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ORIGINAL PAPER

In Vitro Assay of Quinoa (*Chenopodium quinoa* Willd.) and Lupin (*Lupinus* spp.) Extracts on Human Platelet Aggregation

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Abstract

Cardiovascular disease (CVD) is the leading cause of death throughout the world. A major risk factor for CVD is platelet aggregation. Various plant extracts exhibit anti-aggregatory action *in vitro*. The dietary intake of traditional plant crops such as quinoa (*Chenopodium quinoa* Willd) and lupin (*Lupinus* spp., Fabaceae family), highly recognized for their high nutritional value, is increasing worldwide. The aim of the study was to assay possible antiplatelet effects of quinoa and lupin bean extracts *in vitro*. The proximate chemical composition of quinoa grains and the three most widely known lupin cultivars: blue (*L. angustifolius)*, yellow (*L. luteus or mutabilis)* and white (*L. albus*) grown in Chile were analyzed. The anti-aggregation activity of the ethanol extracts of the crops was assayed using flow cytometry in ADP-stimulated human platelets, and their inhibition of the maximal platelet aggregation was measured. All the lupin extracts exhibited a significant anti-aggregatory effect ($p < 0.0001$), while quinoa extracts did not exert this effect compared to control platelets. In conclusion, lupin beans extracts exhibited an anti-aggregatory effect on activated human platelets.

Keywords *Chenopodium quinoa* Willd. · *Lupinus* spp. · Antiplatelet · Cardiovascular risk · Plant extracts · Plant crops · Platelet aggregation

Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
CVD	Cardiovascular disease
PGE1	Prostaglandin E1
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PBS	Phosphate-buffered saline
TRAP-6	Thrombin receptor activator peptide
SD	Standard deviation
SEM	Standard error of the mean

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Introduction

Cardiovascular disease (CVD) is the leading cause of death throughout the world. One of the triggers of CVD is thrombosis, produced by a clot that occurs inside a blood vessel, as a consequence of an increase in platelet activity, generating aggregation. Activated platelets have major roles in primary hemostasis: they adhere to the sub-endothelium of a damaged vessel, trigger coagulation and formation of a stable coagulum that prevents bleeding and facilitates vessel repair. However, an exacerbation of this process may enhance the formation of thrombus [1]. Various pharmacological agents are used to prevent CVD, represented mainly by drugs exhibiting antiplatelet activity. This approach involves various adverse effects, though, such as hemorrhages, hematologic reactions, and dyspnea [2]. Consequently, new strategies to modulate platelet activity are needed.

Food intake plays a key role in reducing the risk of CVD, and over 30% of all deaths could be prevented through dietary

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changes, mainly by increasing the consumption of plant foods [3]. The current interest on the recuperation of food heritage and the use of ancestral crops has increased, and some traditional crops that have been largely recognized for their high nutritional value may be considered in the search for their beneficial effect on platelet activity. In this study, two gluten-free grains that have traditionally been consumed are studied: *Chenopodium quinoa* Willd (quinoa, a pseudocereal), and a dicotyledononus legume, *Lupinus* spp. (lupin bean). The high nutritional value of quinoa is related to the high amount and excellent quality of proteins, a moderate lipid content, as well as the presence of a variety of minor phytochemicals [4]. In fact, quinoa exhibits a low glycaemic index [5]; contains enzyme inhibitors, which inhibit α-amylase and α-glucosidase, allowing for better control of glucose absorption [6]. It is a good source of phenolic compounds, carotenoids, betalains, phytosterols, squalene, fagopyritols, phytoecdysteroids, saponins, among others [4]. Most phenolic compounds display a high antioxidant activity [7] and include isoflavones, mainly genistein and daidzein, which exhibit a wide range of beneficial effects [8]. The quality and amount of protein in seeds has also led to the search of bioactive peptides, among which antihypertensive angiotensin I converting enzyme (ACE) inhibitory action has been demonstrated [9]. On the other hand, *Lupinus* spp. (lupin bean) includes over 450 species, of which *L. albus* (white), *L. luteus* (yellow) or *mutabilis*, and *L. angustifolius* (blue, narrow-leaf) are economically important. The bitter taste of lupin kernels has been reduced in modern varieties (sweet lupin) to very low levels through conventional breeding techniques. It contains a high amount of proteins and supplies dietary fiber [10]. Lupin bean is also a good source of phytochemicals, including enzyme inhibitors (proteases, amylases), lectins, phytosterols, phenolic compounds, phytates, saponins [11, 12]. An interesting feature of lupin bean is the very low (or nil) content of phytate, tannins, lectins, protease inhibitors and isoflavones [13, 14].

There is a global trend among individuals towards the substitution of animal with plant proteins for CVD prevention, and the grains selected for this study are among those currently considered as healthy products. Both quinoa and lupin beans are traditional crops for which a revival is evidenced: quinoa has been part of the traditional diet in the Andean zones, while the use of white lupin as food has been very common in the Mediterranean area for centuries. The common quinoa grains found in the Chilean food market are pre-washed (to reduce saponins), while the variety of lupin beans more consumed is *L. albus*. Most of the produced lupin beans are still used for animal feed, mainly in the fish farming industry, although there is a re-growth of the market for these plant foods.

A number of food plants extracts exhibit potential anti-platelet activity [3]. However, no anti-thrombotic effects have been documented for quinoa or lupin crops. Since the intake of these grains is increasing worldwide, due to the search for high quality plant foods that replace animal based foods, the present study aimed to determine the antiplatelet effects of quinoa and lupin bean extracts *in vitro* and describe some of the possible mechanisms of action involved.

Materials and Methods

Samples. Quinoa (*Chenopodium quinoa* Willd) var. Coastal was obtained from a small organic producer in Petorca, Region of Valparaiso (−33.0309° latitude, −71.5031° longitude), Chile. Two samples of the same cultivar were assayed: in its natural form and pre-washed grains, which denotes an abrasive peeling procedure commonly used to reduce the saponins content from the surface of the grains. The samples were grown in the same field, and were harvested in 2017. The three lupin bean cultivars analyzed (*L. angustifolius*, *L. mutabilis* and *L. albus*) were provided by Seeds von Baer®, Temuco, Region of Araucania (−38.7396° latitude, −72.5984° longitude), Chile. The three varieties are classified as sweet, due to their low content of alkaloids (< 0.05%). The grains were stored between 9 and 11 °C. The grains were ground into flour using a kitchen processor (Moulinex®) and sieved to 0.5 mm, then frozen in sealed plastic bags at −20 °C until analysis.

Chemical Analysis The proximate composition of grains was determined using AOAC methods [15]. Protein content was determined by Kjeldahl assay (AOAC 920.54) in a nitrogen digestor DK6 (VELP) and a nitrogen distiller UDK 129 (VELP), using a factor of 6.25 to convert nitrogen to proteins. Crude fat was assessed using the AOAC method 920.39, moisture was determined using the AOAC method 945.15, ash was determined using the AOAC method 942.05 and dietary fiber was measured using the enzymatic-gravimetric method 991.43.

Preparation of Extracts The extracts from quinoa and lupin were obtained according to Fuentes et al. [16]. Briefly, the samples were blended and mixed with ethanol, then sonicated at 60 kHz (Auto Science, USA) for 15 min. The level of water in the ultrasonic bath was monitored and maintained at room temperature (25 ± 1 °C). Samples were centrifuged for 10 min at 700 *g* (Eppendorf Centrifuge 5804) and the supernatant were filtered (0.22 μm), lyophilized (Freeze Dryer LGJ-10C) at 45 °C, and stored at −80 °C.

Preparation of Human Platelets Six healthy young male volunteers (ages 20–30) participated in the study. The protocol was authorized by the Ethics Committee of the Universidad de Talca in accordance with the Declaration of Helsinki [17]. Once all the participants read and signed the informed consent, 10 mL venous blood samples were obtained in 3.2%

citrate tubes (9:1 *v*/v) by phlebotomy using a vacuum tube system (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) with a 21 G needle [18]. Each sample (5 mL) was centrifuged (DCS-16 Centrifugal Presvac RV) at 240 g for 10 min to obtain platelet-rich plasma (PRP). Then, two-thirds of PRP was removed and the rest was centrifuged (10 min at 650 g) to obtain platelet-poor plasma (PPP). Other 5 mL were centrifuged to obtain PRP and then again centrifuged for 10 min at 650 g. The rest of the pellet was washed with HEPES-Tyrode's buffer containing Prostaglandin E1 (PGE1, 120 nmol/L). PRP and washed platelets were adjusted at a concentration between 200 and 300×10^6 platelets/mL (Bayer Advia 60 Hematology System, Tarrytown, NY).

Platelet Aggregation Assay Platelet aggregation was monitored by light transmission according to Born and Cross [19], using a lumi-aggregometer (Chrono-Log, Havertown, PA, USA). Briefly, 480 mL of PRP (200 to 300×10^9 platelets/L) were pre-incubated with 20 μL saline or each different extract (1 mg/mL) for 5 min. Following this, 20 μL of agonist (adenosine diphosphate ADP, 4 μM, thrombin receptor activator peptideTRAP-6, 10 μM, and collagen 1 μg/mL) were added and platelet aggregation was registered for 6 min. All measurements were performed in triplicate. Platelet aggregation results were determined by AGGRO/LINK (Chrono-Log, Havertown, PA, USA). The extract inhibition of the maximal platelet aggregation was expressed as a percentage of the control (saline).

Flow Cytometry Study Expressions of P-selectin on platelet surface were analyzed by double-label flow cytometry by the method previously described [20]. Briefly, 480 μL of washed platelets (200 to 300×10^9 platelets/L) were incubated with the different extracts (1 mg/mL) for 5 min. Then, each sample was treated for 6 min at 37 °C with ADP 4 μM. An aliquot of 50 μL was taken and mixed with saturated concentrations of anti-CD62P-PE and anti-CD61-FITC for P-selectin expression. The sample was incubated for 25 min in the dark. Platelet populations were gated on cell size using CD61 positivity and forward scatter *vs*. side scatter and analyzed over 5000 events in an Accuri C6 flow cytometer (BD, Biosciences, USA). The results represent the mean and standard error of the mean (SEM) of three independent determinations.

Statistical Analysis The chemical analyses of the grains were performed in triplicate; each replicate was quantified in duplicate. All data given represent mean values ± standard deviation (SD). The platelet aggregation assays data were analyzed using Prism 6.0 software (GraphPad Inc., San Diego CA, USA) and expressed as mean ± SEM. Three or more independent experiments were performed for the different assays. Results were expressed as % inhibition or as a % of control (100%). Differences between groups were analyzed by the Student paired or unpaired *t*-test and a one-way analysis of variance (ANOVA) using Tukey's *post-hoc* test. *p* values lower than 0.05 were considered significant.

Results and Discussion

The intake of grains is growing worldwide as a consequence of a tendency towards a more sustainable diet, replacing meats for plant sources of good quality proteins, lipids, high supply of dietary fiber and a variety of phytochemicals, which are increasingly recognized for their health beneficial effects.

Quinoa and Lupin Bean Proximate Composition The first part of the study comprised the proximate analysis of the crops assayed, in order to determine their general contents of protein and lipids, the main macronutrients present in the grains, as well as their dietary fiber and total mineral content (ashes). The composition of the crops is shown in Table 1. No difference was observed in the chemical composition of natural and pre-washed quinoa grains, except that the treated grains contained less fiber ($p < 0.05$), due to the abrasive peeling applied to eliminate saponins. The three cultivars of lupin beans analyzed exhibited differences in their moisture, lipids, fiber and ashes content, while proteins were similar in *L. angustifolius* and *L.albus* samples, being higher in *L. mutabilis* ($p < 0.05$). The results demonstrate that quinoa is a good source of proteins (higher than cereals), while lupin beans (legume) are an excellent source of them, all varieties exhibiting over 30 g/100 g. The amount of lipids was higher in *L. mutabilis*, which also exhibited the lowest content of fiber.

Effects of Quinoa and Lupin Bean Ethanol Extracts on Human Platelet Aggregation Several researchers have linked the biological effects of these crops with CVD prevention. Quinoa consumption has been mainly associated with a decrease in triglycerides [21], while blue lupin whole grain intake has a hypocholesterolemic effect probably associated to impaired intestinal cholesterol absorption caused mainly by phytosterols [22] and proteins [23], along with a reduction of LDL-cholesterol [24] and bioactive peptides contained in lupin proteins [25–27]. A diet containing lupin bean has also been associated with the maintenance of insulin levels and blood pressure [9, 28].

In order to evaluate possible antiplatelet effects *in vitro*, different agonists may be used such as ADP, collagen, ristocetin, adrenaline, thrombin receptor activating peptide, thromboxane A2, or arachidonic acid, in adequate experimental conditions. Consequently, the maintenance of normal platelet aggregation or the reduction of the aggregation induced by an agonist appear to be supportive for the substantiation of a health claim in the context of reduction of platelet

Table 1 Proximate composition of quinoa and lupin beans (g/100 g)

Sample	Moisture	Lipids	Proteins	Fiber	Ashes
Ch. quinoa 1	8.99 ± 0.05[a]	5.71 ± 0.27[a]	12.38 ± 0.18[a]	10.12 ± 0.29[a]	3.01 ± 0.07[a]
Ch. quinoa 2	11.12 ± 0.08[a]	5.73 ± 0.06[b]	13.75 ± 0.43[a]	8.30 ± 0.10[a]	3.67 ± 0.17[b]
L. angustifolius	10.38 ± 0.09[1]	5.05 ± 0.04[1]	32.35 ± 0.47[1]	40.90 ± 0.13[1]	2.76 ± 0.13[1]
L. mutabilis	9.27 ± 0.13[2]	13.94 ± 0.10[2]	40.68 ± 0.29[2]	18.82 ± 0.05[2]	4.56 ± 0.02[2]
L. albus	8.05 ± 0.04[3]	7.65 ± 0.09[3]	33.19 ± 0.56[1]	35.31 ± 0.08[3]	3.93 ± 0.04[3]

1: natural; 2: pre-washed. $n = 3$. X ± SD. Different letters for quinoa and different numbers for lupin beans indicate significant difference ($p < 0.05$)

aggregation by using accepted experimental protocols, such as the model applied in this study. We determined whether ethanol extracts exerted any inhibition of platelet aggregation triggered by various platelet agonists [ADP (4 μM), TRAP-6 (10 μM) or collagen (1 μg/mL)] (Figs. 1, 2, 3). In general, the extracts inhibited platelet aggregation induced by ADP more effectively than those induced by the other agonists.

The effect of ethanol extracts of natural and pre-washed Chilean quinoa and lupin beans (*L. albus*, *L. angustifolius* and *L. mutabilis*) was assayed on platelet aggregation (Fig. 1). For this purpose, PRP was preincubated for 5 min with each extract and platelets were activated using 4 μM ADP (Fig. 1a). The extracts exhibited different inhibitory actions on ADP-stimulated platelet aggregation. In general, natural and pre-washed quinoa extracts exerted a minimal inhibitory activity, with an inhibition of around 10%. All lupin bean extracts induced a notorious decrease of platelet aggregation percentage, producing an inhibition of almost 53%, showing a significant difference with respect to control (Fig. 1b). The inhibition percentages for the three varieties studied were 47.3, 58.7, and 50.4% for *L. albus*, *L. angustifolius* and *L. mutabilis*, respectively. Under these conditions, lupin extracts clearly inhibited the platelet aggregation induced by ADP. As a control of the inhibition of platelet aggregation, we used PGE1, which stimulates adenyl cyclase activity in platelets and increases cyclic AMP (cAMP) concentrations, causing an inhibition of platelet aggregation.

We further evaluated the inhibition of platelet aggregation using two platelet agonists: TRAP-6 10 μM and collagen 1 μg/mL. For this purpose, the extracts were incubated with the platelets in the same conditions as described above. As shown in Figs. 2 and 3, all the analyzed extracts [natural and pre-washed quinoa and lupin beans (*L. albus*, *L. angustifolius,* and *L. mutabilis*)] induced a percentage of inhibition of platelet aggregation <10%, which reveals that these extracts did not exhibit any antiplatelet activity against the agonists.

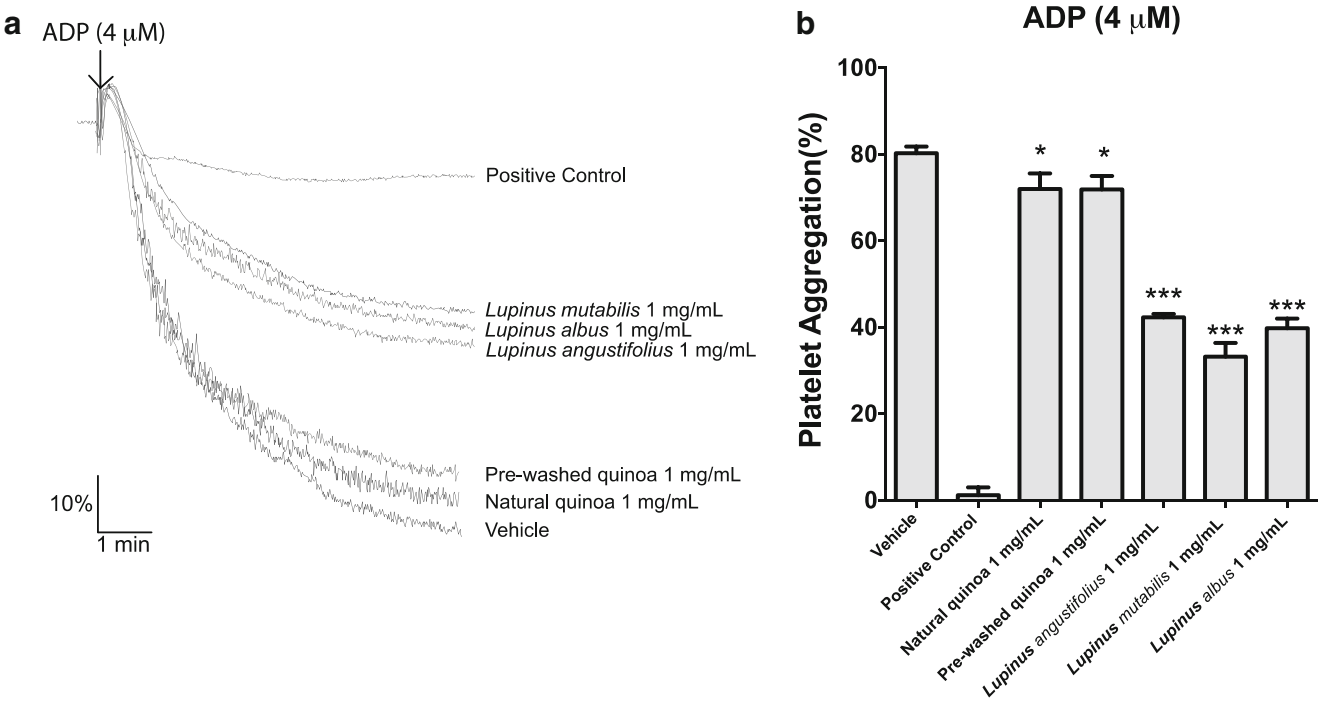

Fig. 1 Effect of quinoa and lupin bean ethanol extracts on human platelet aggregation induced by ADP (4 μM) (**a**) traces represent the kinetic of aggregation (**b**) summarizes the maximal aggregation expressed as percentage (mean ± SEM; $n = 6$). ***$p < 0.0001$ compared with control analyzed by ANOVA using Tukey's *post-hoc* test. Vehicle, phosphate-buffered saline (PBS); positive control, prostaglandin E1 (PGE1, 20 μM)

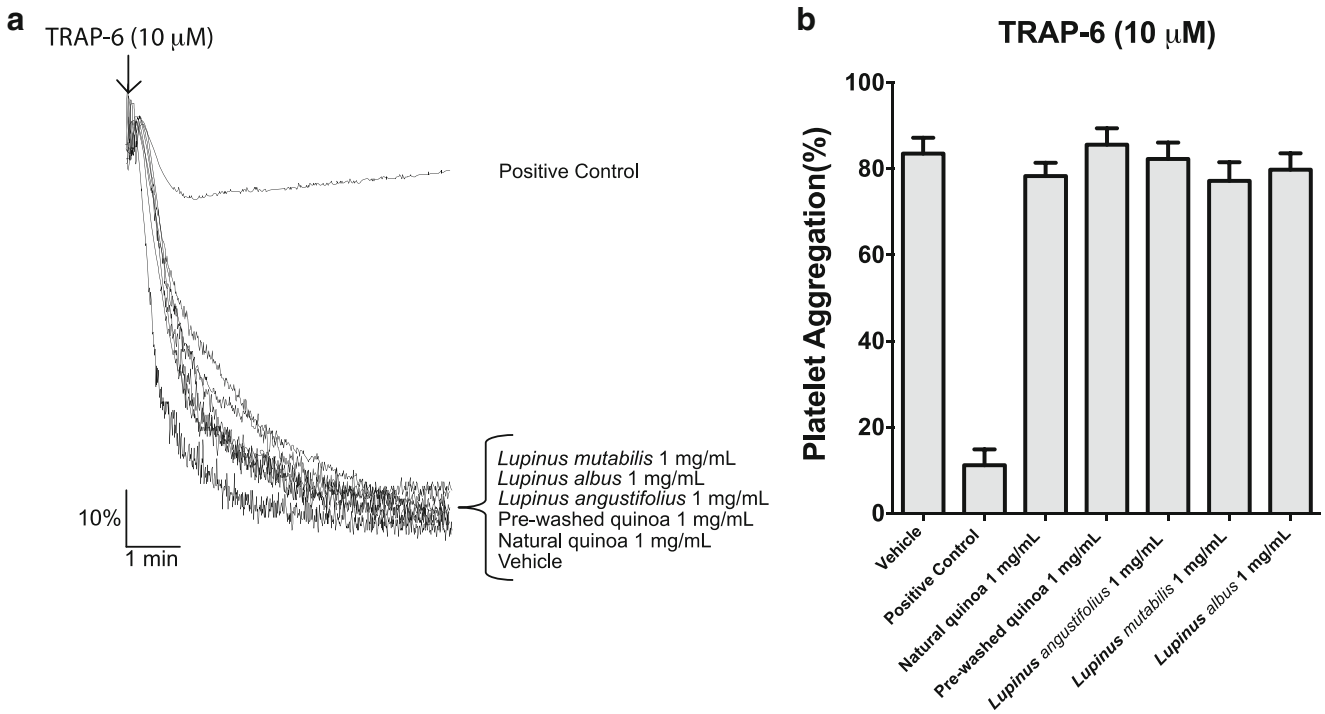

Fig. 2 Effect of quinoa and lupin bean ethanol extracts on human platelet aggregation induced by TRAP-6 (10 μM) (**a**) traces of the aggregation for each extract (**b**) summarizes the maximal aggregation expressed as percentage (mean ± SEM; $n = 6$). ***$p < 0.0001$ compared with control analyzed by ANOVA using Tukey's *post-hoc* test. Vehicle, phosphate-buffered saline (PBS) positive control, prostaglandin E1 (PGE1, 20 μM)

The different agonists used in platelet aggregation (ADP, TRAP-6, and collagen) activate different signal transduction pathways, determining the capacity and degree of inhibition of the extracts used. It has been shown that certain molecules present in different extracts have a specific and selective anti-platelet action in response to some agonists (*i.e.*, ADP, and not

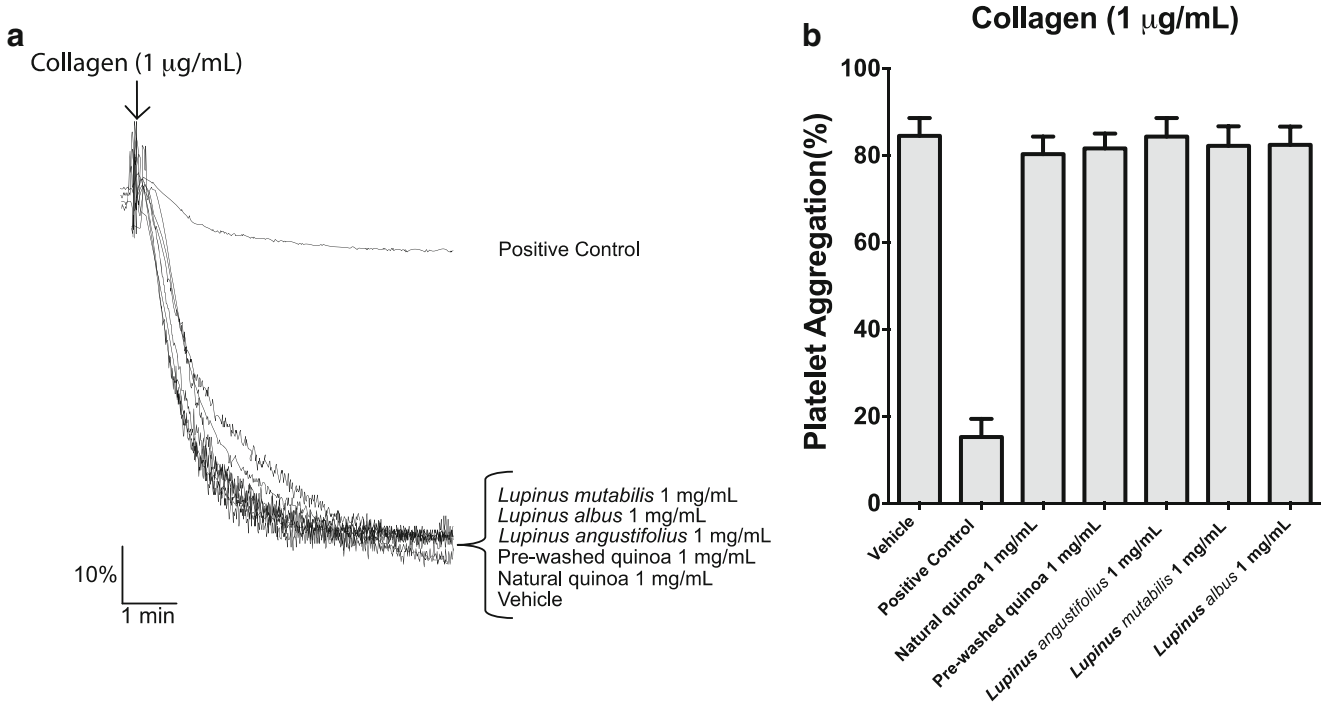

Fig. 3 Effect of quinoa and lupin bean ethanol extracts on human platelet aggregation induced by collagen (1 μg/mL) (**a**) aggregation for each extract (**b**) summarizes the maximal aggregation expressed as a percentage (mean ± SEM; $n = 6$). ***$p < 0.0001$ compared with control analyzed by ANOVA using Tukey's *post-hoc* test

thrombin or collagen), while other compounds are effective against more than one platelet agonist [29, 30]. For example, coffee extract inhibited platelet aggregation induced by ADP, while no effect was observed on collagen-induced platelet aggregation. Therefore, future assays could specifically determine which compounds present in the different lupin extracts are bioactive in order to examine their mechanisms of action.

Effects of Lupin Beans on P-Selectin Expression The third objective of this study was to determine whether the different extracts exerted an inhibitory effect on platelet activation triggered by platelet agonists using ADP 4 μM (Fig. 4). ADP was used as a platelet agonist for the lupin beans extracts in the flow cytometry assay, since these extracts showed antiplatelet activity with this agonist.

The main component expressed in the pathogenesis of vascular lesions is P-selectin. In addition to participating in the development of the atherosclerotic lesion, it is also involved in the development of arterial thrombosis, since it allows platelet-endothelial cell adhesion [31] and platelet-leukocyte aggregates [32]. It also allows the adhesion of platelets and white cells (neutrophils or monocytes) to the endothelium, triggering a series of events, such as inflammation and atherogenesis [31]. It is important to highlight the importance of its detection, since it is only expressed in activated cells [33], and high levels of platelet expression has been detected in stroke [34], diabetes, hypertension, and acute coronary syndromes [34, 35]. Therefore, the increase in P-selectin levels in platelets is associated with a higher risk of atherosclerosis and vascular pathologies.

Washed platelets were pre-incubated for 5 min with the different extracts of lupin beans and subsequently activated with ADP 4 μM in order to evaluate the expression of P-selectin on platelet membranes. The figures and values showed in Fig. 4a are representative. Platelets were washed and purity was determined by flow cytometry using anti-CD61 (around 98% positive for this marker). We compared the activated conditions with ADP 4 μM *versus* the basal condition (not activated), observing a significant difference in both conditions: 75.2 ± 2.1% and 8.3 ± 3.1%, respectively.

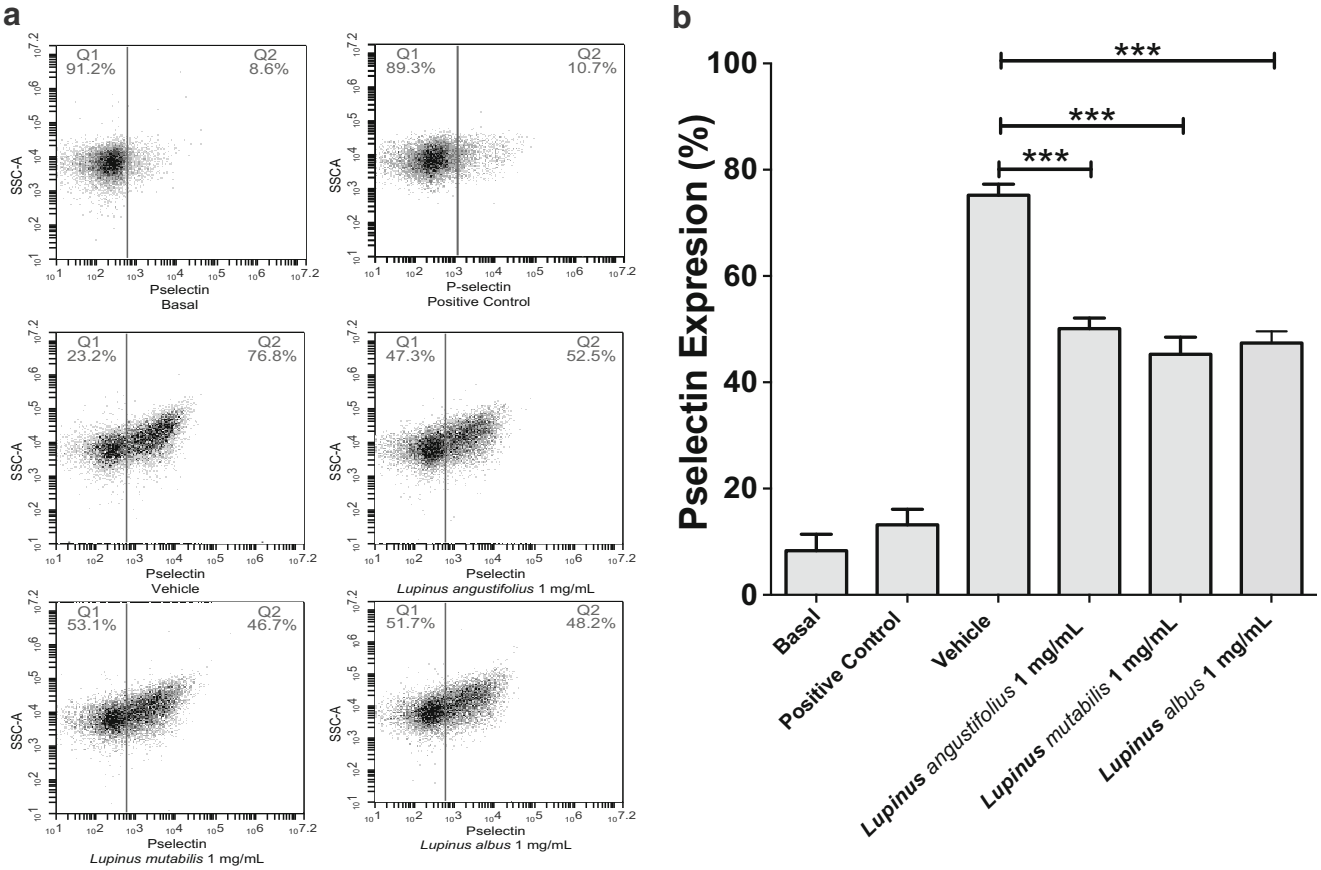

Fig. 4 Effects of lupin bean ethanol extracts on human platelet activation. Washed platelets were incubated with the extracts of lupin beans (*L. angustifolius, L. mutabilis, and L. albus*), pre-incubated for 6 min. All conditions were activated by ADP (4 μM) as a platelet agonist (not activated is basal condition) (**a**) different distribution of platelets are separated by side scatter and positivity for P-selectin expression on human platelet surface induced by ADP (4 μM) (**b**) maximal P-selectin expression is expressed as a percentage (mean ± SEM; $n = 6$). Results were obtained from six volunteers (each donor performed as single triplicates). ***$p < 0.0001$, **$p < 0.001$. Vehicle, phosphate-buffered Saline (PBS); positive control, prostaglandin E1 (PGE1, 20 μM)

There was also a considerable reduction in the expression of P-selectin in the three extracts of lupin beans, from 75.2 ± 2.1% to 50.1 ± 2.0% (1.7 fold), 45.3 ± 3.2% (1.2 fold), and 47.4 ± 2.2% (1.2 fold), respectively (Fig. 4b). All the data were obtained from six volunteers and represent mean ± SEM. The figures indicate that these extracts are able to reduce the expression of P-selectin and, consequently, inhibit platelet activation.

In conclusion, both quinoa grains and lupin beans grown in Chile are highly nutritive plant foods, although an anti-aggregatory effect on activated platelets was exhibited only by ethanol extracts of lupin beans, which represents their ability to decrease this major risk factor for CVD. This evidence supports the substantiation of the recommendation of the inclusion of quinoa and lupin beans in a healthy, highly nutritious diet, while lupin beans may additionally be considered to be included as part of a cardioprotective diet.

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Compliance with ethical standards

Conflict of Interest The authors declare no potential conflicts of interest.

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