace

We first analyzed the fatty acid profile of tomato pomace, aiming to determine if unsaturated fatty acids, the substrate for the formation of NO_2-FA, were present and at which concentration. The results show that unsaturated fatty acids are predominant in the sample, representing more than 75 % of the total fatty acids (Table 1). The most abundant fatty acid in tomato pomace is LA (C18:2Δc9c12), constituting almost 50 % of the total fatty acids. This is followed by OA (C18:1Δc9, approximately 22 %), both essential fatty acids.Tomato pomace contains

Table 1
Tomato pomaceś fatty acid profile. Results express the mean ± SEM of the fatty acids content in the tomato pomace sample.

Fatty acids	Tomato pomace (g/100gofextract)
C13:0	1.69 ± 0.05
C14:0	0.17 ± 0.02
C15:0	ND
C16:0	14.87 ± 1.25
C16:1	0.31 ± 0.15
C17:0	1.03 ± 0.08
C18:0	5.34 ± 0.12
C18:1Δc9(c18:1ω9)	22.21 ± 1.27
C18:1Δc11	1.23 ± 0.08
C18:2Δc9c12(C18:2ω6)	49.36 ± 1.35
C20:0	0.43 ± 0.03
C18:3Δc9c12c15(C18:3ω3)	2.06 ± 0.50
C22:0	0.22 ± 0.10
C23:0	ND
C24:0	0.43 ± 0.01

ND: not detected.

a significant amount of α-LnA (C18:3Δc9c12c15), an omega-3 fatty acid. Tomato pomace's saturated fatty acid content is relatively low, comprising about 25 % of the total fatty acids. The most abundant saturated fatty acid in tomato pomace is palmitic acid (C16:0) (Table 1). Once the presence of unsaturated fatty acids in our starting material was confirmed, we performed *in vitro* nitration under gastric conditions.

The phenolic fraction resulting from the methanolic cleaning of the pomace, both from the non-nitrated tomato pomace sample and the lipid fraction to be nitrated, was quantified. In both cases, the remaining phenolic content of the nitrated and non-nitrated tomato pomace was similar (Table I. Supporting Information).

3.2. Characterization of NO_2-FA from tomato pomace by HPLC-MS/MS

Tomato pomace is a good source of fatty acids associated with cardiovascular protection as it can be a substrate for forming the protective NO_2-FA molecules [4]. To characterize the formation of NO_2-FA in tomato pomace, fatty acid nitration of this agroindustrial waste was performed under acidic conditions in the presence of NO_2^-, a mechanism similar to that which operates in the gastric compartment during digestion [37].

The detection of the NO_2-FA was done by HPLC-MS/MS in the negative ion mode using ^{13}C labeled standards and ^{15}N labeled nitroalkenes [28,40,41]. Fragmentation analysis and elution profiles of the specific fragments provided the structural information to assign the position of the nitro group (-NO_2) in the NO_2-FA present in tomato pomace.

The MRM scanning mode targeted transitions corresponding to potential nitrated fatty acids. Fig. 1 shows the chromatographic behavior as well as the *m/z* transitions consistent with the presence of i) nitrated linolenic acid (NO_2-LnA, *m/z* 322/46, Fig. 1 panel I), ii) nitrated linoleic acid (NO_2-LA, *m/z* 324/46, Fig. 1 panel II), and iii) nitrated oleic acid (NO_2-OA, *m/z* 326/46, Fig. 1 panel III). The detection and identification of NO_2-LnA, NO_2-LA, and NO_2-OA were consistent with tomato pomace's most abundant unsaturated fatty acids (Table 1). Moreover, identification was performed by their capacity to react through electrophilic Michael-addition reactions with thiols. The samples were treated with excess β-ME, eliminating all NO_2-FA peaks. The corresponding β-ME Michael adducts of NO_2-LnA, NO_2-LA, and NO_2-OA were formed (Fig. 1C), confirming the electrophilic nature of the observed NO_2-FA.

Identification of the nitrated isomers of NO_2-LA was performed using previously reported fragmentation patterns using mass spectrometry [5, 39,54]; and shown in Supporting Information Figure S.1. MS spectra

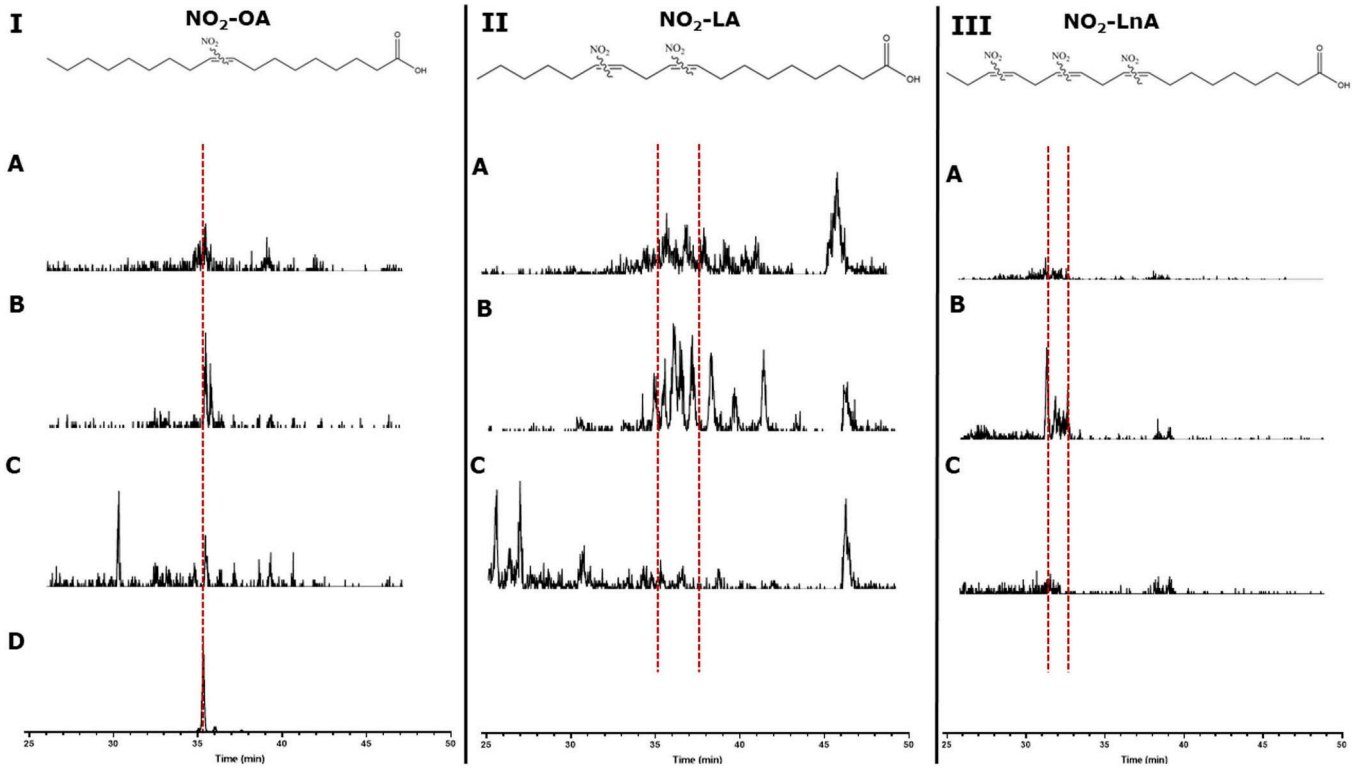


Fig. 1. Chromatographic profiles of the NO_2-FA present in tomato pomace. The presence of different NO_2-FA was analyzed by HPLC-MS/MS. **Panel I** corresponds to the identification of NO_2-OA (*m/z* 326/46); **Panel II** corresponds to the identification of NO_2-LA (*m/z* 324/46); and **Panel III** corresponds to the identification of NO_2-LnA (*m/z* 322/46). Chromatographic profiles shown are of (**A**) the non-nitrated tomato pomace; (**B**) the gastric acidic conditions nitrated tomato pomace; (**C**) the covalent adducts after treatment with β-ME: *m/z* 404.4/78, BME-NO_2-OA; *m/z* 402.4/78, BME-NO_2-LA; *m/z* 400.4/78, BME-NO_2-LnA;and (**D**) the NO_2-OA internal standard,$^{13}[C]_{18}$-NO_2-OA (*m/z* 344/46).

allowed us to identify the 12-NO_2-LA (Fragment I) and 9-NO_2-LA (Fragments II and III) isomers. Fragmentation of 9-NO_2-LA results in forming a fragment ion with *m/z*of 168 (Supporting Information Figure S.1 Scheme II and III) [4]. Its structure was confirmed by detecting the specific fragmentation ions with *m/z* of 168 and 224. The mechanistic pathway leading to the formation of an ion with *m/z* of 168 is common between 9-NO_2-OA and 9-NO_2-LA and corresponds to the cleavage of the C_9-C_{10} double bond, with a net transfer of one or both oxygen of the NO_2 group to its β-carbon (C_{10}), in other words it indicates the presence of a double bond at the C_9 position and NO_2 groups at C_9 and C_{10}, respectively [4].

Fragmentation of 12-NO_2-LA resulted in ions with *m/z* of 157 (Supporting Information Figure S.1 and Scheme I). The difference in 14 Da between ions with *m/z* of 157 and 171 corresponds to the neutral formation of six- and five-membered heterocycles [4]. Finally, Scheme IV in Supporting Information Figure S.1 shows the ion with *m/z* 171, a fragment that may be characteristic of 13-NO_2-LA or 12-NO_2-LA. For this reason, we could not unequivocally assign this signal according to the MS analysis.

On the other hand, we identified and quantified NO_2-OA using the internal standard with ^{13}C (*m/z* 344, Supporting Information Figure S.2). The structure shown in A and B (*m/z* 326) was analyzed by LC-MS/MS after organic extraction following the neutral loss of the NO_2 group (loss of *m/z* 46). The red dashed line indicates thatthe NO_2-OA detected in the nitrated tomato pomace exhibited the same chromatographic retention time as the standard (A-C). Quantification was performed as the ratio of NO_2-OA/[$^{13}C_{18}$]NO_2-OA (black bars), and most of the nitrated fatty acid was esterified to triacylglycerides or phospholipids as the amount of NO_2-OA increased after enzymatic release (D).

3.3. Modulation of platelet aggregation and activation by NO_2-FA present in tomato pomace

Before conducting biological tests on the samples, it is necessary to determine whether nitrated and non-nitrated tomato pomace is cytotoxic. None of the evaluated fractions exhibited cytotoxicity (Supporting Information Figure S.3).

The efficacy of inhibiting platelet aggregation by NO_2-FA formed after nitration of fatty acids present in tomato pomace was evaluated by turbidimetry. Platelet aggregation inhibition was assessed in platelets stimulated by TRAP-6 (5 μM) and collagen (2 μg/mL) at various concentrations of NO_2-FA from tomato pomace (0.5, 1, 2, 4, and 6 mg/mL). The presence of NO_2-FA in nitrated tomato pomace can support the differences in its efficacy in inhibiting platelet aggregation induced by different platelet agonists, and its potential was higher than in non-nitrated tomato pomace (Fig. 2). The antiplatelet activity of nitrated tomato pomace was concentration-dependent when platelet aggregation was induced by TRAP-6 (IC_{50}= 1.18 ± 0.04 mg/mL) and collagen (IC_{50}= 0.07 ± 0.02 mg/mL). These results demonstrate that the antiplatelet potential of nitrated tomato pomace was more significant against collagen.

3.3.1. Potentiation of the activity of nitrated tomato pomace components as mediators of antiplatelet effects

To evaluate the potentiation of antiplatelet effects exerted by NO_2-FA in nitrated tomato pomace compared to non-nitrated tomato pomace, we conducted experiments by mixing nitrated and non-nitrated components. These experiments aimed to determine if additive, cooperative, or synergistic actions influenced tomato pomace's antiplatelet activity (Fig. 3).

The evaluated mixtures (M1, M2, and M3) had a synergistic effect on

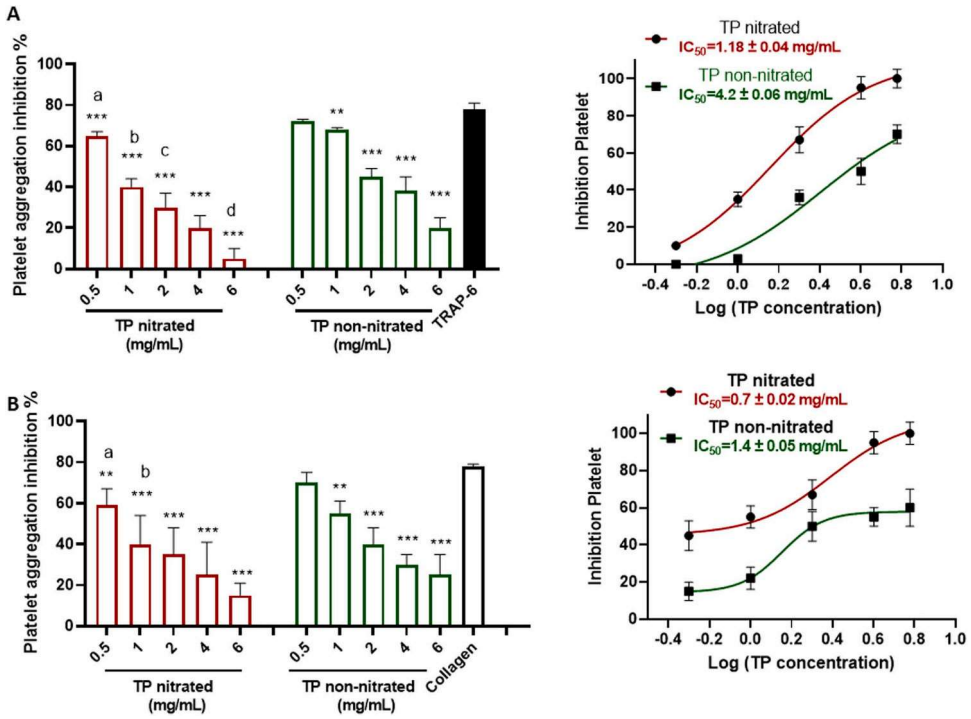


Fig. 2. Modulation of platelet aggregation by NO_2-FA in tomato pomace. Platelet aggregation was induced by TRAP-6 (A) or Collagen (B) in the absence or presence of tomato pomace (TP), and IC_{50} values were obtained for each fraction (nitrated and non-nitrated tomato pomace) and agonist. Platelets were preincubated for 6 minutes with nitrated and non-nitrated samples, and then aggregation was induced with the indicated agonist. Results were expressed as mean ± SEM, n = 6. ** $p < 0.01$ and *** $p < 0.001$ vs activated control (TRAP-6 or collagen). Basal: platelets + DMSO (0.2 %). a, b, c, d, and e denote statistically significant differences regarding nitrated tomato pomace vs. non-nitrated tomato pomace at different concentrations (a: nitrated tomato pomace vs. non-nitrated tomato pomace at 0.5 mg/mL, b: nitrated tomato pomace vs. non-nitrated tomato pomace at 1 mg/mL, c: nitrated tomato pomace vs. non-nitrated tomato pomace at 2 mg/mL, d: nitrated tomato pomace vs. non-nitrated tomato pomace at 4 mg/mL, and e: nitrated tomato pomace vs. non-nitrated tomato pomace at 6 mg/mL). Comparisons between the two groups were made with the Tukey test.

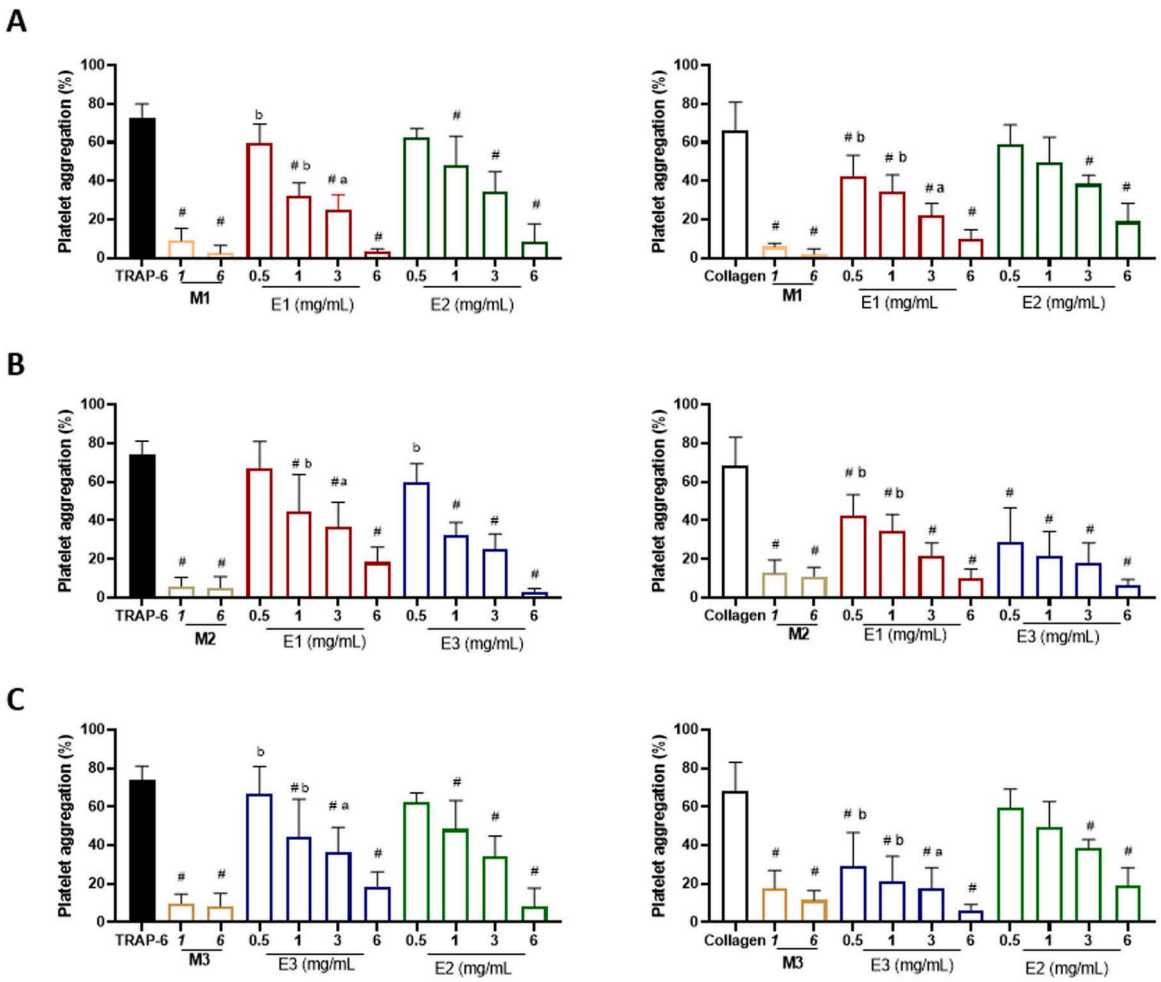


Fig. 3. TRAP-6 or collagen-induced platelet aggregation. Platelets were preincubated for 6 minutes with nitrated and non-nitrated samples, and then aggregation was induced with the indicated agonist. Results were expressed as mean ± SEM, n = 3. #: shows statistically significant differences compared to the platelet activation control (TRAP-6 or collagen), a: denotes statistically significant differences compared to the mixture at the concentration of 6 mg/mL (to the compounds at 6 and 3 mg/mL), b: denotes statistically significant differences compared to the mixture at the concentration of 1 mg/mL (to the compounds at 1 and 0.5 mg/mL). Legends: **E1**: Tomato pomace is taken and the nitration is carried out after phenolic cleaning with methanol, subsequently nitration and extraction with n-hexane; **E2**: Tomato pomace is taken after a previous phenolic cleaning with methanol, and the nitration conditions are simulated without adding the $NaNO_2$, after this time the extraction is carried out with n-hexane; **E3**: Tomato pomace is taken and nitrated, subsequently phenolic cleaning and extraction with hexane are carried out to the nitrated matrix free of phenols.The mixes were: **M1** = E1 + E2; **M2** = E1 + E4; and **M3** = E4 + E2.

antiplatelet activity; the combination of components intensified their effects individually. A concentration-dependent effect was confirmed. Additionally, the antiplatelet activity varied depending on the agonist (TRAP-6 or collagen), corresponding to the different activation pathways involved in platelet aggregation inhibition.

M2 and M3 exhibited more significant antiplatelet potential when platelet aggregation was stimulated by TRAP-6, unlike M1, which demonstrated more excellent antiplatelet activity against collagen. When nitrated tomato pomace (E1) was mixed with non-nitrated tomato pomace (E2), a synergistic effect was verified, indicating that nitrated tomato pomace enhances the antiplatelet potential of this mixture (M1), which is particularly beneficial in nutritional terms.

Our results suggest that consuming tomato pomace alongside a balanced diet rich in NO_2^- and nitrate NO_3^- from vegetables enhances the antiplatelet effect.

3.3.2. *Inhibition of platelet activation and secretion*

The impact of nitrated and non-nitrated samples on the expression of platelet activation markers (P-selectin, CD63, and fibrinogen binding) was evaluated in response to stimulation with collagen or TRAP-6. Nitrated tomato pomace inhibited P-selectin expression up to the lowest concentration evaluated (0.5 mg/mL) when platelets were stimulated with TRAP-6 and collagen (Fig. 4A and 4B). The effect of non-nitrated tomato pomace on P-selectin expression was significantly lower. Furthermore, CD63 secretion significantly decreased to 1 mg/mL of nitrated pomace, with lower effects observed for non-nitrated tomato pomace (Figs. 4C and 4D). Nitrated tomato pomace exhibited a more substantial effecton fibrinogen binding when platelets were activated with collagen compared to the same concentrations of non-nitrated tomato pomace (Fig. 4F). However, similar results were obtained when the agonist was TRAP-6 (Fig. 4E), supporting different mechanisms of action regarding the platelet activation agonist.

3.4. *Decreased thrombosis by nitrated tomato pomace*

Due to the antiplatelet potential exhibited by NO_2-FA in tomato pomace, we hypothesized that these compounds, present in nitrated tomato pomace, may possess antithrombotic properties. The impact of NO_2-FA from tomato pomace on thrombus formation was investigated *in vitro* under physiological arterial flow conditions. Blood was treated with the vehicle and perfused onto collagen-forming thrombi within the expected parameters during the 6-minute test period. In contrast,

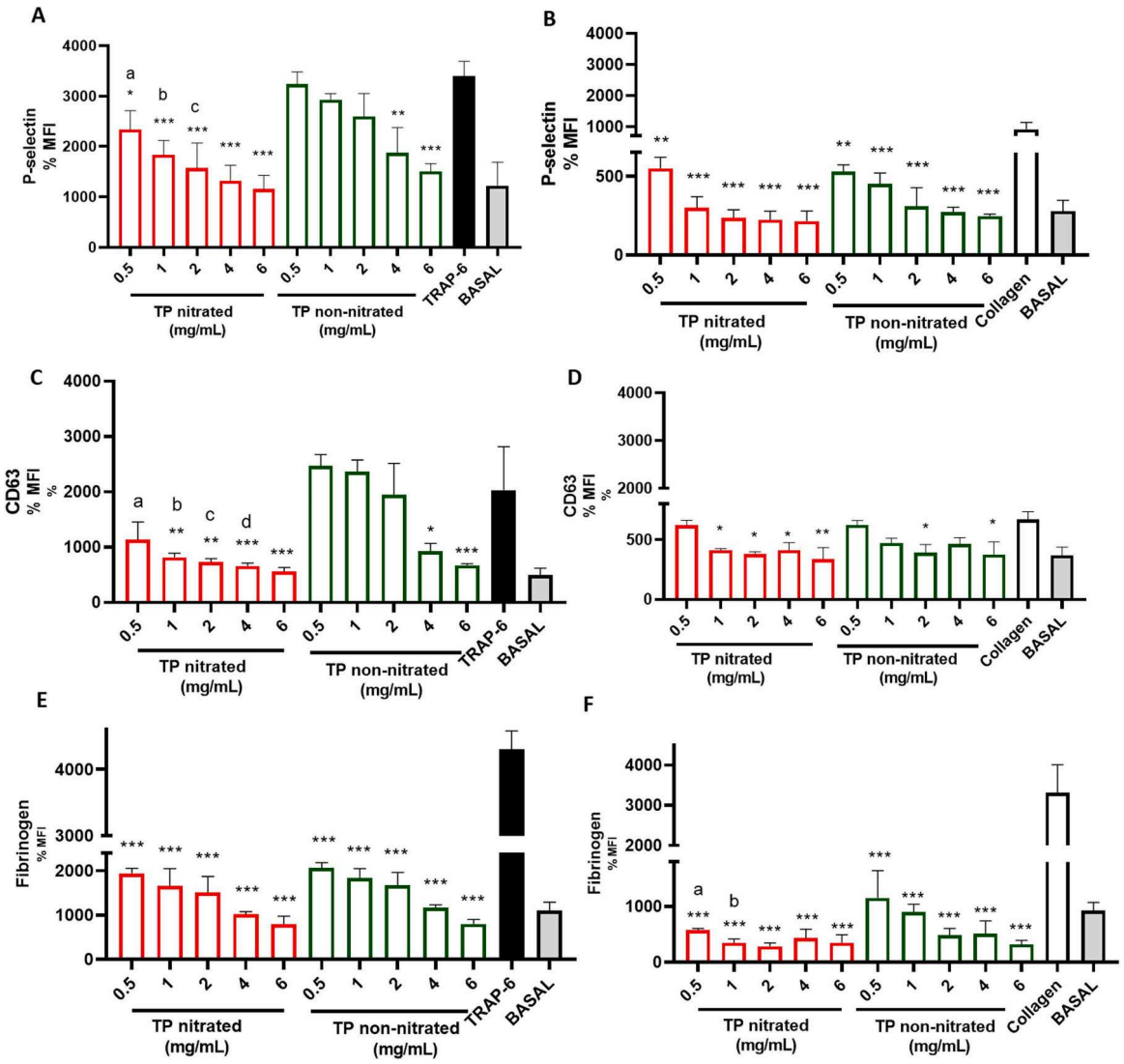


Fig. 4. Platelet activation markers were modulated by NO_2-FA present in nitrated tomato pomace (TP). (**A** and **B**) Effect on the expression of P-selectin. (**C** and **D**) Effect on the expression of CD63. (**E** and **F**) Impact onthe fibrinogen binding. Platelets were stimulated with TRAP-6 (5 μM) and collagen (2 μg/mL). Platelets were identified by flow cytometry as a CD61+ population. Statistical analysis was performed using ANOVA (Tukey's test). Results were expressed as mean ± SEM, n = 6. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs activated control (TRAP-6 or collagen). Basal: platelets + DMSO (0.2 %). a, b, c, d, and e denote statistically significant differences regarding nitrated tomato pomace vs. non-nitrated tomato pomace at different concentrations (a: nitrated tomato pomace vs. non-nitrated tomato pomace at 0.5 mg/mL, b: nitrated tomato pomace vs. non-nitrated tomato pomace at 1 mg/mL, c: nitrated tomato pomace vs. non-nitrated tomato pomace at 2 mg/mL, d: nitrated tomato pomace vs. non-nitrated tomato pomace at 4 mg/mL, and e: nitrated tomato pomace vs. non-nitrated tomato pomace at 6 mg/mL). Comparisons between the two groups were made with the Tukey test.

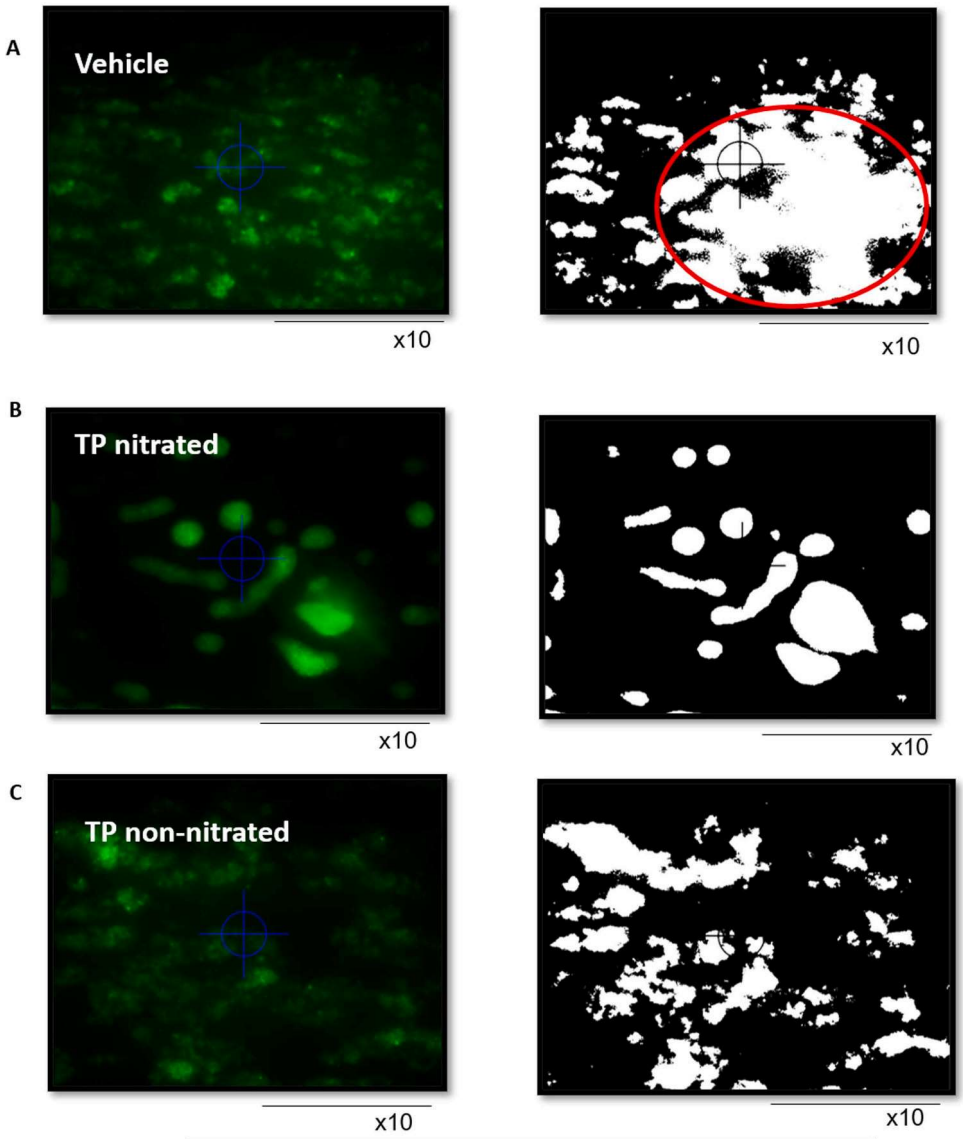
treatment with NO_2-FA from tomato pomace significantly reduced the area of formed thrombi compared to the vehicle-treated counterpart (14,563.20 ± 404.38 μm^2 vs 37,765.57 ± 546.47 μm^2) (Fig. 5). The nitrated tomato pomace, administered at the IC_{50} concentration for inhibiting platelet aggregation (0.07 ± 0.02 mg/mL), exhibited a notable reduction in thrombus area compared to the untreated blood sample, a decrease of 60 %. Furthermore, this antithrombotic effect was more pronounced when compared to the non-nitrated tomato pomace (thrombus area: 20,087.52 ± 879.76 μm^2, a 42 % reduction). These findings substantiate the antithrombotic efficacy of NO_2-FA derived from tomato pomace in impeding thrombus formation under physiological arterial flow conditions on a collagen matrix.

4. DISCUSSION

The discussion regarding the role of platelet aggregation and thrombus formation in CVD [18] emphasizes the necessity to investigate specific fatty acid compositions within tomato pomace due to its potential variability due to factors such as tomato variety, growing conditions, and extraction processes employed [47].

NO_2-FA has emerged as a promising avenue for reducing CVD risk [22,36], given their ability to inhibit platelet aggregation [19] and possess antioxidant and anti-inflammatory properties [24]. Controlled synthesis of NO_2-FA enables exploration of their therapeutic benefits, including their potential to mitigate platelet aggregation and associated cardiovascular risks, facilitating the further exploration of their properties and applications in various fields such as biomedical research, nutrition, and the food industry.

Our study focused on the formation of NO_2-FA in tomato pomace under gastric conditions, identifying NO_2-OA, NO_2-LA, and NO_2-LnA as significant formations. Literature supports the cardiovascular benefits of NO_2-FA [35,50], particularly NO_2-OA and NO_2-LA [3,42], which regulate vascular function and exhibit protective effects against various cardiovascular and metabolic conditions. NO_2-LA can regulate vascular



Samples	Total Area (μm²)	%Area
Vehicle	37765.573±546.47	34.386±0.42
TP nitrated	14563.195±404.38	13.260±0.37***
TP non-nitrated	20087.521±879.76	18.290±0.80**b

Fig. 5. Antithrombotic effect *in vitro* of nitrated tomato pomace. NO_2-FA from tomato pomace inhibits thrombosis *in vitro*. Labeled whole blood was incubated with 4 mM Calcein-AM, (**A**) Vehicle (DMSO, 0.2 % v/v), (**B**) nitrated tomato pomace at 0.07 ± 0.04 mg/mL, and (**C**) non-nitrated tomato pomace at 1.18 ± 0.04 mg/mL, before perfusion through collagen-coated Vena8 biochip channels. The images were captured by fluorescence microscopy. Fluorescence images were analyzed using ImageJ software (version 1.26 t, NIH, USA). The area values (μm^2) of the formed thrombi are shown in the table. Results were expressed as mean ± SEM, n = 2. ** $p < 0.01$ and *** $p < 0.001$ denote differences compared to the vehicle (DMSO, 0.2 %). "a" and "b" denote statistically significant differences between nitrated tomato pomace and non-nitrated tomato pomace. A green platelet is observed on the left side, and on the right side, the area selected for measuring the thrombus size is observed.

function by inhibiting thrombin-stimulated platelet activation, attenuation of calcium mobilization, and induction of vasodilator-stimulated phosphorylation of phosphoproteins through cAMP-dependent mechanisms [8]. Current evidence supports the dual regulation of platelet adenylyl cyclase and phosphodiesterase E activities by NO_2−FA [3]. Structural, functional, and biological characterization of NO_2−FA has revealed clinically relevant protection against inflammatory injury in various cardiovascular and metabolic experimental models, including angioplasty-induced restenosis [7], cardiac ischemia−reperfusion (I/R) injury [32], diabetes [44], hypertension [55], pulmonary hypertension [21], vascular inflammation [53] and atherosclerosis [35].

The Mediterranean diet, rich in fruits and vegetables, has been associated with cardiovascular benefits, including the inhibition of platelet aggregation [40]. Data from the literature showing the formation of NO_2-FA from food sources has unveiled a positive correlation between the formation of NO_2-FA from extra virgin olive oil, reduction in fat accumulation, and improvement in mitochondrial function in non-alcoholic fatty liver disease through the inhibition of respiration

mobility [40].

The Food and Agriculture Organization of the United Nations (FAO) has shown that tomato harvest and production increase worldwide every year, with the leadingproducers being: China, America, India, Turkey, Egypt, Italy, Iran, Spain, Brazil, and Mexico, mainly [26]. Only a relatively small part of the tomato is consumed as a fresh product, while a large amount is processed into juice and paste. Therefore, the elimination or use of tomato pomace is an inevitable and significant problem for the food industry worldwide [20,26].

Tomato pomace, a by-product of tomato processing, poses a significant disposal challenge globally. In the Maule region of Chile, where substantial tomato processing occurs, tomato pomace accounts for a considerable portion of industrial waste [11,31]. Currently, its utilization is limited, resulting in environmental contamination and the loss of potentially valuable bioactive compounds [11,23].

Our findings underscore the antiplatelet potential of NO_2-FA derived from tomato pomace, surpassing non-nitrated pomace's in inhibiting platelet aggregation. Furthermore, NO_2-FA demonstrated inhibitory effects on platelet activation markers and promoted •NO production, further contributing to their antiplatelet activity [6,8,50,52]. NO_2-FA may add or potentiate protective effects to those observed for other antioxidants or antiplatelet agents, a hypothesis that warrants further exploration. NO_2-FA may interact with other compounds in food or the body, potentially influencing their antiplatelet activity [13].

Our results demonstrated an antithrombotic effect of NO_2-FA formed in tomato pomace under acidic gastric conditions. However, this evidence requires further evaluation *in vivo* by supplementing with tomato pomace. It is essential to analyze the levels of circulating NO_2-FA and determine if the physiologically achieved levels correlate with those used in the current study. Therefore, future research through *in vivo* studies is necessary to elucidate the mechanisms of action at physiologically relevant concentrations and validate the therapeutic potential of NO_2-FA.

Additionally, future research should focus on diet personalization, as individual responses may vary depending on genetic profiles or metabolism.

5. Conclusions

In conclusion, the growing emphasis on maintaining a balanced and nutritious diet has prompted research into harnessing bioactive compounds from agricultural byproducts. This study provides valuable insights into the antiplatelet potential of NO_2-FA synthesized and formed from industrial tomato byproducts, particularly prevalent in Chile's Maule region. Notably, primary NO_2-FA identified, including OA, LA, and LnA derivatives, exhibit robust, concentration-dependent antiplatelet activity, as demonstrated through our investigation.

Future research should focus on determining the optimal dosage to achieve therapeutic plasma levels of bioactive lipids from tomato pomace consumption or dietary supplementation and assessing their clinical significance in CVD and thrombosis. A comprehensive understanding of the circulating concentrations achieved, mechanisms of action, and cellular targets will be pivotal in advancing the development of functional foods or nutraceuticals derived from tomato pomace with antiplatelet properties. This knowledge will contribute to broader efforts in preventive and therapeutic strategies against CVD.

CRediT authorship contribution statement

Francisca Telleria: Investigation, Formal analysis. **Ivan Palomo:** Writing – review & editing. **Eduardo Fuentes:** Writing – review & editing, Supervision, Conceptualization. **Andrés Trostchansky:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Lyanne Rodríguez:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Felipe Lagos:** Formal analysis. **Mauricio Mastrogiovanni:** Methodology, Investigation, Formal analysis. **Ana Flores:** Investigation. **Andrea Plaza:** Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.117154.

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