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Effects of Pomegranate Juice and Extract Polyphenols on Platelet Function

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ABSTRACT Several studies have shown that polyphenols reduce cardiovascular accidents in high-risk patients; in particular, the inhibition of platelet function may be responsible for part of this benefit. This research studied the antiplatelet effect of Wonderful variety pomegranate (*Punica granatum*) products, which contain primarily hydrolyzed tannins such as ellagitannins. We have investigated *in vitro* the effects of treatment with either pomegranate juice (PJ) or the polyphenol-rich extract from pomegranate fruit (POMx) on platelet aggregation, calcium mobilization, thromboxane A_2 production, and hydrogen peroxide formation, induced by collagen and arachidonic acid. PJ and POMx reduce all the platelet responses studied. POMx showed a stronger action in reducing platelet activation; moreover, POMx is active at the concentration that it is possible to obtain after polyphenol-rich food intake (2 μM). These results demonstrated that the cardiovascular health benefits of pomegranate may in part be related to the ability of polyphenols to inhibit platelet function. In fact, PJ and pomegranate extract have similar effects at concentrations expected for normal intake.

KEY WORDS: • ***Antioxidant*** • ***platelet activation*** • ***polyphenols*** • ***pomegranate extract*** • ***pomegranate juice***

INTRODUCTION

SEVERAL CLINICAL AND *IN VITRO* studies showed that polyphenols are able to inhibit platelet activation, preventing cerebro- and cardiovascular disease.[1] The Mediterranean diet includes fruit and vegetables rich in polyphenols known as effective protective agents.[2] Fruits and vegetables contain polyphenols such as tannins found in red wine and phenolic acid, flavonones, anthocyanin, flavonols, stilbenes, and lignans found in pomegranates as protective agents from the hot Mediterranean sunshine.[3] Those properties may vary at minimal structural modifications. Epidemiological studies have shown that a polyphenol-rich diet is able to protect humans against degenerative diseases such as cancer and cardiovascular diseases. Cancer and cardiovascular protection,[4] reduction of thrombotic risk, and better quality of life are the most well-known properties of polyphenols. In the past, the more scientifically supported opinion was that glycoside polyphenols were not as effective as protective agents. Polyphenol bioavailability depends on chemical and structural factors and, above all, on intra-individual variability of metabolism pathways. *In vivo*, after moderate polyphenol consumption, their plasma concentration is between 0 and 4 μmol/L,[5] even if the local concentration may be higher than 3 mmol/L[4] in the intestine.

Interestingly, some of the natural polyphenols present in the Mediterranean diet might inhibit the platelet activation pathway,[6] even if it is not clear if their *in vivo* biological activities are exerted by the native compounds, by their metabolites, or by a combination of both. In any event, the polyphenols present in the Mediterranean diet may partially explain the "French paradox."[7]

The polyphenols contained in pomegranate juice (PJ) are hydrolyzed tannins, such as ellagitannins and anthocyanins, but PJ is also rich in sugar and organic acids. The polyphenols from the polyphenol-rich extract from pomegranate fruit (POMx) primarily consist of hydrolyzed tannins with anthocyanins, or almost pure hydrolyzed tannins. Although the accurate characterization of all PJ compounds is very complex, organic chemical studies by physical methods (electron paramagnetic resonance, nuclear magnetic resonance, infrared, and high-performance liquid chromatography) have permitted identification and quantification of nearly all pomegranate polyphenols.[8]

The aim of the present study is to evaluate the effects of PJ on human platelet function at very low concentrations, like those that are possible to find *in vivo* after a moderate consumption of PJ. Moreover, in order to understand whether other non-polyphenolic PJ compounds could be effective as antiplatelet agents, we have also used POMx, an extract of polyphenols from fresh pomegranate fruit with the same polyphenolic constituent of PJ,[8] lacking free sugar and in which levels of all non-polyphenolic compounds are greatly reduced. This work is meant to verify the effective biological action of polyphenols, in their main natural form.

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The mixture obtained had the same concentration and type of polyphenols as the juice.

MATERIALS AND METHODS

Chemicals

Unless otherwise specified, chemicals were from Sigma Chemical Co. (St. Louis, MO). Fura-2 acetoxymethyl ester and carboxydichlorodihydrofluorescein (CDCFH)-diacetate (DA) were from Molecular Probes® (Eugene, OR). Collagen reagent was from Mascia-Brunelli (Milan, Italy). Arachidonic acid was from Helena BioSciences Europe (Gateshead, Tyne and Wear, UK). PJ (0.36% polyphenols) and purified polyphenol mix (POMx) (90% polyphenols) were from POM Wonderful (Los Angeles, CA). As POMx is a dry polyphenol solution, it was reconstituted in water at the same concentration of polyphenols as PJ (20 m*M*).

Blood samples

Human blood was obtained from drug-free healthy volunteers and anticoagulated with acid citrate dextrose. Informed consent was obtained from all blood donors.

Platelet-rich plasma was prepared by centrifugation at 200 *g* for 15 minutes at room temperature, washed twice by centrifugation at 800 *g* for 15 minutes, and resuspended in Tyrode's buffer containing 0.2% bovine serum albumin, 5 mmol/L glucose, and 10 mmol/L HEPES, pH 7.35. In all experiments performed, washed platelets were incubated for 30 minutes at room temperature with PJ (from 6.6 μmol/L to 20 μmol/L) or POMx (from 1 μmol/L to 20 μmol/L) before agonist addition.

Platelet aggregation

In order to assess platelet aggregation, washed platelets (250×10^9/L) were stimulated using collagen and arachidonic acid as agonists. It was monitored with the Born method in an aggregometer (AggRAM, Helena Biosciences) at 37°C under continuous stirring at 1,000 rpm.

Platelet aggregation was evaluated in platelets treated with increasing concentrations of PJ, POMx, or solvent alone for 5 minutes after agonist addition considering the percentage of light transmission with platelet suspension as the baseline value and Tyrode's buffer as 100%. Results are expressed as the maximal percentage of platelet aggregation; dose-dependent curves were obtained for collagen activation, whereas for arachidonic acid twice the threshold concentration was used, as we obtained a very small difference between threshold and maximal concentration.

Calcium mobilization

Calcium mobilization was monitored in a fluorimeter (model SFM25, Kontron, Zurich, Switzerland), using Fura-2 as the fluorimetric probe. Platelet-rich plasma was treated with 3 μmol/L tracer for 30 minutes at 37°C, and then the excess of probe was eliminated by double centrifugation. Untreated samples and samples treated with either PJ or POMx were activated with collagen (5 mg/L) or arachidonic acid (0.050 mmol/L) at 37°C and under continuous stirring. Excitation wavelength was set at 340 nm, and emission was set at 510 nm. Results were calculated according to the formula of Grynkiewicz *et al.*[9]: concentration (n*M*) = $K_D \cdot (F_x - F_{min})/(F_{max} - F_x)$, where K_D is the Fura-2 calcium binding dissociation constant (224 n*M*), F_x is the experimental fluorescence obtained, F_{min} is the baseline fluorescence of the Ca^{2+}-free Fura-2 obtained after addition of digitonin (50 m*M*), EGTA (0.1 *M*), and Tris base (2 *M*), and F_{max} is the fluorescence of the Fura-2 saturated with Ca^{2+} after addition of $CaCl_2$ (0.1 *M*). The results are expressed as the change in cytosolic calcium concentration obtained after platelet stimulation. Absolute absence of interference between the Fura-2 probe and various dilutions of PJ and POMx was checked.

Thromboxane (Tx) A_2 production

To assess the potential effects of PJ or POMx on agonist-induced TxA_2 synthesis, platelets were activated with collagen (5 mg/L) or arachidonic acid (0.050 mmol/L) for 5 minutes at 37°C under continuous stirring. Indomethacin and acid citrate dextrose were added at the end of the platelet activation to block TxA_2 production. Samples were centrifuged, and levels of TxA_2 were measured in the supernatant, as TxB_2, a stable metabolite of TxA_2, by a commercial enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI).

Hydrogen peroxide production

H_2O_2 formation was studied using the fluorescent probe CDCFH. Washed platelets (0.5×10^8 cells/mL) were incubated with the cell-permeant probe, CDCFH-DA (10 μmol/L), for 10 minutes at 37°C and centrifuged to eliminate the excess of probe. Untreated CDCFH-loaded platelets and those treated with PJ and POMx were stimulated for 2 minutes with both collagen and arachidonic acid at minimal concentrations able to induce measurable responses (8 mg/L and 0.05 mmol/L, respectively). In the presence of H_2O_2, CDCFH is oxidized to carboxydichlorofluorescein (CDCF), which is highly fluorescent. The results are reported as the percentage of inhibition of CDCF fluorescence (arbitrary units) between platelets treated with variable pomegranate dilutions versus untreated cells.

To avoid CDCFH oxidation by cyclooxygenase-1 activity the platelets were treated with aspirin (100 μmol/L for 10 minutes at 37°C). In each experiment diphenyleneiodonium (DPI)-dependent inhibition of CDCF fluorescence induced by collagen and arachidonic acid was performed in order to evaluate a possible residual activity of cyclooxygenase-1; when the fluorescence was not reduced by DPI (10 μ*M*) more than 90% the platelet preparation was dis-

charged. Possible interference among each pomegranate dilution and the fluorescent probe was tested, and none of the pomegranate dilutions interfered with the probe.

Statistical analysis

Results are reported as mean ± SD values obtained from different platelet samples. Statistical comparison of untreated cells and platelets treated with different pomegranate polyphenol concentrations was achieved using the one-way analysis of variation test.

RESULTS

Platelet aggregation

PJ affected platelet aggregation in a dose-dependent manner up to a polyphenol concentration of 10 μmol/L. The 50% effective concentration (EC_{50}) values obtained were 0.9 mg/L and 0.7 mg/L for platelets treated with PJ at a polyphenol concentration of 20 μmol/L and 10 μmol/L versus 0.25 mg/L obtained in untreated platelets (Fig. 1a).

POMx was able to reduce platelet aggregation at lower concentrations of phenols than PJ. In fact, the EC_{50} values were 0.8 mg/L, 0.65 mg/L, and 0.28 mg/L, respectively, for platelets treated with POMx at polyphenol concentrations of 6.6 μmol/L, 3.3 μmol/L, and 2 μmol/L versus 0.20 mg/L obtained in untreated platelets (Fig. 1b).

Likewise, POMx inhibited arachidonic acid-induced platelet aggregation more efficiently than PJ. In fact, both pomegranate products reduced platelet aggregation induced by twice the arachidonic acid threshold concentration, in a dose-dependent manner, up to a polyphenol concentration of 10 μmol/L for PJ and of 3.3 μmol/L for POMx (Fig. 2).

POMx used at concentrations of 10 μmol/L and 20 μmol/L showed only a slightly higher inhibition compared to 6.6 μmol/L in response to both collagen (EC_{50} of 1.0 μmol/L and 1.1 μmol/L, respectively) and arachidonic acid (maximal percentage of platelet aggregation, 58 ± 11% and 53 ± 14%).

Calcium mobilization

In our study calcium mobilization was studied in platelets both untreated and treated with scalar doses of either PJ or POMx and activated with either collagen (5 mg/L) or arachidonic acid (0.050 mmol/L). Figure 3 reports the results obtained, expressed as change in cytosolic calcium concentration in untreated and PJ- or POMx-treated platelets.

In platelets stimulated with collagen, PJ showed a significant reduction of calcium mobilization up to a polyphenol concentration of 6.6 μmol/L for both collagen and arachidonic acid activation.

POMx showed a higher efficiency in reducing calcium responses induced by either collagen or arachidonic acid, with a polyphenol concentration of 2 μmol/L capable of reducing both collagen- and arachidonic acid-induced calcium mobilization.

TxA_2 production

In order to understand whether the polyphenol compounds are able to reduce platelet function through TxA_2 production, we studied both collagen- and arachidonic acid-induced TxA_2 formation in untreated platelets and those treated with POMx. The experiments were performed using only POMx at high dilution rates as it was suggested that high concentrations of phenols reduce the action of anti-cyclooxygenase-1, a compound used to block TxA_2 production.[10] The results obtained (Fig. 4) clearly showed that

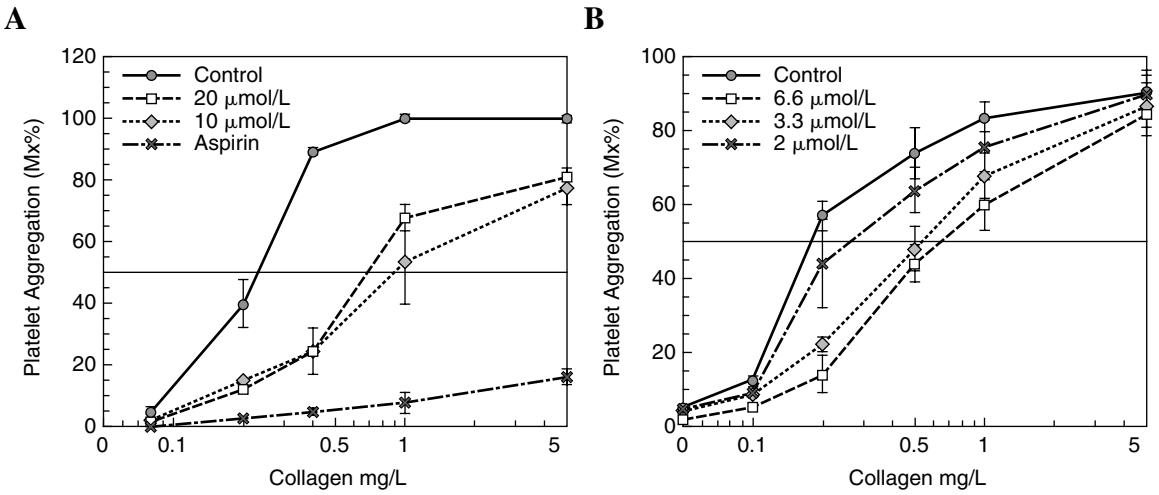

FIG. 1. Effects of (**a**) aspirin (100 μmol/L) or PJ used at scalar doses of polyphenol concentrations of 20 μmol/L and 10 μmol/L and (**b**) POMx used at polyphenol concentrations of 6.6 μmol/L, 3.3 μmol/L, and 2 μmol/L on collagen-induced platelet aggregation. The results are reported as dose–response curves of the maximal platelet aggregation (Mx%) obtained after the addition of scalar doses (from 0.1 to 5 mg/L) of collagen in six different experiments performed.

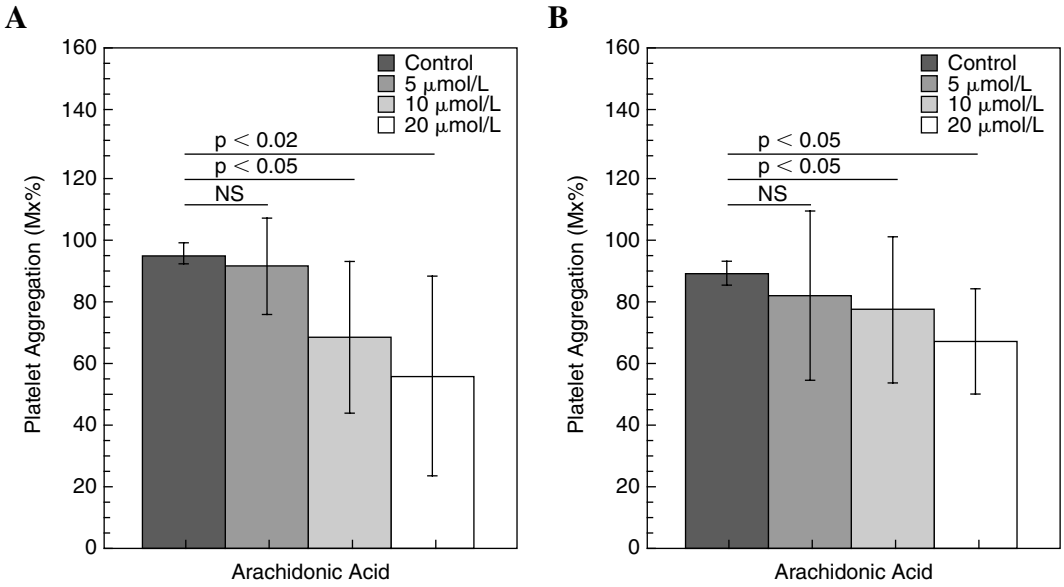

FIG. 2. Effects of (**a**) PJ used at scalar doses of polyphenol concentrations of 20 μmol/L, 10 μmol/L, and 5 μmol/L and (**b**) POMx used at polyphenol concentrations of 6.6 μmol/L, 3.3 μmol/L, and 2 μmol/L on arachidonic acid (two times the threshold concentration)-induced platelet aggregation. Data are mean ± SD values of the maximal platelet aggregation (Mx%) obtained in seven different experiments performed. NS, difference not significant.

POMx reduces TxA_2 formation in response to collagen (13,166 ± 2,441 pg/10^8 cells for 2 μmol/L and 19,330 ± 10,504 pg/10^8 cells for 1 μmol/L vs. 22,800 ± 8,600 pg/10^8 cells for untreated platelets) and arachidonic acid (17,750 ± 6,420 pg/10^8 cells for 2 μmol/L and 26,330 ± 11,540 pg/10^8 cells for 1 μmol/L vs. 28,750 ± 14,000 pg/10^8 cells for untreated platelets).

Hydrogen peroxide production

Collagen- and arachidonic acid-induced platelet reactive oxygen species production was significantly reduced by both PJ (Fig. 5a) and POMx (Fig. 5b). POMx showed a stronger effect in inhibiting reactive oxygen species production; in fact, it reduced collagen- and arachidonic acid-

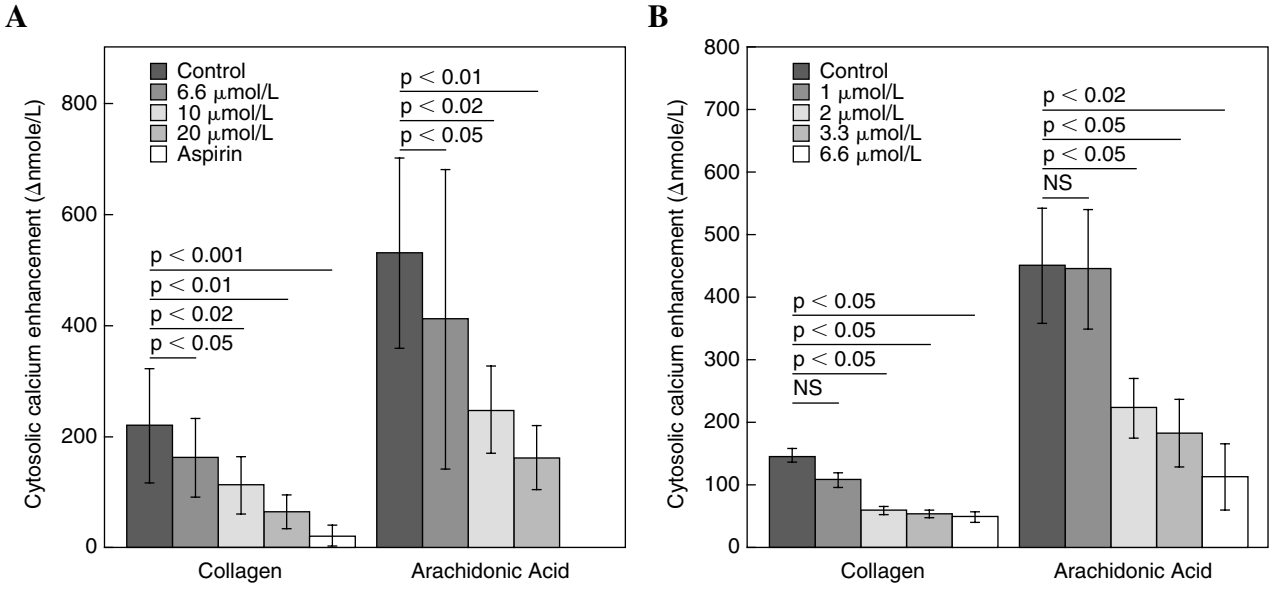

FIG. 3. Effects of (**a**) PJ used at scalar doses of polyphenol concentration of 20 μmol/L, 10 μmol/L, and 6.6 μmol/L and (**b**) POMx used at polyphenol concentrations of 6.6 μmol/L, 3.3 μmol/L, 2 μmol/L, and 1 μmol/L on the changes in intraplatelet calcium concentrations after stimulation with both collagen (5 mg/L) and arachidonic acid (0.05 mmol/L). Data are mean ± SD values of the change in cytosolic calcium concentration obtained in five different experiments performed. NS, difference not significant.

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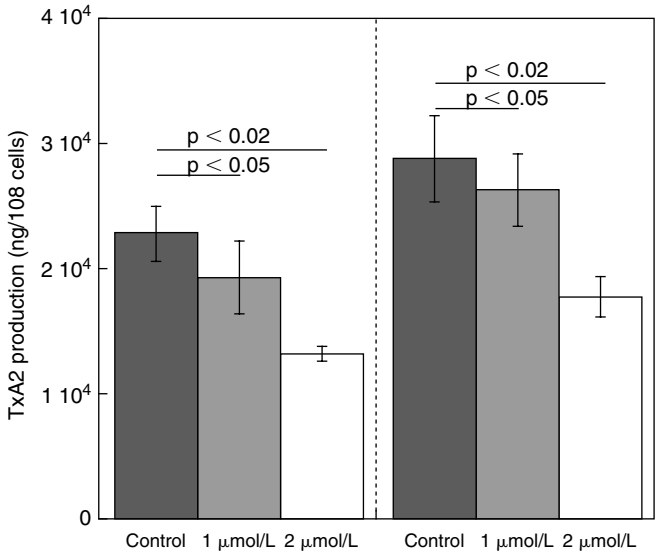

FIG. 4. Effects of POMx used at polyphenol concentration of 2 μmol/L and 1 μmol/L on TxA_2 formation after stimulation with both collagen (5 mg/L) and arachidonic acid (0.05 mmol/L). Data are mean ± SD values of the TxB_2 formation 4 minutes after agonist addition concentration obtained in four different experiments performed.

induced CDCF fluorescence up to a polyphenol concentration of 2 μmol/L versus the 10 μmol/L obtained in platelets treated with PJ. Figure 5 reports the results obtained expressed as a percentage of CDCF fluorescence inhibition compared to untreated platelets. In all experiments platelets were aspirated in order to avoid the enhancement of the CDCF due to prostaglandin H-synthase peroxidase activity.

DISCUSSION

The experiments reported in this paper clearly point out that PJ is able to inhibit platelet activation. In fact, we have shown that PJ reduces every step of platelet activation, such as platelet aggregation, calcium mobilization, hydrogen peroxide formation, and TxA_2 production induced by collagen and arachidonic acid. More interesting results were obtained using POMx, which showed a stronger action in reducing platelet activation at a lower concentration than PJ (2 μM vs. 20 μM). The capability of polyphenols to reduce platelet function has been already demonstrated (see Kuhnau[11] for review), although in the previous studies only single or sometimes two phenols were used.[12]

Moreover, the amount of each phenol necessary to achieve an antiplatelet function was very high, and it is not available *in vivo*, even after absorption of food rich in phenols. These data confirm previous ones showing that three phenols (catechin, caffeic acid, and resveratrol) are able to inhibit platelet activation at a concentration of 2 μM, a level that is quickly available during daily consumption.[13,14]

Both PJ and POMx inhibit an early event of platelet biochemical pathways, as they are more efficient in reducing collagen- and arachidonic acid-induced Ca^{2+} mobilization, TxA_2 production, and H_2O_2 formation than aggregation. The large variety of the polyphenols present in both PJ and POMx does not allow us to understand the exact mechanism of action and requires further *ad hoc* studies. However, we can hypothesize that the reduction of hydrogen peroxidase production is the mechanism responsible for such inhibition because POMx is a more powerful antioxidant than PJ and because collagen- and arachidonic acid-induced hydrogen peroxidase production levels were reduced.

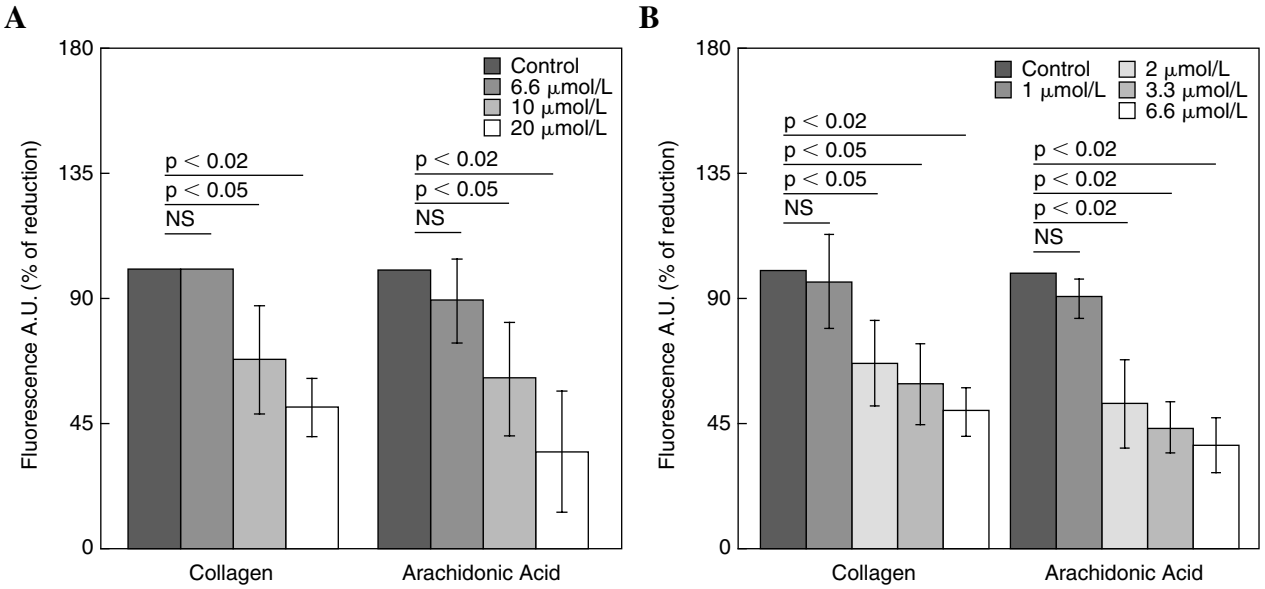

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FIG. 5. Effects of (**a**) PJ used at scalar doses of polyphenol concentrations of 20 μmol/L, 10 μmol/L, and 6.6 μmol/L and (**b**) POMx used at polyphenol concentrations of 6.6 μmol/L, 3.3 μmol/L, 2 μmol/L, and 1 μmol/L on collagen (8 mg/L)- and arachidonic acid (0.05 mmol/L)-induced hydrogen peroxide production. Data are mean ± SD values of the percentage of inhibition of CDCF fluorescence (arbitrary units [A.U.]) compared to the control. NS, difference not significant.

The mixture of polyphenol hydrolyzed tannins, the major phenol components of both PJ and POMx,[8] could have a role for this phenomenon, and to our best knowledge this is the first time that there has been a demonstration that food rich in a mixture of hydrolyzed tannins is effective in inhibiting platelet function. A new aspect that we have explored in this paper is that glycoside phenols are still able to inhibit platelet function because POMx is particularly rich (more than 90%) in glycoside phenols, whereas in previous papers only aglycone compounds were used. Absorption of glycoside polyphenols following flavonoid-rich food intake,was recently demonstrated,[5] and because platelet function was reduced after 2 weeks of PJ daily consumption,[15] we can suggest that the glycoside flavonoids could act with such inhibition also *in vivo*.

It has been recently demonstrated that after flavonoid-rich food intake[5] glycoside polyphenols were found in human plasma. It has also been shown that after 2 weeks of PJ daily consumption[15] platelet function is reduced. Our data, taken together with others previously published, strongly suggest that *in vivo* the pomegranate polyphenols are the active compounds responsible for platelet inhibition.

PJ is rich in glycoside polyphenols with several hydroxyl groups in specific positions, showing significant biological activities[16]; we can speculate that these hydroxyl compounds may have an important role in reducing platelet function.

Finally, our data indicate that the beneficial healthy effects of moderate consuption of PJ or POMx are also due to the antiplatelet activity of the heterogeneous hydrolyzed tannin polyphenol antioxidants present in PJ and POMx.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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