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https://doi.org/10.3390/app14135707

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Antiplatelet Activity of Phenolic Compounds-Fortified Merlot Wine and Pure Phenolic Compounds

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Abstract: Red wines and their pomace are valuable sources of phenolic compounds (PCs), which have been proposed as potential contributors to their cardioprotective effect through the inhibition of platelet aggregation. The antiplatelet activity of an extract depends on its chemical composition, specifically the presence of certain phenolic compounds, as well as the interactions between them affecting biological activity. In order to assess the effect on platelet aggregation, we investigated the effect of the grape pomace PC enrichment of a Merlot wine, as well as the effect of the five major phenolic compounds present in wine extracts: caffeic acid, gallic acid, quercetin, epicatechin, and catechin. We analyzed how their combination influenced platelet aggregation. We found that the fortified wine sample with the highest PC content (W8) exhibited a potent antiplatelet effect in aggregation and platelet activation assays induced by the agonists TRAP-6, collagen, and ADP, with its activity being most potent against the latter agonist (78 ± 4%). Among the evaluated phenolic compounds, quercetin showed the highest antiplatelet potential against all three agonists studied, while gallic acid showed minimal antiplatelet effect. These findings suggest that the cardioprotective effect of wines is related to their chemical composition and the synergy among phenolic compounds. However, further research is required to fully understand the underlying mechanisms and clinical relevance of this activity.

Keywords: wine extracts; platelet aggregation; phenolic compounds

Citation: Rodriguez, L.; Muñoz-Bernal, Ó.A.; Fuentes, E.; Alvarez-Parrilla, E.; Palomo, I. Antiplatelet Activity of Phenolic Compounds-Fortified Merlot Wine and Pure Phenolic Compounds. *Appl. Sci.* **2024**, *14*, 5707. https://doi.org/10.3390/app14135707

Academic Editor: Dimitris P. Makris

Received: 21 May 2024
Revised: 11 June 2024
Accepted: 24 June 2024
Published: 29 June 2024

1. Introduction

The incidence and prevalence of chronic non-communicable diseases (NCDs) [1] and lifestyle and eating habits are key factors influencing our overall health and well-being. The incidence and prevalence of chronic non-communicable diseases (NCDs) [2].

Platelets play a crucial role in the development of CVDs, such as coronary heart disease, myocardial infarction, and stroke. The formation of blood clots, or thrombosis, is a normal process and is essential to stop bleeding in case of injury. However, in people with cardiovascular disease, this process is disrupted, and platelets can aggregate excessively and form unnecessary clots in the arteries, obstructing blood flow and potentially leading to an acute cardiovascular event [3]. Through the inhibition of aggregation processes, antiplatelet therapy has proven to be a cornerstone in the prevention and treatment of cardiovascular events [4]. Current approaches to improve antiplatelet efficacy have been based on oral and intravenous pharmacotherapies; the main antiplatelet agents are aspirin (reversible COX-1 inhibitor), clopidogrel (irreversible P2Y12 inhibitor), abciximab, and eptifibatide (GPIIb/IIIa inhibitors). The existing range of antiplatelet drugs presents shortcomings, therapeutic constraints, and side effects like dyspnea, ventricular pauses, and hyperuricemia. Additionally, they cause suffering from a slow onset of action and a risk of

bleeding [5]. The search for superior and safer antiplatelet agents constitutes a significant therapeutic challenge.

Cohorts from the USA, Japan, and Northern and Southern Europe show that nutrition is the cornerstone that promotes health, highlighting the importance of the Mediterranean Diet [6], which has been characterized by a high consumption of vegetables, legumes, fruits, nuts, cereals, fish, shellfish, and poultry as a source of protein, and olive oil, nuts, and a low or moderate consumption of red wine [6,7]. Diets rich in polyphenols have shown a great impact on the vascular system, improving both platelet and endothelial functions [8]. It is important to highlight that low or moderate wine consumption is understood to be between one and two drinks/day or ~150 and 300 mL/day [6,9]. It is important to consider that new guidelines have been published in Europe, indicating that consumers should control their drinking habits without exceeding the healthy threshold of alcohol consumption (30–40 g of alcohol/day in men and 10–20 g of alcohol/day in women) [10].

Phenolic compounds play an important role in enology since they are related to the organoleptic characteristics of wine, such as color, aroma, astringency, and bitterness [11]. The final phenolic content and the phenolic profile of the wines depend on the viticulture processes and the environmental conditions associated with the grape-growing region [12,13]. The antioxidant capacity of phenolic compounds depends on their chemical structures and the interactions between them [14]. Anthocyanins and flavonols are the main fractions of phenolic compounds in wine. The content of these compounds depends on several factors, and the specific profile of anthocyanins depends on the crop and is related to its genetic information [12].

The consumption of phenolic compounds found in red wines and grape pomace is associated with a lower prevalence of cardiovascular diseases, although the preventive mechanism is still not fully understood [15]. It has been proposed that the phenolic compounds present in red wines may enhance endothelium-dependent vasodilation, increase plasma antioxidants, and inhibit platelet aggregation [16]. The cardioprotective mechanisms associated with moderate wine consumption involve not only an increase in high-density lipoprotein cholesterol (HDL-C) levels and the regulation of blood lipids but also an improvement in glucose metabolism and endothelial function, decreasing inflammation and platelet aggregation as well as exerting antioxidant effects [1].

Previously, we studied the antiplatelet activity of these extracts and their relationship with the phenolic content. The results indicated that the antiplatelet effect of the most potent extracts (Merlot and Petit Verdot wine extract, as well as Petit Verdot grape pomace extract) could be related to their phenolic profile [17]. From these results, a PC-fortified wine was formulated by extracting PCs from Cabernet Sauvignon to fortify a Merlot wine (2 g of gallic acid equivalents (GAE)/L) at two PC levels: 4 and 8 g of GAE/L). These wines were sensory characterized, and the effect of PC fortification on the in vitro PC bioaccessibility was evaluated [13]. However, the antiplatelet effect of this PC fortification was not evaluated; for this reason, in the present study, the antiplatelet potential of Merlot wine and the two PC-fortified Merlot wines was evaluated. Considering that there is evidence that the interactions of PCs can generate a synergistic, additive, or antagonistic effect on different biological activities (Mercado–Mercado), the synergistic, additive, or antagonistic effect of the main phenolic compounds found in Merlot wines (control and fortified) was evaluated. The results of this study could have important implications for the formulation of natural therapies and supplements that seek to harness the potential benefits of phenolic compounds for the prevention and treatment of CVDs and other disorders related to platelet aggregation.

2. Materials and Methods

2.1. Chemicals

Adenosine 5′-diphosphate (ADP), thrombin receptor activating peptide-6 (TRAP-6), tubes of 3.2% citrate, and phosphate buffer solution (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents used for antiplatelet activity were of HPLC

quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA). Collagen was purchased from Havertown, USA. Pure compounds were HPLC grade (Sigma), and the extracts were lyophilized and dissolved in phosphate-buffered saline (PBS) for platelet aggregation studies.

2.2. Preparation of Fortified Wines

Four (F4) and eight (F8) g of GAE/L-fortified Merlot wines were prepared by adding the corresponding amount of 40% ethanol and Cabernet Sauvignon grape pomace PC extract, as previously reported [18]. A total of 100 mL of wine was freeze-dried and stored at −20 °C until antiplatelet evaluation.

2.3. Obtaining Human Platelets

This study involved six healthy volunteers of both sexes (aged between 20 and 45 years) who abstained from using anti-inflammatory medications for a week. The donors previously signed an informed consent according to the protocol approved by the Scientific Ethics Committee of the University of Talca (No. 29-2021) in accordance with the Declaration of Helsinki [19]. Blood samples were removed by phlebotomy in 3.2% sodium citrate tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). Briefly, blood samples were centrifuged at 240 g for 10 min at room temperature to obtain platelet-rich plasma (PRP). Once the PRP was separated, it was centrifuged again, this time at 800 g, to obtain platelet-poor plasma (PPP). PPP was used as a blank in subsequent platelet aggregation assays and to dilute PRP to the desired concentration (200–300 $\times$ 10^9 platelets/L). Platelet counts were performed on a Bayer Advia 60 Hematology System (Tarrytown, NY, USA) [20].

2.4. Antiplatelet Activity of Wine Extracts

Platelet aggregation studies were performed by turbidimetry in an aggregometer (AggRAM Module) following the methodology previously described by Born and Cross [13,21]. The wine samples were dissolved in PBS for analysis. First, the adjusted PRP (200–300 $\times$ 10^9 platelets/L) was incubated with PBS (negative control, maximum platelet aggregation). The study on platelet aggregation was carried out by incubating the PRP for 4 min with the extracts of wine at a concentration of 1 mg/mL. Platelet aggregation was induced by adding ADP (4 µM), TRAP-6 (10 µM), or collagen (1 µg/mL) for 6 min at 37 °C. Once the extracts were evaluated at 1 mg/mL, the samples with the highest antiplatelet potential, % platelet inhibition above 50%, were selected to continue platelet aggregation studies at lower concentrations. The concentration necessary to reduce platelet aggregation by 50% (Ic_{50}) was obtained from the concentration curves of the extracts (concentrations 0.1, 0.25, 0.50, 0.75, and 1 mg/mL) [22].

The antiplatelet activity of the phenolic compounds quercetin, epicatechin, catechin, gallic acid, and caffeic acid was also evaluated. Compounds were dissolved in DMSO. For these studies, the PRP was incubated with the compounds (0.1, 0.25, 0.50, 0.75, and 1 mg/mL) or vehicle (DMSO, 0.2%) and, subsequently, the platelet agonists (ADP, TRAP-6, or collagen) were added.

Platelet aggregation was measured as the increase in light transmission over 6 min. Results were obtained as the mean ± SEM of 6 volunteers provided by AGGRO/LINK software (Chrono-Log, Havertown, PA, USA). The inhibition of platelet aggregation was calculated as follows: % Inhibition of platelet aggregation = 100 − ((% Platelet aggregation of the extracts or compounds * 100)/% Platelet aggregation of the negative control) [13].

2.5. Activation Markers and Platelet Secretion

The expression of p-selectin and the activation of GP IIb/IIIa were evaluated by flow cytometry (BD FACSLyric), following the methodology previously described by Rojas et al., 2021 [20]. The study involved three healthy volunteers of both sexes (aged between 20 and 45 years) who abstained from using anti-inflammatory medications for a week. PRP

(200–300 × 10^9 platelets/L) was incubated with lyophilized wine (control and fortified) and pure phenolic compounds at different concentrations (0.1, 0.25, 0.50, 0.75, and 1 mg/mL) or with the vehicle (positive platelet activation control). Platelet aggregation was stimulated with collagen (1 µg/mL), TRAP-6 (10 µM), and ADP (4 µM) for 6 min at 37 °C. Then, to assess p-selectin expression, the sample was incubated with CD62-PE for 30 min in the dark. To evaluate the expression of GP IIb/IIIa in platelets, the sample was incubated with PAC-1-FITC under the same conditions. Measurements were performed on platelets from 3 healthy volunteers in duplicate. Platelet populations were selected according to cell size by forward scatter (FSC) versus side scatter (SSC). Platelets were identified with anti-CD61-PE. In a similar way, the study of platelet activation markers for phenolic compounds was carried out.

2.6. Antiplatelet Effect of Binary Interactions of Phenolic Compounds

In order to study the antiplatelet effect of binary interactions between the different phenolic compounds, gallic acid, caffeic acid, quercetin, catechin, and epicatechin, ten different combinations of these phenolic compounds were made in equal proportions ((50A:50B%, 50% of each compound) and evaluated at a final concentration of 1 mg/mL. Percent inhibition (Inh%) of the individual phenolics was compared to that of the binary mixtures to determine if there was a synergistic, additive, or antagonistic effect. For this, six replicates of each combination were made, and the results are expressed as the mean ± SEM.

2.7. Statistical Analysis

Antiplatelet aggregation data were analyzed using Prism 8.0 software (GraphPad Inc., San Diego, CA, USA) and are expressed as the mean ± standard error (SEM) of the individual experiments. Platelet inhibition results were analyzed by one-way ANOVA and Dunnet's t post-hoc test to determine the significant differences between samples. The level of significance used for the analyses was $p < 0.05$ [21].

3. Results

3.1. Antiplatelet Activity of Lyophilized Wines and Phenolic Compounds

In this study, an in vitro assay was performed to evaluate the antiplatelet activity of wine samples at a concentration of 1 mg/mL (CW, F8, and F4). Table 1 shows the results of the antiplatelet activity of three lyophilized wine samples (CW, F8, and F4) stimulated by collagen, TRAP-6, and ADP, as well as the vehicle used as control. Platelet aggregation (PA) and the inhibition of platelet aggregation (Inh) values are expressed in percentage (%). Compared to the vehicle, the F8 extract showed significant inhibition of platelet aggregation stimulated by collagen, TRAP-6, and ADP, with 52 ± 13%, 55 ± 9%, and 78 ± 4% inhibition, respectively. On the other hand, extracts CW and F4 did not present significant differences in the inhibition of platelet aggregation compared to the vehicle.

Table 1. Antiplatelet activity of wine extracts stimulated by collagen, TRAP-6, and ADP.

Extracts	Collagen 1 µg/mL		TRAP-6 10 µM		ADP 4 µM	
	PA (%)	Inh (%)	PA (%)	Inh (%)	PA (%)	Inh (%)
CW	91 ± 6 ^{ns}	4 ± 2	87 ± 17 ^{ns}	12 ± 5	91 ± 2 ^{ns}	1 ± 2
F8	45 ± 12 ***	52 ± 13	44 ± 10 ***	55 ± 9	20 ± 4 ***	78 ± 4
F4	88 ± 4 ^{ns}	7 ± 1	85 ± 7 ^{ns}	14 ± 6	74 ± 8 ^{ns}	19 ± 6
Vehicle	95 ± 5	0	99 ± 1	0	93 ± 3	0

Results are expressed as mean ± SEM, $n = 6$. Data were analyzed by one-way ANOVA. Post hoc analyses were performed using Dunnet's test. *** $p < 0.001$ and denote statistically significant differences compared to the negative control (vehicle). CW: control Merlot wine. F8: 8 g GAE/L fortified Merlot wine. F4: 4 g GAE/L-fortified Merlot wine. ns: denotes non-statistical differences with respect to the vehicle. ADP: Adenosine diphosphate, Inh: Inhibition, PA: Percentage of platelet aggregation, SEM: Standard error, TRAP-6: Thrombin-6 receptor activating peptide.

These results indicate that the F8 extract possesses potent antiplatelet activity, especially in response to TRAP-6 stimulation, suggesting its potential utility in the context of the prevention of platelet aggregation-related cardiovascular disorders.

Considering that F8 showed antiplatelet activity greater than 50%, a dose-dependent antiaggregant evaluation effect of this sample was carried out based on the concentration of the F8 extract, and results are shown in Figure 1. It was observed that this wine extract exhibited a greater concentration-dependent antiplatelet potential when TRAP-6 and ADP stimulated platelet aggregation, with inhibition percentages of IC_{50} = 0.30 ± 0.08 mg/mL and IC_{50} = 0.39 ± 0.04 mg/mL, respectively (Table 2). However, when platelet aggregation was induced by collagen, the extract only showed activity at the highest concentration tested (1 mg/mL).

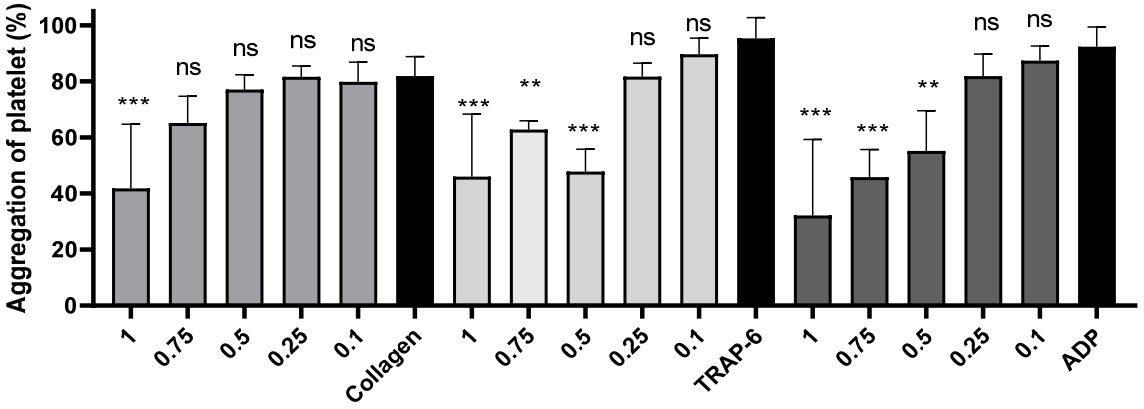

Figure 1. Effect of lyophilized F8 fortified wine on the platelet aggregation induced by collagen, TRAP-6, and ADP. PRP was previously incubated with vehicle or lyophilized wine (0.1, 0.25, 0.50, 0.75, and 1 mg/mL). After 4 min of incubation at 37 °C, platelets were stimulated with the agonist to initiate platelet aggregation for 6 min. The negative control is PBS (absence of the extracts). The bar graph indicates maximum aggregation expressed as the percentage (mean ± SEM; n = 3 for duplicate). A one-way ANOVA and Dunnet's post-hoc test for statistical analyses were used. *** $p < 0.001$ and ** $p < 0.01$ denote statistically significant differences compared to the vehicle; ns: non-statistical difference with respect to the vehicle.

Table 2. Inhibitory effect of wine extract on platelet aggregation stimulated by collagen, TRAP-6, and ADP.

Extract	Collagen 1 µg/mL	TRAP-6 10 µM	ADP 4 µM
	IC_{50}	IC_{50}	IC_{50}
CW	>1 mg/mL	>1 mg/mL	>1 mg/mL
F4	>1 mg/mL	>1 mg/mL	>1 mg/mL
F8	0.87 ± 0.23 mg/mL	0.30 ± 0.08 mg/mL	0.39 ± 0.04 mg/mL
Epicatechin	>1 mg/mL	>1 mg/mL	0.98 ± 0.19 mg/mL
Catechin	>1 mg/mL	>1 mg/mL	0.94 ± 0.20 mg/mL
Gallic acid	>1 mg/mL	>1 mg/mL	>1 mg/mL
Caffeic Acid	>1 mg/mL	>1 mg/mL	0.83 ± 0.23 mg/mL
Quercetin	0.81 ± 0.31 mg/mL	0.92 ± 0.11 mg/mL	0.65 ± 0.62 mg/mL

IC_{50} values (mg lyophilized wine/mL) are presented as the mean ± SEM of 3 independent experiments in duplicate.

These results indicate that the F8 extract has concentration-dependent activity, being more effective in inhibiting platelet aggregation induced by TRAP-6 and ADP at lower concentrations, while a higher lyophilized wine concentration is required to obtain significant antiplatelet activity in response to collagen stimulation. This specificity in the response may have important implications in terms of the extract's selectivity and effectiveness in controlling platelet aggregation in different physiological or pathological situations.

Once the in vitro antiplatelet activity of the F8 wine sample was evaluated, the antiplatelet activity of some of the main phenolic compounds reported in Merlot (control and fortified), including quercetin, epicatechin, catechin, caffeic acid, and gallic acid, was evaluated (Table 2). The antiplatelet potential of these compounds was studied at different concentrations (0.1, 0.25, 0.50, 0.75, and 1 mg/mL) in PRP (Figure 2). The results revealed that all phenolic compounds, except gallic acid, had antiplatelet activity depending on the agonist used. Specifically, epicatechin and catechin, both isomers, inhibited platelet aggregation induced by TRAP-6 and collagen. Catechin specifically had a concentration-dependent antiplatelet potential when platelets were stimulated by collagen.

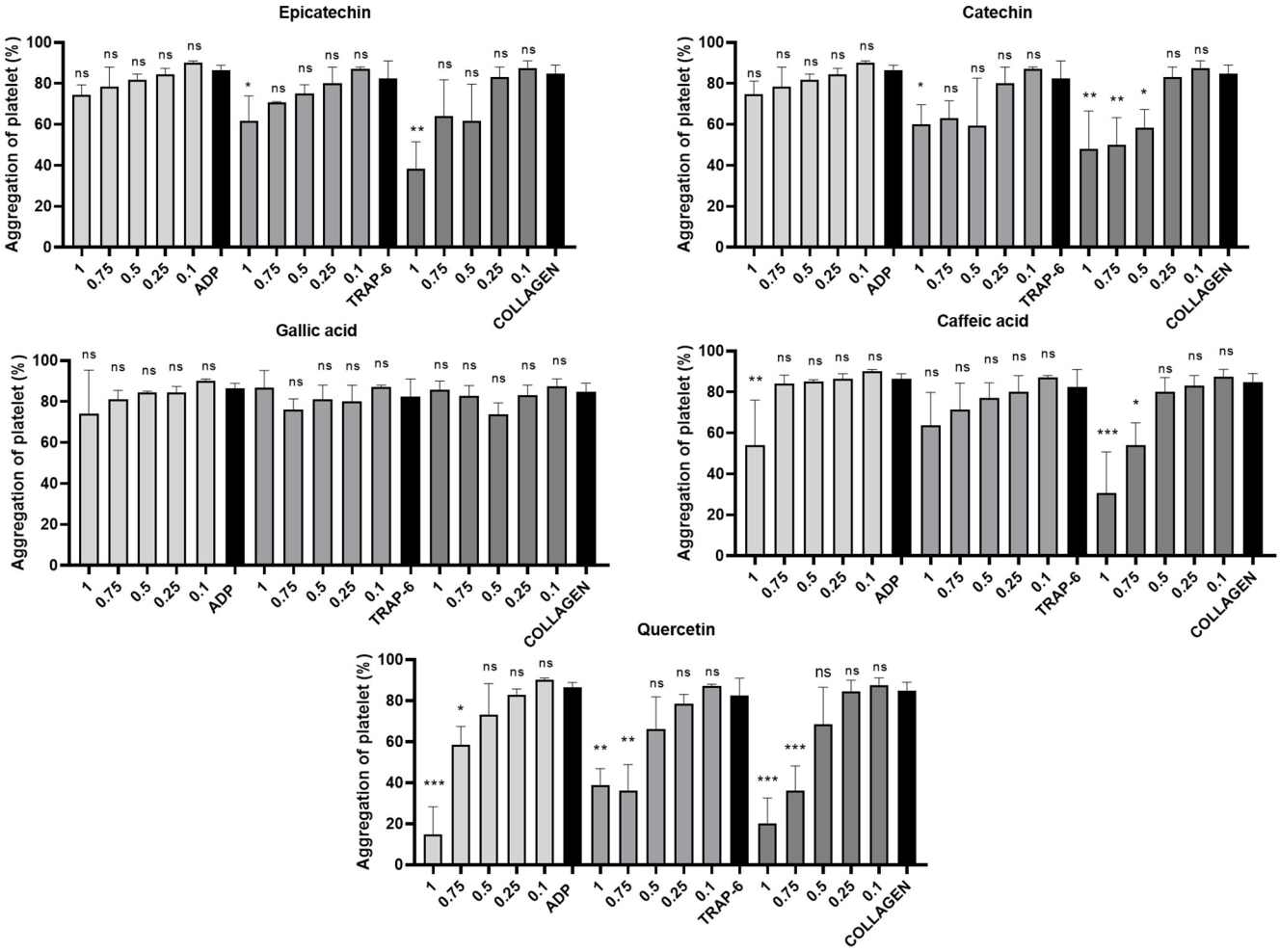

Figure 2. Study of platelet aggregation of free pure phenolic compounds induced by collagen, TRAP-6, and ADP. PRP was incubated with vehicle or phenolic compounds (0.1, 0.25, 0.50, 0.75, and 1 mg/mL). After 4 min of incubation at 37 °C, PRP was stimulated with the agonist to initiate platelet aggregation for 6 min. The negative control is DMSO 0.2% (absence of the extracts). The bar graph indicates maximum aggregation expressed as a percentage (mean ± SEM; n = 3 for duplicate). Differences between groups were analyzed by one-way ANOVA using Dunnet's post-hoc test. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ denote statistically significant differences compared to the vehicle; ns: non-statistical difference with respect to the vehicle.

For its part, gallic acid did not show antiplatelet activity, while caffeic acid inhibited platelet aggregation stimulated by ADP and collagen, being more effective against the latter agonist (IC_{50} = 0.83 ± 0.23 mg/mL). On the other hand, quercetin showed antiplatelet potential against the three agonists studied, exhibiting potent inhibition in

each case (IC_{50} = 0.81 ± 0.31 mg/mL, collagen; IC_{50} = 0.92 ± 0.11 mg/mL, TRAP-6; and IC_{50} = 0.65 ± 0.62 mg/mL, ADP).

These findings suggest that the phenolic compounds present in the F8 wine sample could be responsible for its antiplatelet activity. The presence of multiple compounds with antiplatelet activity may contribute to the synergistic effect observed in the F8 wine sample, reinforcing its positive potential in the inhibition of platelet aggregation and its possible implications for cardiovascular health.

3.2. Activation Markers and Platelet Secretion of Wine Extracts and Phenolic Compounds

Platelet activation markers of the most active fortified wine sample (F8) and of the phenolic compounds epicatechin, catechin, quercetin, and caffeic acid were evaluated. Gallic acid was not evaluated since no statistical activity was observed compared to the control.

A high level of P-selectin in platelets is associated with an increased risk of atherosclerosis and vascular pathologies, so we evaluated the expression of this cell adhesion molecule. The results of the study showed that the F8 wine sample, which was the most active in terms of antiplatelet activity, inhibited p-selectin expression induced by the three agonists, collagen, TRAP-6, and ADP, only at 1 mg/mL, the highest evaluated concentration (Figure 3A–C). However, at lower concentrations, the extract did not show significant inhibition of p-selectin expression compared to the platelet-activated condition. In addition, the F8 wine sample at the highest concentration (1 mg/mL) also inhibited ADP-induced GPIIb/IIIa activation (Figure 3C–E). GPIIb/IIIa is an important marker of platelet activation related to aggregation and blood clot formation.

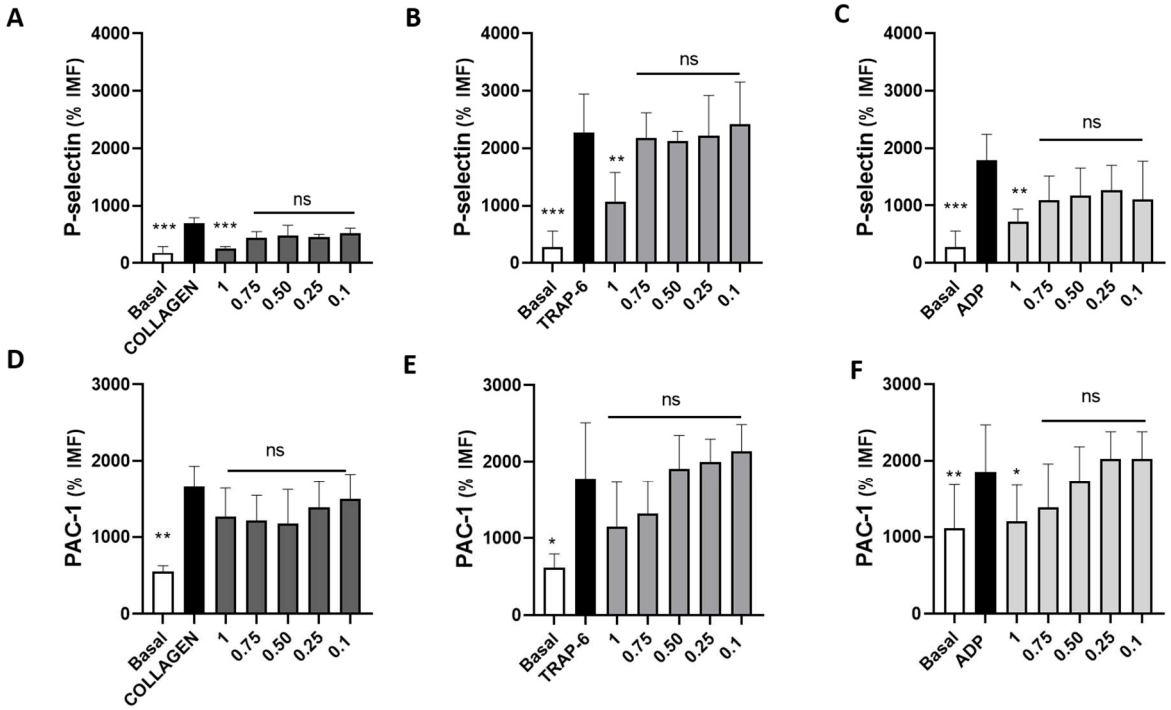

Figure 3. Effect of lyophilized fortified wine (F8) on the secretion of p-selectin (**A**–**C**) and activation of GP IIb/IIIa (**D**–**F**) induced by collagen, TRAP-6, and ADP. Platelet activation and secretion markers were determined by flow cytometry using different lyophilized wine concentrations (0.1, 0.25, 0.5, 0.75, and 1 m/mL). Negative control, phosphate-buffered saline (PBS). Platelets were identified as the $CD61^+$ population. Statistical analysis was performed by one-way ANOVA (Dunnet's test). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. vehicle vs. activated control (agonist) ($n = 3$ for duplicate).

These findings suggest that F8 wine may exert its antiplatelet effect by inhibiting the expression of p-selectin, a marker of platelet activation related to the interaction between platelets and leukocytes during the inflammatory response and clot formation.

Furthermore, the F8 wine sample also showed GPIIb/IIIa inhibition, which may have important implications in the modulation of platelet aggregation. These results provide a better understanding of the mechanisms involved in the antiplatelet activity of the F8 wine sample.

Additionally, the effect of phenolic compounds on platelet activation markers was evaluated (Figure 4). The results of our study revealed that the phenolic compounds evaluated, including catechin, quercetin, and caffeic acid, showed the ability to modulate the expression of p-selectin, a cell adhesion molecule associated with the risk of atherosclerosis and vascular diseases.

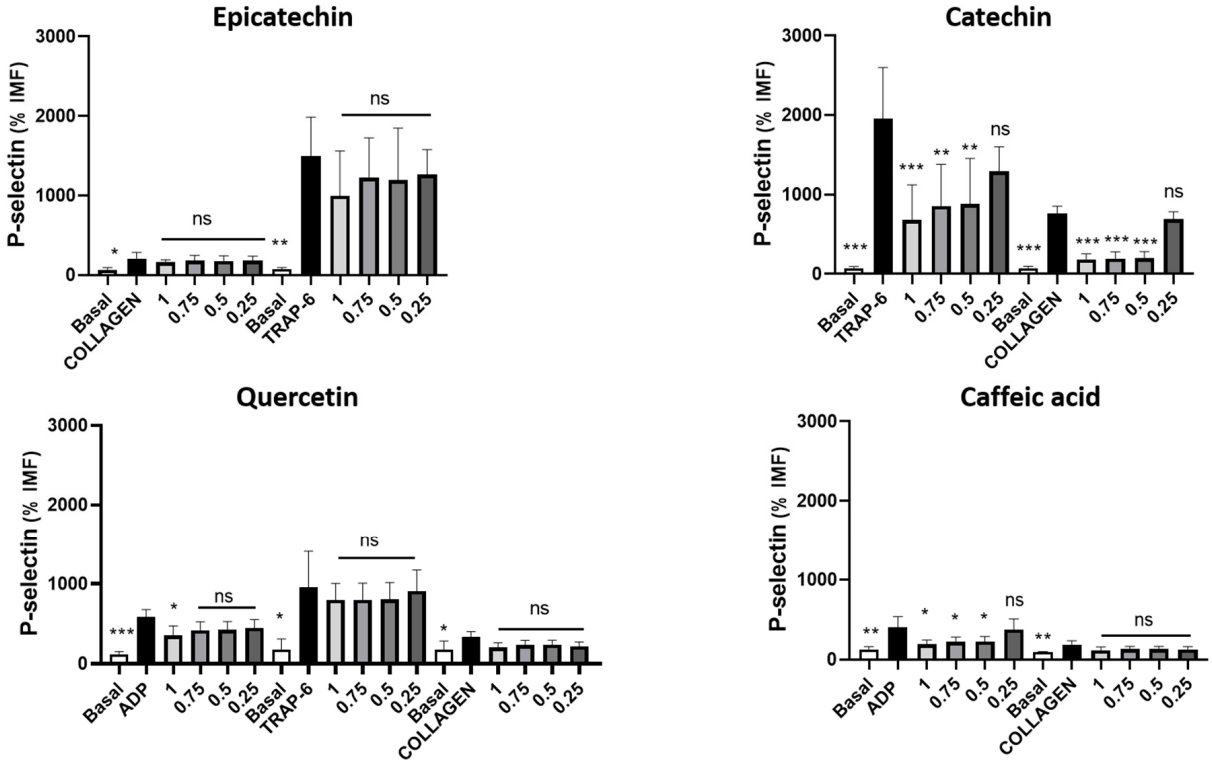

Figure 4. Effect of pure free phenolic compounds on the secretion of p-selectin induced by collagen, TRAP-6, and ADP. Platelet activation and secretion markers were determined by flow cytometry using different phenolic compound concentrations (0.25, 0.5, 0.75, and 1 m/mL). Negative control is DMSO 0.2%. Platelets were identified as a $CD61^{+}$ population. Statistical analysis was performed by one-way ANOVA (Dunnet's test). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. vehicle vs. activated control (agonist) ($n = 3$ for duplicate).

Interestingly, catechin significantly inhibited p-selectin expression when platelets were stimulated with TRAP-6 and collagen at concentrations higher than 0.5 mg/mL. On the other hand, epicatechin did not show a significant effect on p-selectin expression in our study. Regarding quercetin, it was found that it inhibited the expression of p-selectin induced by ADP at a concentration of 1 mg/mL. This indicates that quercetin can regulate the expression of this adhesion molecule in response to ADP stimulation. Caffeic acid also showed an inhibitory effect on ADP-stimulated p-selectin expression, and this effect was concentration-dependent. A significant decrease in the expression of p-selectin was observed up to a concentration of 0.5 mg/mL of caffeic acid. These results suggest that caffeic acid may have an impact on the regulation of p-selectin expression in response to ADP stimulation. Once again, and in agreement with platelet aggregation results shown in Figure 2, gallic acids showed no p-selectin activity, confirming that this phenolic compound does not seem to show any anti-platelet activity.

In general, these findings support the idea that the phenolic compounds evaluated may be responsible for the inhibition of platelet aggregation observed in the F8 wine sample. However, it is important to highlight that further research is needed to better understand the underlying mechanisms and the clinical relevance of the effects of phenolic compounds on the regulation of p-selectin expression and their contribution to the antiplatelet activity of the fortified wine samples.

3.3. Anti-Plateral Effect of Binary Interactions between Phenolic Compounds

The results of the interactions between phenolic compounds on the inhibition of platelet aggregation revealed interesting and varied patterns (Figure 5). Higher inhibition results were observed for practically all combinations, suggesting that combinations of phenolic compounds may have synergistic or additive effects that modulate antiplatelet activity, which could have important implications for the potential therapeutic application of these compounds in the management of CVDs.

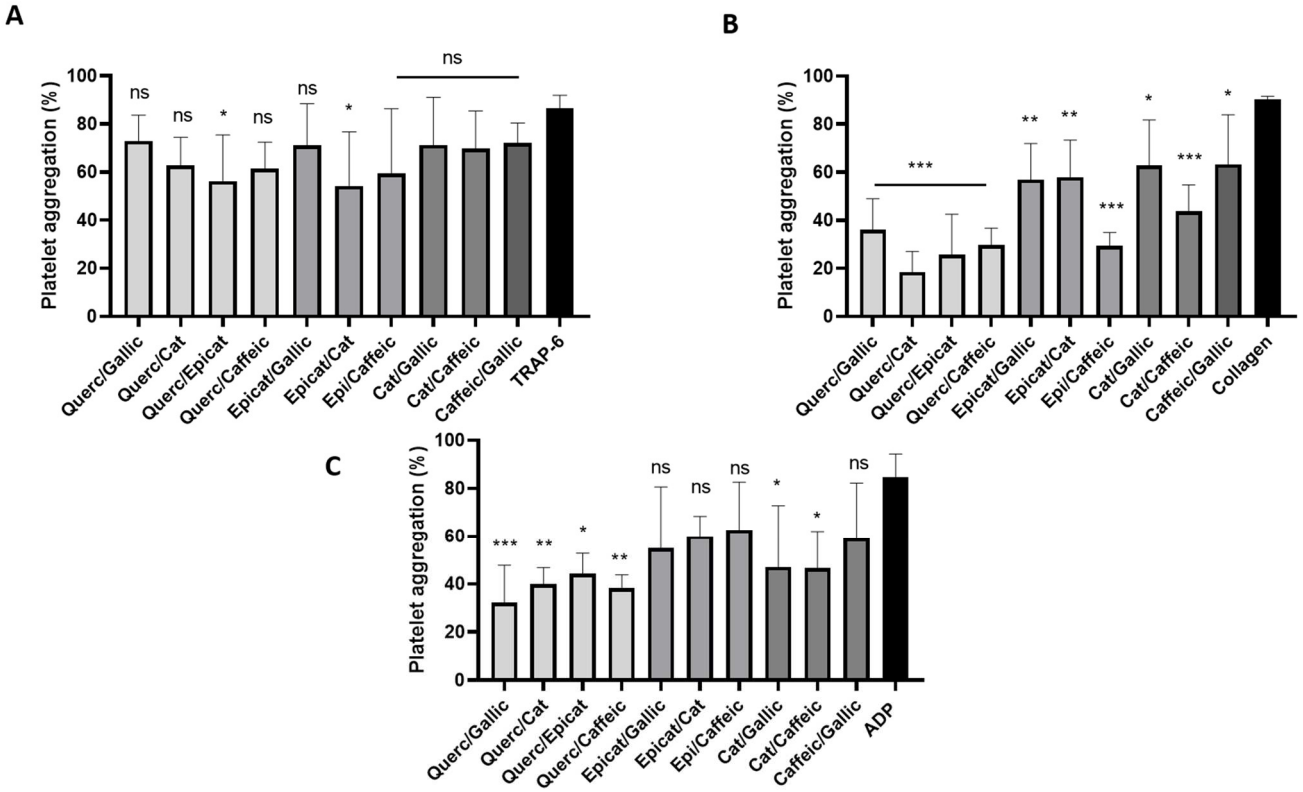

Figure 5. Effect of the binary combination of phenolic compounds (Caffeic: Caffeic acid, Cat: Catechin, Epicat: Epicatechin, Gallic: Gallic acid, Querc: Quercetin) on platelet aggregation. Platelets were stimulated with (**A**) TRAP-6, (**B**) collagen, and (**C**) ADP. The PRP was previously incubated with vehicle or binary mixtures of the phenolic compounds (50:50%, 1 mg/mL). After 4 min of incubation at 37 °C, it was stimulated with the agonist to initiate platelet aggregation for 6 min. The negative control is DMSO 0.2% (absence of phenolic compounds). Each phenolic compound concentration was 50% of each compound at a total final concentration of 1 mg/mL. The bar graph indicates the maximum aggregation expressed as a percentage (mean ± SEM; $n = 3$ in duplicate). Differences between groups were analyzed by ANOVA using Dunnet's post-hoc test. *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ denote statistically significant differences with respect to the vehicle; ns: non-statistical difference with respect to the vehicle.

When platelet aggregation was induced with TRAP-6, a slight but significant antiaggregant effect was observed in the Querc/Epicat and Epicat/Cat combinations (36 ± 4% platelet inhibition and 38 ± 5% platelet inhibition, respectively). On the other hand, the

antiplatelet effect of the independent compounds was only greater for Quercetin 1 mg/mL and 0.75 mg/mL (53 ± 2 and 56 ± 3% platelet inhibition, respectively), while Epicatechin and Catechin (26 ± 2 and 28 ± 1% platelet inhibition, respectively) had less potential than their respective combinations with quercetin (Figure 5A).

Stimulation of platelet aggregation with collagen showed that all phenolic compounds, except gallic acid, significantly inhibited platelet aggregation at 1 mg/mL. Quercetin revealed the strongest effect, and this inhibition remained statistically significant up to 0.75 mg/mL. In addition, all the binary combinations tested showed potent inhibition of platelet aggregation, with the mixtures including quercetin being especially notable since they presented more potent antiplatelet effects than the independent compounds (Querc/Gallic, 60 ± 3, Querc/Cat, 80 ± 2, Querc/Epicat, 71 ± 4 and Querc/Caffeic, 67 ± 2% platelet inhibition). This suggests a possible synergistic effect between quercetin and other phenolic compounds in the inhibition of collagen-stimulated platelet aggregation (Figure 5B).

When platelet aggregation was evaluated with ADP, only caffeic acid and quercetin compounds showed significant antiplatelet activity. As expected, combinations of quercetin with other phenolic compounds showed significant inhibitory effects. Interestingly, Cat/Gallic and Caffeic/Gallic also revealed a slightly significant inhibitory effect compared to the control. These results are interesting, considering that gallic acid showed no antiplatelet activity, suggesting that the combination of gallic acid with other phenolic compounds may potentiate their antiplatelet activity in a synergistic way. This suggests that although some individual compounds may not be effective in inhibiting platelet aggregation against a specific agonist, when combined with other compounds they may have a synergistic or additive effect and potentiate its activity (Figure 5C).

In conclusion, our results indicate that the combinations of phenolic compounds present in wine may have synergistic effects on the inhibition of platelet aggregation. These findings are relevant to better understanding the mechanism of action of these compounds and could have implications in the development of more effective and safe antiplatelet therapies for the management and prevention of CVDs. However, it is important to take into account that these results were obtained in vitro and that additional studies, such as clinical trials, are required to confirm and validate the biological potential under real physiological and clinical conditions.

4. Discussion

Parallel to the improvement in life expectancy, deaths from CVD have been increasing [23]. Platelet hyperactivity has been increasingly linked to the development and complication of certain CVDs, including atherosclerosis, thrombosis, peripheral arterial disease, myocardial infarction, and ischemic stroke [8]. Through inhibition of these activations and aggregation processes, antiplatelet therapy has proven to be a mainstay in the prevention and treatment of CVD events [4].

Polyphenolic compounds present in products of plant origin play a central role in many scientific studies due to their potential health benefits, mainly due to their positive impact on CVDs [8,24]. Phenolic compounds have been shown to have antiplatelet capabilities and a positive impact on CVD management, exerting prominent antioxidant, anti-inflammatory, antitumor, cardioprotective, antihyperglycemic, and antimicrobial effects [25]. Quercetin, after ingestion, is metabolized into several compounds, including isorhamnetin and tamarixetin. Research has shown that these metabolites retain potent antithrombotic activities. They effectively inhibit platelet aggregation and thrombus formation, similar to quercetin itself [26]. On the other hand, catechin is rapidly metabolized into glucuronide and sulfate conjugates after absorption. These metabolites have been shown to maintain bioactivity and contribute to the beneficial cardiovascular effects associated with catechin intake [27,28]. The absorption and metabolism of these dietary phenolic compounds determine the extent of their health benefits, which in turn are determined by their structure (conjugation with other phenols, degree of glycosylation/acylation), solubility,

and molecular size. Its effectiveness decreases with the replacement of the hydroxyl groups in its structure by sugars; therefore, aglycones exhibit more potent activities than their corresponding glycosides [8].

The antiplatelet activity of an extract is strongly influenced by its chemical composition. It has been described that the presence of bioactive compounds is responsible for the activity of different samples [20,22]. In particular, phenolic compounds have been largely attributed to extensive cardiovascular benefits [29]. Its effects are strongly related to its chemical structure and the interactions that the compounds can exert, whether synergistic (when the effect increases), additive (when the effect is maintained), or antagonistic (when the effect decreases) [14]. The antiplatelet ability of phenolic compounds in vivo is poor. Therefore, despite the current advances, more studies are needed to confirm the cardioprotective potential of phenolic compounds towards their use alone or in combination with conventional drugs for effective therapeutic intervention [13].

In our study, we evaluated different pathways of platelet activation induced by platelet agonists: collagen, TRAP-6, and ADP. As expected, there were differences in antiplatelet potential. Platelets have previously been shown to be activated by different activators through complex signaling pathways [26,30]. Thrombin, thromboxane A, collagen, and ADP are some of the key agonists of platelet activation that lie at the intersection of a plethora of inflammatory pathways that modulate proinflammatory and coagulation processes [3]. ADP interacts with the family of purinergic receptors (P2Y1, P2Y12, and P2X1), while TRAP-6 acts on the thrombin receptor PAR1 [31]. Wine sample 8 showed a potent antiplatelet effect against the agonists TRAP-6, collagen, and ADP, with its activity being more potent against the latter agonist (78 $\pm$ 4% percentage of platelet inhibition). Although F8 showed greater antiplatelet activity than the rest of the wines evaluated, this effect was indistinct depending on the agonist that triggered platelet aggregation, with a lower IC_{50} compared to TRAP-6 (0.30 $\pm$ 0.08). The results suggest that our extract does not inhibit platelet activation through glycoprotein VI (GPVI), which is the main collagen receptor, while its activity seems to be related to activation pathways through purinergic receptors and PAR1 (ADP and TRAP-6, respectively) [26]. F8 also inhibited the expression of p-selectin induced by the three agonists and the expression of GPIIb/IIA induced by ADP. Similar results were shown by Rein et al., 2000; a procyanidin-enriched cocoa drink together with the administration of dealcoholized red wine resulted in a significant reduction in platelet activation (p-selectin and GPIIb/IIa) [32]. However, we would need additional studies to elucidate the way by which the wine extract induces the inhibition of platelet aggregation.

Also, it was observed that individual phenolic compounds exhibit antiplatelet effects, with quercetin being the most prominent against the three agonists studied, while gallic acid did not show any antiplatelet effect. In addition to antiplatelet activity, the expression of the cell adhesion molecule p-selectin, which is associated with the risk of atherosclerosis and vascular disease, was assessed. Both the F8 wine sample and some of the phenolic compounds evaluated showed the ability to inhibit agonist-induced p-selectin expression, suggesting that this mechanism could contribute to the regulation of platelet aggregation.

Research on the synergistic, additive, and antagonistic effects between phenolic compounds is also relevant to understanding how to optimize their use in therapeutic applications. Overall, these results support the importance of phenolic compounds in promoting a healthy diet and their potential benefit for cardiovascular health [33,34]. Previous studies have shown that binary combinations of phenolic compounds have additive or antagonistic effects on the DPPH [14]. In this study, ten combinations of five independent phenolic compounds were evaluated to understand how they interact and whether they exhibit synergistic, additive, or antagonistic effects in inhibiting platelet aggregation. The phenolic compounds analyzed were quercetin, epicatechin, catechin, caffeic acid, and gallic acid.

In our case, when platelet aggregation was stimulated with TRAP-6, there was an antagonistic effect, both for the flavonoid–flavonoid and flavonoid–phenolic acid binary combinations; the Querc/Epicat and Epicat/Cat mixtures showed a slight inhibition effect,

and this was less than the independent quercetin. On the other hand, when aggregation was stimulated with ADP, the flavonoid–flavonoid and flavonoid–phenolic acid binary combinations generally showed a synergistic effect, while phenolic acid–phenolic acid had an antagonistic effect. When we studied the ADP-stimulated platelet aggregation of the individual pure compounds, caffeic acid and quercetin showed antiplatelet activity; as expected, the quercetin mixtures showed antiplatelet potential, as did the Cat/Gallic and Cat/Caffeic combinations.

While a synergistic effect was observed against collagen, all combinations had activity, in fact, more potent than the independent compounds. Although gallic acid had no activity against collagen, the combinations of this phenolic acid with other phenolic acids (caffeic acid) and flavonoids (quercetin, epicatechin, and catechin) potentiated the inhibition of platelet aggregation, showing the synergistic effect of these mixes. It has been reported that when gallic acid is mixed with another phenolic compound, the 3-OH substitution results in a higher torsion angle and loss of coplanarity [14,35]. Some investigations have also observed that there is oxidation in the combination of phenolic compounds, which could generate an effect on the antiplatelet capacity of the ions (antagonism/additive) [36,37].

This research provides valuable insights into the interactions between phenolic compounds and their impact on the inhibition of platelet aggregation. The phenolic compounds present in wine show great potential as therapeutic agents for the management and prevention of CVD. The focus on combinations of phenolic compounds opens new perspectives for the development of more effective and safer antiplatelet therapies in the future.

However, it is essential to continue investigating and understanding the mechanisms of action of these phenolic compounds, as well as their specific contributions to the antiplatelet effect of wine extract. It is necessary to conduct in vivo studies to confirm if the metabolites identified after the consumption of enriched wine can present any antiplatelet activity. Additionally, preclinical studies and clinical trials are required to confirm the antiplatelet activity of these combinations of phenolic compounds and to evaluate their safety and efficacy in humans. Future research should focus on these in vivo studies to validate the physiological relevance of our findings. On the other hand, our strategies to exploit the therapeutic potential of wine phenols would focus on developing extracts and supplements. This involves isolating the phenolic compounds from wine and grape pomace and creating concentrated extracts or supplements that can be administered in a safe and controlled manner.

5. Conclusions

These findings are relevant to research in the field of antiplatelet therapies. Platelet aggregation plays a crucial role in the formation of blood clots and the development of CVD, such as atherosclerosis, thrombosis, and myocardial infarction. The ability of the phenolic compounds present in wine to inhibit platelet aggregation may have a significant impact on the prevention and treatment of these diseases. The results suggest that combinations of phenolic compounds may have advantages over the use of individual compounds. Mixtures that mainly showed synergistic effects might have higher potency in inhibiting platelet aggregation, which might allow lower doses of each compound to achieve the same therapeutic effect. This could reduce potential side effects associated with high doses of individual compounds and improve treatment efficacy. However, it is important to highlight that these results were obtained in vitro studies, which means that further studies are still needed to validate these effects under real physiological and clinical conditions. Future research should focus on developing supplements or medications based on these phenolic compounds to take advantage of their beneficial properties without the risks associated with alcohol consumption. Furthermore, we propose to perform in vivo studies and clinical trials to confirm the efficacy and safety of these compounds in inhibiting platelet aggregation and their potential in the prevention and treatment of CVD.

Author Contributions: Conceptualization, E.A.-P. and I.P.; methodology, L.R. and Ó.A.M.-B.; software, L.R. and Ó.A.M.-B.; formal analysis, L.R.; investigation, L.R. and Ó.A.M.-B.; data curation, L.R. and Ó.A.M.-B.; writing—original draft preparation, L.R. and Ó.A.M.-B.; writing—review and editing, E.F., I.P. and E.A.-P.; supervision, I.P. and E.A.-P.; funding acquisition, E.A.-P. All authors have read and agreed to the published version of the manuscript.

Funding: CONAHCYT Mexico (Project CB-2016-286449).

Institutional Review Board Statement: The protocol was approved by Scientific Ethics Committee of the University of Talca, following the Declaration of Helsinki (Protocol No. 19/ 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated from this study are available to the corresponding authors upon request.

Conflicts of Interest: The authors declare no conflict of interest.

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