Table of contents entry

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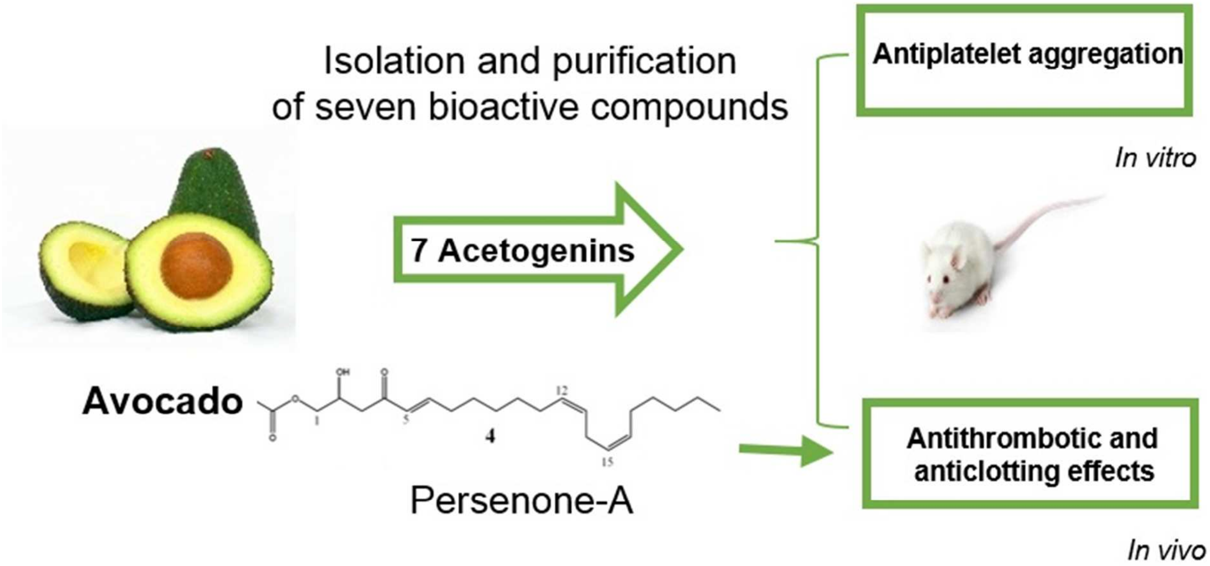

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Bioassay-guided isolation of acetogenins from avocado pulp with *in vitro* antiplatelet aggregation and *in vivo* antithrombotic effects in CD1 mice.

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Isolation and chemical identification of lipid derivatives from avocado (*Persea americana*) pulp with antiplatelet and antithrombotic activities

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Platelets play a pivotal role in physiological hemostasis. However, in coronary arteries damaged by atherosclerosis, enhanced platelet aggregation, with subsequent thrombus formation, is a precipitating factor in acute ischemic events. Avocado pulp (*Persea americana*) is a good source of bioactive compounds, and its inclusion in the diet as a source of fatty acid has been related to reduced platelet aggregability. Nevertheless, constitutes of avocado pulp with antiplatelet activity remain unknown. The present study aims to characterize the chemical nature of avocado constituents with inhibitory effects on platelet aggregation. Centrifugal partition chromatography (CPC) was used as a fractionation and purification tool, guided by an *in vitro* adenosine diphosphate (ADP), arachidonic acid or collagen-platelet aggregation assay. Antiplatelet activity was initially linked to seven acetogenins that were further purified, and their dose-dependent effect in the presence of various agonists were contrasted. This process led to the identification of Persenone-C (3) as the most potent antiplatelet acetogenin (IC_{50}=3.4 mM), among evaluated compounds. *In vivo* evaluations with Persenone A (4), demonstrated potential protective effects against arterial thrombosis (25mg/kg of body weight), as coagulation times increased (2-fold with respect to vehicle) and thrombus formation was attenuated (71% versus vehicle). From these results, avocado may be referred to as functional food containing acetogenins compounds that inhibits platelet aggregation with a potential preventive effect on thrombus formation, as those that occur in ischaemic diseases.

Introduction

According to the World Health Organization, cardiovascular diseases (CVD) are the leading cause of mortality and morbidity, representing about 23% of deaths worldwide[1]. CVD, including acute myocardial infarctions, cerebrovascular diseases are clinical manifestations of acute arterial thrombosis,[2] after disruption of the hemostatic balance. Blood platelets, endothelial wall, coagulation and fibrinolytic factors are key regulators of this delicate balance.

Platelets are essential in physiological hemostasis because they can be activated and mobilized rapidly to control bleeding through blood clot formation.[3] However, in pathological conditions of the cardiovascular system, rupture or erosion of atherosclerotic plaques can occur, leading to vascular injury. Subsequent platelet interaction with the exposed sub-endothelial extracellular matrix at sites of injury triggers their activation, uncontrolled aggregation and thrombus formation. The thrombus blocks blood flow resulting in ischemic tissue injury and organ dysfunction.[4]

Hence, pharmacologic agents with antiplatelet actions are considered as fundamental therapies in the prevention of atherothrombotic events.[5] However, antiplatelet agents, such as aspirin, are often associated with an increased risk of bleeding and frequently require gastric protection medications.[6] Therefore, exploration of alternative sources of compounds that inhibit platelet aggregation continues to be necessary.

In this regard, avocado pulp (*Persea americana*) is a good source of bioactive compounds such as monounsaturated fatty acids and sterols, which may have a protective role against arterial thrombosis.[7-9] Intervention trials in human subjects using avocado pulp as a source of monounsaturated fatty acids have demonstrated a significant decrease in *ex vivo* platelet aggregation induced by ADP.[10] However, no information is available regarding the precise identity of avocado molecules associated with antiplatelet action.

In the present study we describe the bioassay-guided isolation and identification of lipophilic constituents from avocado pulp, based on their, *in vitro*, ADP-induced platelet aggregation inhibitory properties. Initial stages of the isolation process involved the evaluation of antiplatelet activity of semi-crude fractions, obtained from partitioning of avocado pulp solids in a liquid-liquid byphasic system and in sub-fractions separated by CPC. Purification and chemical identifications were subsequently performed for compounds contained in the active fractions. Antiplatelet activity of purified compounds was then characterized, using different agonists to induce aggregation. Finally, the *in vivo* effects of one of the active molecules on blood clotting time and thrombosis were evaluated in CD1 mice.

Materials and Methods

Plant material

Avocado fruits (*Persea americana* Mill, cv. var. Hass) were kindly provided by Avomex International (Sabinas, Coah., México) and were obtained from the region of Uruapan, Mich., México (19º25′0′′N 102º4′0′′O). Avocado pulp was manually separated from seeds, pureed, vacuum packed and stored at-80ºC prior to use, to avoid enzymatic activity, since it has been observed that enzyme lipoxygenase oxidizes acetogenins.

Isolation of semi-purified fractions for preliminary activity screening

An acetone extract **E001** was obtained from freeze-dried avocado pulp, which was partitioned in a heptane:methanol byphasic system, and further fractionated by centrifuge partition chromatography (CPC). Bioactive fractions were grouped to finally obtain seven fractions (coded as **GF01** to **GF07**), following grouping procedures previously described.[11] Subsequently, evaluation of ADP-induced platelet aggregation inhibitory properties of these seven fractions was performed as further described. Chromatographic profiles and identity of the compounds present in fractions with higher antiplatelet activity were obtained using the HPLC-PDA/TOF method described in next section.

Large scale isolation and purification of active constituents

Preparation of an acetogenin-enriched extract. In order to further validate the potential antiplatelet activity of compounds present in fractions with higher antiplatelet activity, a large scale isolation and purification process was developed to recover them (**Fig. 1**). An acetone extract **E001** from freeze-dried avocado pulp (240 g) was obtained and partitioned in a two non-miscible solvent system comprised of heptane:methanol (1:1 v/v), according to Rodriguez-Sanchez et al .[11] Phase **E002** (upper) and phase **E003** (lower) were separated and washed with methanol-saturated with heptane or heptane-saturated with methanol (1:1 v/v), respectively. Phases were separated and concentrated under reduced pressure, yielding heptane- and methanol- soluble semicrude subfractions: **E004** and **E005** (derived from **E002**, respectively), and **E006** and **E007** (resulting from **E003**, respectively). Chromatographic profiles of sub-fractions **E001** to **E007** were obtained using the HPLC-PDA method further described in section.

CPC fraction of an acetogenins-enriched extract. After HPLC-PDA evaluation, **E005** and **E006** sub-fractions were mixed and further fractionated in a 1L CPC system (Kromaton Technologies, Angers, France) using heptane:ethyl acetate:methanol:water (8:2:8:2) as solvent system. Upper phase (UP) of the solvent system served as stationary phase (SP) and after hydrodynamic equilibrium establishment, the lower phase (LP) accounted for 18% of the total column volume. Extracts **E005-E006** (12.73±0.77 g), dissolved in 30 mL of UP and 80 mL of LP were injected into CPC column. Based on elution-extrusion approach,[12] LP was used to elute fractions during 170 min, and then UP was pumped during 100 more min, both at a 10 mL/min flow rate. A total of 240 fractions (10 mL/fraction) were collected and their corresponding partition coefficients (K_D) were calculated as described by Berthod et al. (2007). Aliquots (0.5 mL) of every 10 fractions were taken, evaporated under stream of nitrogen and resuspended in isopropanol (0.5 mL), for further HPLC-PDA and HPLC-MS-TOF analysis as further described. At the end of the CPC run, the column was entirely filled with the SP, so it was ready to be reequilibrated again by pumping LP, and subsequent chromatographic fractionations (4) were carried as previously described.

Acetogenin purification. Consecutive HPLC separations were carried out in a preparative Phenomenex Prodigy C_{18} column (250 x 20 mm, 5 µm), using water 100% (A) and methanol 100% (B) as elutants, at a 20 mL/min flow rate. Solvent gradient was: 0-4 min, 75-85% B linear; 4-22 min, 85% B isocratic; 22-24 min, 85-95% B linear; 24-32 min, 95% B isocratic. Photodiode (PDA) detector was set at 220 nm. Final purification was conducted in a semi-preparative Phenomenex Synergi Hydro-RP column (250 x 4.6 mm, 4 µm), using water 100% and methanol 100% as mobile phases (A and B, respectively) at a flow rate of 1 mL/min. Isocratic methods were optimized for each peak. The platelet aggregation (induced by ADP, collagen and arachidonic acid) inhibitory activity of purified molecules was evaluated as further described. One of the purified molecules exhibiting antiplatelet activity was selected for further evaluation of its performance as antiplatelet agent in *in vivo* studies.

Identification of active compounds

For chemical identity the compounds were subjected to HPLC-PDA and HPLC-MS analysis as previously described by Rodriguez-Sanchez.[11] Chemical identity was assigned by comparison of spectroscopic data with values reported in the literature and with data from standards isolated in our laboratory from avocado seed.[11,13]

***In Vitro* Assays**

Platelet Aggregation. This study was conducted in accordance with the Helsinki Declaration. All human donors from the blood bank of Instituto Nacional de Cardiología Ignacio Chávez previously signed informed consent. Blood samples were taken from healthy volunteers who had not taken any medications for at 2 weeks, or ingested any alcohol for at least 24 h prior to sample collection. Blood was collected by venipuncture into Vacutainer (BD Diagnostics, Plymouth, UK) tubes containing sodium citrate 3.8% as anticoagulant (at a 9:1 ratio, v/v). Platelet-Rich Plasma (PRP) and Platelet-Poor Plasma (PPP) were obtained as earlier reported by De la Peña.[14] The assays were carried out within 2 h after the blood had been drawn.
Evaluation of platelet aggregation was performed by turbidimetric measurement accordingly to Falkenberg [15] with the some modifications. PRP adjusted with PPP to a platelet count of 2.5 x 10^8/mL (215 µL) was pre-incubated at 37°C for 10 min, with the evaluated sample (10 µL) and, at various concentrations. Platelet aggregation was initiated by the addition of 25 µL of platelet agonist. Final concentrations of agonists in the reaction mixture were 20µM ADP, 500 µM arachidonic acid or 5 µg/mL collagen, all obtained from Chrono-PAR Corporation (Havertown, PA, USA). Aggregation response was recorded during 10 min using a Chrono-log Model 700 Whole Blood/Optical Lumi-Aggregometer (Chrono-Log, Havertown, PA, USA). Maximal aggregation (MA) observed for samples containing avocado extracts, at different degrees of purification, were compared to those of vehicle controls (DMSO 2M) evaluated under the same experimental conditions. Percent aggregation inhibitions, for all samples, were calculated using the following equation: %Inhibition = (MA vehicle control − MA extract)/MA vehicle control * 100%.

To verify the possible platelet cytotoxic effects of avocado extracts, at different degrees of purification, cell viability was measured by CellTiter-Blue (Promega, Madison, WI, USA) and by trypan blue exclusion, counting live/dead platelets.

***In Vivo* Assays**

Animal use and treatment. Experiments were conducted in accordance to the Mexican National Protection Laws on Animal Protection and the General Health Law Related to Health Research (NOM-062-Z00-1999). All procedures were approved by an ethics committee. Male adult CD1 mice weighing 25–35 g were used (obtained from Animal Care Unit from Facultad de Medicina. UNAM).

Animals were distributed among groups according to a balanced design based on body weight (3 animals per group in each experiment). Room temperature was kept constant (21–24°C), and with light–dark cycles of 12h. Food and water were given *ad libitum*. Persenone-A was dissolved in DMS0 (vehicle) and administered intraperitonally (i.p.). Control animals only received the vehicle (1.66 mL/kg).

Blood clotting time. Groups of animals received a single i.p. administration of Persenone A (1, 10 or 100 mg/kg of body weight, respectively) or vehicle. Blood clotting time was measured 24 h after administration, as previously described.[16] The tail of the animal was warmed in water bath at 37°C for 3 min. The tail was dried and transected at 8mm from the tip with a scalpel. Briefly, a 25µL blood sample was collected from the bleeding tail tip into microhematocrit glass capillary tube. Capillary tube was alternatively tilted to angles of +60º and -60º with respect to the horizontal plane, allowing blood to flow by gravity between two marks, separated by 45 mm. Time was counted from the instant that blood first made contact with the glass capillary tube and until the blood ceased to flow. The blood clotting time data was presented as relative increase elicited by Persenone A as a percentage of that obtained in samples treated with vehicle.

Experimental thrombosis model. Groups of animals received a single i.p. administration of Persenone A (25 mg/kg of body 208 weight) or vehicle. After 24 h of treatment mice were anesthetized with phenobarbital (80mg/kg). Thrombosis was induced by a surgical model, by tightening two sutures separated by 1 cm for 1 h to cause vascular occlusion in the right leg femoral vasculature. The vascular segments were then removed, fixed in formalin, dehydrated and embedded in paraffin. 4µm thick sections were stained with Masson's trichrome and hematoxylin-eosin, and scored by a pathologist.

Statistical Analysis

Data are representative of at least three independent experiments. Results were expressed as means ± SEM. Statistical significance among groups were analyzed employing one-way analysis of variance (ANOVA) and differences between the control and the treated group were estimated by Dunnet's or LSMean Student's as appropriate. Differences were considered significant at a level of $P<0.05$. Half-maximal inhibitory concentrations (IC_{50}, µM) of platelet aggregation and half-maximal effective concentrations (EC_{50}, mg/kg body weight) of blood clotting times were determined by nonlinear regression analysis using a sigmoidal concentration-response equation. Statistical calculations were performed using the GraphPad Prism software, version 5.0 (GraphPad software, San Diego, CA, USA).

Results and discussion

Preliminary activity screening

A previous study evaluated the inclusion of avocado pulp as a fat source (2.75%) in the diet of Type 2 diabetic patients (avocado cultivar was not reported). Diets were administered during a four week period, and were formulated to provide adequate energy supplies that came from carbohydrates (50%), fats (30%) and proteins (20%).[10] Authors reported that patients consuming avocado showed significantly lower levels of platelet aggregation (~30%) when compared with patients who did not consume avocado. Based on these prior observations, and preliminary tests with avocado pulp crude extracts, the present screening study was undertaken with the purpose of separating molecules present in crude extracts. Fractionation was conducted based on the hypothesis that compounds called acetogenins, present in avocado pulp and seed, could potentially exhibit antiplatelet aggregation activities, based on their chemical structure. To test the hypothesis, acetone soluble compounds were extracted from avocado seed, dried, and fractionated by using CPC. Fractions, with similar HPLC-PDA chromatographic profiles (at 220nm), were grouped together, as previously described.[11] Seven different avocado pulp sub-fractions, enriched in acetogenins, were obtained and designed as **GF01** to **GF07**. Calculated K_D values ranged from 0.14-0.40, 0.83-1.13, 1.59-1.72, 2.03-2.41, 4.12-5.82, 7.37-11.67 and 14-∞, for fractions **GF01** to **GF07**, respectively. Potential inhibitory effects on platelet aggregation, for fractions **GF01** to **GF07** (at 500 µg solids dry-weight (dw)/mL) were measured turbidimetrically on platelets induced by ADP. As shown in **Fig. 2**, fractions **GF03** to **GF07** significantly inhibited ($P<0.01$) platelet aggregation in reference to the control vehicle. However, **GF03** exhibited lower inhibition values (38 ± 4.15%) than the rest of the fractions, which resulted in levels of over 75% inhibition.

In the presence of equal concentrations of solids from each fraction (500 µg solids dw/mL), platelet viability was found to be >80%, for fractions **GF02** to **GF07** (data not shown), suggesting that platelets integrity appeared to be not affected by compounds present in those fractions at concentrations evaluated in the platelet aggregation assay. However, compounds present in fraction **GF01** appeared to have a negative effect on platelet function resulting in 62.5 ± 9.68% viability. Based on the higher activity and minimal effects on platelet viability, **G004** to **G007** were further characterized to determine the nature of the phytochemicals therein contained.

Mass spectrometry analysis of chromatographic peaks that were contained in the four fractions (**GF04** to **GF07**), which presented the highest platelet aggregation inhibitory effects, consistently presented a similar ion pattern of $[M+Na]^+$ and $[M+H]^+$ molecular ions. In addition, fragment ions showing successive losses of H_2O and/or acetic acid ($C_2H_4O_2$) from the $[M+H]^+$ ion were also present. This pattern was in accordance with the characteristic ion pattern reported for acetogenins,[17] and more specifically for acetylated acetogenins derived from avocado fruit.[18,19] With that information it was possible to assign the chemical identity to the chromatographic peaks by comparison of their mass spectra with values reported in the literature, and with data from standards isolated in our laboratory from avocado seed.[13] As shown in **Table 1**, compounds were identified as: 1-acetoxy-2,4- dihydroxy-n-heptadeca-16-ene (**1**)[18]; Persediene (**2**)[13]; Persenone-C (**3**)[13]; Persenone-A (**4**)[18]; Persenone-B (**5**)[20], Persin (**6**)[21] and 1-acetoxy-2,4-dihydroxy-heneicosa-12,15-diene (**7**).[19] Data from the preliminary screening study suggests, for the first time, the inhibition of platelet aggregation in the presence of fractions containing acetogenins obtained from avocado pulp.

Isolation and purification of avocado pulp acetogenins

In order to further validate the potential antiplatelet activity observed for fractions that contained acetogenins, a large scale isolation and purification process was developed (**Fig. 1**). Through this process it was possible to recover larger amounts of purified compounds (4-130 mg, depending on their naturally occurring concentration) and to conduct the subsequent studies; which included the evaluation of dose-response relationship of purified molecules in platelet aggregation induced by different agonist and *in vivo* studies.

Primary extraction was then optimized to increase yields. For this task, a new acetone crude extract **E001** was prepared and partitioned in a biphasic system comprised of heptane:methanol. Heptane and methanol phases were separated and washed with methanol or heptane, respective. Phases were separated and concentrated, yielding heptane- and methanol- soluble semicrude subfractions: **E004** and **E005** (derived from **E002**), and **E006** and **E007** (resulting from **E003**), respectively. HPLC-PDA analysis of these subfractions indicated that acetogenins were preferentially concentrated in **E005** and **E006** (data not shown**).**

Consequently, they were mixed and subjected to sequential CPC-fractionation. For that purpose, based on CPC principles,[12] the proper solvent system was selected to ensure correct separation of compounds; considering that extract **E005-E006** contained less impurities than the sample chromatographed in preliminary evaluation (section 2.2 in materials and methods), the same system used back then would not perform the same for this new sample. Mass spectrums from CPC fractions were obtained (every 10 fractions), and used as a tool to track the location of compounds **1** to **7**, and selectively isolate them from the fractions in which they were preferably enriched (**Fig. 3**); which corresponded to fractions 74-120, with K_D values ranging from 0.90-1.52. Based on their higher relative concentrations, compounds **1** to **4** and **6**, were then selected for further purification by preparative and semi-preparative HPLC sequential runs.

Characterization of platelet aggregation inhibitory properties of purified acetogenins

Platelets normally circulate in a resting state and upon vascular injury they interact with components of the sub-endothelial matrix, particularly collagen and von Willebrand factor (vWF) via their respective receptors glycoprotein (GP) VI and GPIb/V/IX. Agonists, such as ADP, epinephrine, thromboxane A_2 (arachidonic acid-derived) and thrombin are then released, or produced to further amplify platelet activation by interacting with their respective membrane receptors. Hence, more circulating platelets from the blood flow are recruited to sustain hemostatic plug growth. The final pathway for all agonists is the activation of the platelet membrane GPIIb/IIIa integrin, leading to thrombus formation through fibrinogen bridges.[22]

The most common antiplatelet agents currently used in the clinical practice for prevention of atherothrombosis are aspirin and clopidogrel.[2] Their mechanisms of action are focused on inhibiting, irreversibly, amplification mechanisms of platelet activation by blocking the interaction of an agonist with its specific cell surface receptor.[23]. For instance, aspirin, as irreversible inhibitor of cyclooxygenase (COX)-1, preventing arachidonic acid from being metabolized to prostaglandins G_2/H_2, and subsequently inhibits thromboxane A_2 formation.[24] Whereas, clopidogrel directly interferes with ADP binding to its P_2Y_{12} receptor.[25] Therefore, to confirm acetogenin contribution to antiplatelet activity, their individual activity was evaluated at different concentrations (0.15 – 15 mM). Aggregation was induced by different agonists, that included collagen (5 µg/mL), ADP (20 µM) and arachidonic acid (500 µM), in an approximation to explore the potential interference of acetogenins with the binding of a particular agonist to its cell surface receptor.

As shown in **Table 2**, Persenone-C **(3)** presented a significantly lower ($p < 0.05$) IC_{50} than the other compounds, for collagen, ADP and arachidonic acid (3.42 ± 1.56, 5.22 ± 1.19, 7.40 ± 1.20 mM, respectively). Compound **1**, also inhibited platelet aggregation induced by all three agonists, but at >1.5-fold higher concentrations than Persenone-C **(3)**. Whereas Persenone-A **(4)** exhibited a similar IC_{50} than Compound **1** against collagen-induced aggregation, but even at the highest concentrations (15mM) tested it was unable to reach the 50% inhibition when aggregation was induced by arachidonic acid. Isolated Persediene (**2**) and Persin (**6**) showed significantly lower platelet aggregation inhibition levels. Persediene (**2**), inhibited collagen-induced aggregation, however, Persin (**6**) did not inhibit platelet aggregation at any of the evaluated concentration. Incubation of the platelets with purified compounds at 15 mM had no effect on the platelet viability (data not shown).

In preliminary screening studies, described in section 3.2, fractions containing mixtures of compounds, evaluated at concentrations of 500 µg solids dw/mL presented inhibitory concentrations of greater than 80% (**Fig. 2**). However purified compounds presented higher IC_{50} values (3.42 mM = 1,207 µg/mL) than semi-pure fractions (**Table 2**). This suggests that isolation of compounds has a detrimental effect on antiplatelet activity, probably due to synergist interactions between compounds in mixture,[26] or that acetogenins are not the only responsible for the activity. However, this is the first evidence linking antiplatelet action, previously observed for avocado pulp, to acetogenins, of which Persenone-C (**3**) was the most potent.

As expected,[23] aspirin markedly inhibited (IC_{50}=0.07±0.01µM) arachidonic acid-induced platelet aggregation with IC_{50} values 5- and 50-fold lower than the observed for collagen- and ADP-induced aggregation, respectively. In contrast, purified acetogenins did not exhibit a so evident selective inhibition for any of the evaluated agonist. This observation suggests that acetogenin do not interfere with binding of the evaluated agonists to their specific receptors on platelet's surface.

The precise mechanism by which acetogenins trigger antiplatelet activity is not clear. However, they may be contribute to increase the negativity of the platelet surface charge. In this context, it has been demonstrated that under normal conditions, when human platelets circulate in a resting state, they carry a net negative surface charge, as determined by their electrophoretic mobility.[27] This charge is mainly attributed to the presence of sialic acid, a negatively charged nine-carbon sugar,[28] attached to proteins and lipids into their outer membranes.[29] Repulsive force of the same negative charged platelets keeps them apart inhibiting their aggregation.[30] Studies have reported that platelets from patients with coronary heart disease present a significantly lower ($p < 0.05$) sialic acid content compared to controls; resulting in a lower negative surface charge, and higher propensity to aggregate.[29]

Upon activation, platelets undergo drastic morphological changes, becoming flatter. Their granules are gathered into the center and the cytoskeleton is rearranged to develop filopods.[22] Additionally, flipase and scramblase are activated, and location of negatively charged phospholipids, mainly phosphatidylserine, changes from the inner to the outer membrane surface, inducing coagulation.[31] Besides structural changes, agonist compounds of dense and α-granules are secreted facilitating the recruitment of additional platelets and thrombus stabilization.[3] Cytosolic and surface calcium concentration increases as result of its influx from the extracellular milieu, and its release from the dense tubular, becoming an important second messenger in the platelet activation cascade.[32] Ultimately a net positive charge is developed.[27,30] The mechanism of the surface charge reversal is not known.[27] However it has been demonstrated that platelet activation occurs when the negative surface charge of resting platelets is neutralized by different means. For example by the addition of cationic molecules[33,34] or when they come in contact with positively charged biomaterials' surfaces.[30]

A recent report showed that increasing oral doses of omega-3 polyunsaturated fatty acids (PUFAS, from 1 to 8 g daily over 24 weeks) in human subjects produced a significant increase ($p<0.05$) in the negative surface charge of resting platelets, compared to baseline.[27] Additionally, an attenuated response to arachidonic acid-induced platelet aggregation was observed. Based on their findings, researchers suggest that the increased negativity of the platelet surface charge produced by PUFAS, may create an additional hurdle that must be overcome before platelets can become fully activated. Thus, the structural similarities between PUFAS and avocado acetogenins[18,35] suggest a similar mechanism, since acetogenins are lipophilic compounds able to accumulate in cell membranes[36] and it has been proposed that they can exist as anionic species, due their activity as mitochondrial uncoupling agents.[36,37] Delocalization of the charge over its structure, facilitated by the presence of a keto group at C-4[37], conjugated to an unsaturation at C5-C6,[36] enables the loss of protons.

In addition, it has been observed that diets with high content of omega-3 PUFAS, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are associated with a lower rate of cardiovascular events.[38] These fatty acids have shown positive effects on platelet function;[39] which have been associated to the metabolic end products derived from them.[40] For instance, araquidonic acid (omega-6 PUFA), hydrolyzed from inner membrane phospholipids by phospholipase A2, is further metabolized to thromboxane A_2, a potent platelet agonist and vasoconstrictor.[41,42] In contrast, omega-3 PUFA are metabolized to thromboxane A_3, considered as a poor platelet agonist and vasoconstrictor.[40] Furthermore, there is a novel family of lipid local mediators (lipoxins, resolvins, protectins, and maresins) generated from omega-3 PUFAS by the action of lipoxygenases and cyclooxygenases.[43] These lipid mediators have recently been associated to resolution of vascular inflammation[43,44] and inhibition of platelet aggregation,[45] both relevant in pathological cardiovascular events.

To our knowledge, no information is available regarding acetogenins metabolism in humans that may be used for comparison to PUFAS-derived metabolic products with protective effect on platelet aggregation. However, it has been reported that Persin (**6**) can be metabolized by lipoxygenases, causing the loss of its antifungal activity.[46] In our results, it can be observed that among the evaluated acetogenins (Compounds **1-4** and **6**), Persediene (**2**) and Persenone-C (**3**) present an unsaturation at C3-C4 from the methyl end, similarly to omega-3 PUFA (**Table 1**); but Compound **2**, additionally presents a terminal vinyl group. Interestingly, Persenone-C (**3**) exhibited the highest antiplatelet activity (**Table 2**), suggesting that the omega-3 unsaturation may be another relevant structural feature for this effect. For instance, Compounds Persenone-C (**3**) and Persenone-A (**4**) contain the enone group, previously proposed as an important feature to form stabilized anions that increase the negativity of the platelets surface; but the lack of the omega-3 unsaturation in Persenone-A, seems to reduce its antiplatelet activity compared to Compound **3**.

The case of Compound **1** is particular, because it does not present none of the mentioned structural features, although it performs somehow better than Persenone-A. Prior reports confirmed that Compound **1**, among other acetogenins extracted from avocado, reduced inflammation in skin cells and UV-induced damage; as it increased cell viability, decreased the secretion of interlukine-6 and prostaglandin E_2 (PGE_2), and enhanced DNA repair.[47] Furthermore, it was suggested that reduced secretion of PGE_2 in keratinocytes was associated with inhibition of phospholipase A_2 activity in skin cells.[48] This may suggest that the mechanism, through which Compound **1** attenuates platelet aggregation, can be related to lower hydrolysis of araquidonic acid from the inner membrane by inhibiting phospholipase A_2 action. However the proposed mechanisms need to be proved.

In vivo Assay

Blood clotting time and thrombosis model. *In vitro* methods used to measure platelet function detect a simultaneous aggregation of most of the platelets in the sample under low shear conditions.[23] Although they represent very valuable tools to measure specific platelet functions and their modification by antiplatelet drugs, these methods do not reflect the physiological processes involved in the formation of a platelet-rich thrombus.[23] Then, trying to confirm if the observed antipletet activity *in vitro* was reflected *in vivo*, blood clotting times of male mice were evaluated 24 h later of i.p. administration of Persenone-A (**4**) (1, 10 and 100 mg/kg of body weight). Persenone-A was not the most potent antiplatelet acetogenin, but it was selected for *in vivo* evaluation because of its higher recovered yields. Considerations to determine the maximum explored dose were based on the few reports available evaluating *in vivo* effect of acetogenins. There is one study that documented the protective effect of Persenone-A **(4)** and Persin (**6**), at doses of 100 mg/kg, on rat liver injury induced by D-galactosamine.[21] Another study suggested that Persin (**6**), produced necrosis of the secretory epithelium of the mammary gland in lactating mice, at 60-100 mg/kg, whereas greater doses, additionally produced necrosis of myocardial fibers.[49]

Our findings indicate that at 100 mg/kg, Persenone-A **(4)** produced a 2-fold increase in blood clotting time. Calculated EC_{50} value for Persenone-A **(4)** was 24.8±1.7 mg/kg of body weight (**Fig. 4**).

Blood coagulation is not only the result of interactions between platelets, but also it is a complex process, is a highly regulated process involving plasma coagulation factors and the vessel wall.[50] It was confirmed that the antiplatelet activity of acetogenins initially observed *in vitro*, was successfully reflected *in vivo* models.

Finally, mice were subjected to an acute ischemic challenge, 24 h after i.p. administration of Persenone-A **(4)**, at a dose of 25mg/kg of body weight, based on its EC_{50} value on blood clotting time increase. With respect to control vehicle **(Figure 5)**, mice treated with Persenone-A (**Figure 5C**) decrease 71% the presence of a thrombus. This provides more evidence that the antiplatelet activity of acetogenins observed *in vitro* assays was positively reflected in animals, ultimately strongly suggesting an efficient antithrombotic effect. However, these interesting results should be explored more detailed in a further report.

No studies have been performed on the bioavailability of Persenones (in vitro or in vivo), which if available could provide some information on the amounts of avocado pulp that a human would need to consume in order obtain specific levels of these bioactive lipids in the blood stream. However, studies on the concentrations of Persenones in avocado pulp (cv Hass) have indicated that it contains aprox. 2,600 ppm of Persenones (A, B and C) and a concentration of total acetogenins of aprox. 2800 ppm (data not shown). Values that are ~110-fold higher than the concentrations found to be active in the vascular oclussion study. Although, acetogenins are lipid derivatives and can possibly be absorbed in a similar manner than other fatty acids such as EPA and DHA, further work needs to be done on their absorption and metabolism in order to extrapolate the bioactivities observed herein to the human metabolism.

Conclusion

In conclusion, a bioassay-guided isolation and purification process, based on *in vitro* antiplatelet properties, led to the identification of seven acetogenins as constituents of the active fractions. Characterization of antiplatelet activities on purified compounds showed inhibitory properties of Persenone-C (**3**) (IC_{50}=3.42-7.40 mM). Acetogenins did not interfere with binding of ADP, arachidonic acid or collagen to their specific receptors on platelet's surface. Therefore contribution to increase the negativity of the platelet surface is suggested as a possible mechanism of action that needs to be explored. Additionally it was observed that the most potent acetogenin exhibited an omega-3 unstauration, similarly to omega-3 PUFAS which are recognized by their cardioprotective effect. Performance of Persenone-A **(4)**, another active acetogenin, was evaluated *in vivo*, suggesting that it may produces protective effects against arterial thrombosis.

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Notes and references

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FIGURE LEGENDS

Figure 1. Flow chart of the process developed to isolate and purify the compounds identified as constituents of sub-fractions (KD=0.71-1.24) that exhibited the higher antiplatelet activity (**GF04** to **GF07**) in the preliminary activity screening.

Figure 2. Percent inhibition of ADP-induced (20 µM) platelet aggregation exhibited by the groups of avocado pulp fractions **GF01** to **GF07** (500 µg solids dry weight /mL), obtained after partition and centrifugal partition chromatography purification of acetone soluble solids from avocado pulp. Acetogenins content in each pulp fraction has been reported in Rodríguez-Sánchez et al. 2013. Each point represents the mean ± S.E.M. (n=4). **P<0.01, ***P<0.001 compared with vehicle control (one-way ANOVA with Dunnet-corrected post hoc analyses).

Figure 3. Total ion intensity values obtained by HPLC-MS analysis of different avocado pulp fractions isolated from an acetogenin-enriched extract. Information was used to track their location, and selectively isolate them from the fractions in which they were preferably enriched (K_D = 0.90-1.52, corresponding to fractions 74-120).

Figure 4. Dose–response relationships of Persenone-A (1 – 100 mg/kg of body weight) on blood clotting time of male mice, 24 h after single i.p. administration. Data was expressed as percentage increase from that obtained with vehicle. Each point represents the mean ± S.E.M. (n=4). *P<0.05 compared with vehicle control (one-way ANOVA with Dunnet-corrected post hoc analyses). N=3 for each treatment.

Figure 5. Persenone A (Compound 4) decreases thrombus induction in male mice. Upper panel, shown a transverse section of the femoral vasculonervous package stained with Masson's trichrome. **(A)** The femoral artery does not present thrombi. Numerous unorganized erythrocytes can be observed in the light. **(B)** Surgical induction of the thrombus. A recent, unorganized thrombus can be observed in the artery. Fibrin accompanies the first step in thrombus organization, which occludes 40% of the vascular light. (**C**) A single i.p. administration of Persenone-A (25mg./kg-1) dissolved in DMSO vehicle 24h before the surgical induction reduces the thrombus formation . In this representative image 10% of the vascular light is occluded. Bottom panel, shown a percentage analysis of thrombus induction. *P<0.05 compared with vehicle control. N=4 for each treatment.

Figure 1

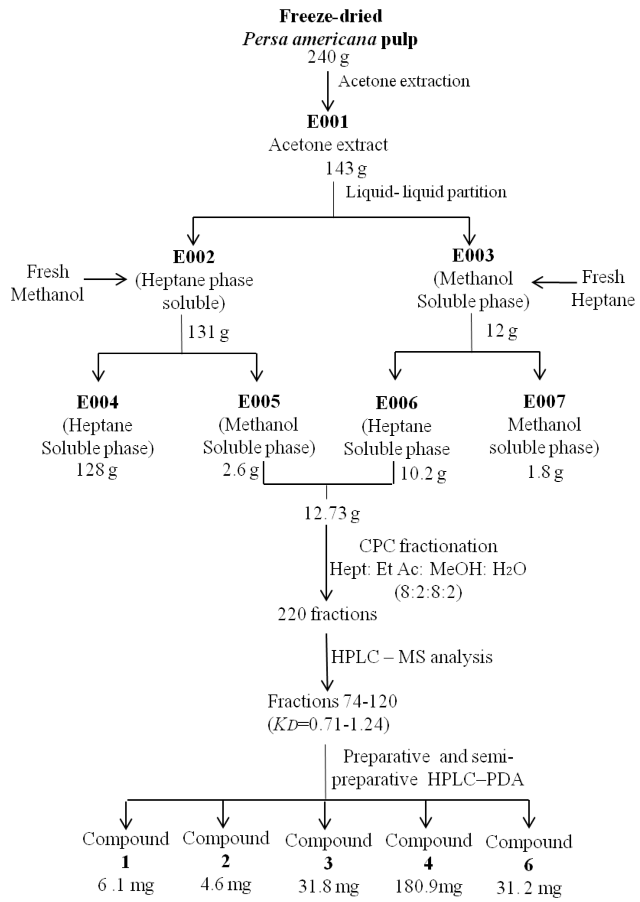


Figure 2

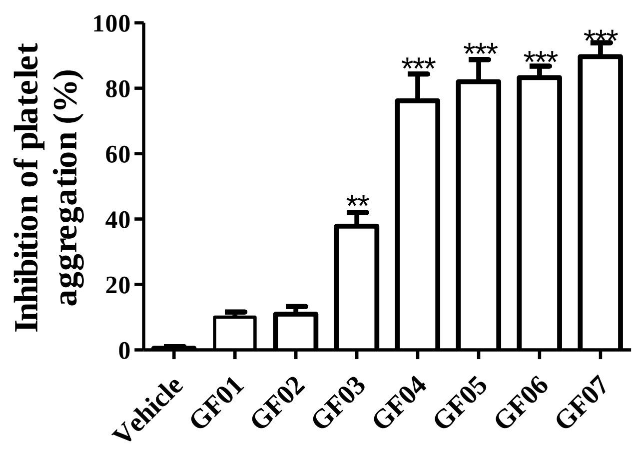


Figure 3

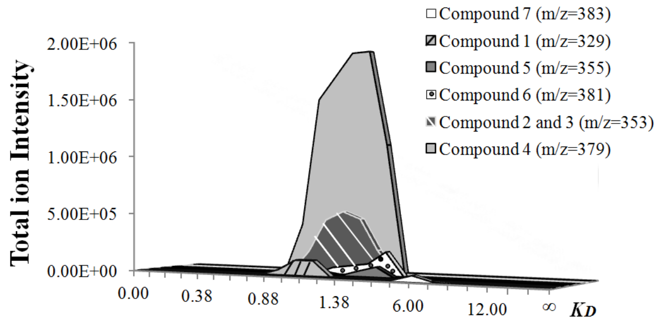


Figure 4

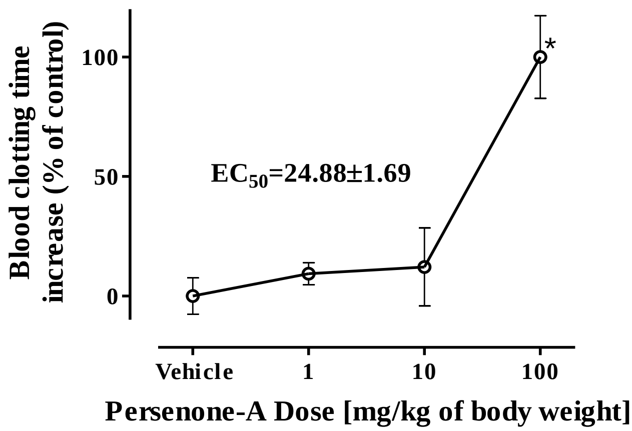


Figure 5

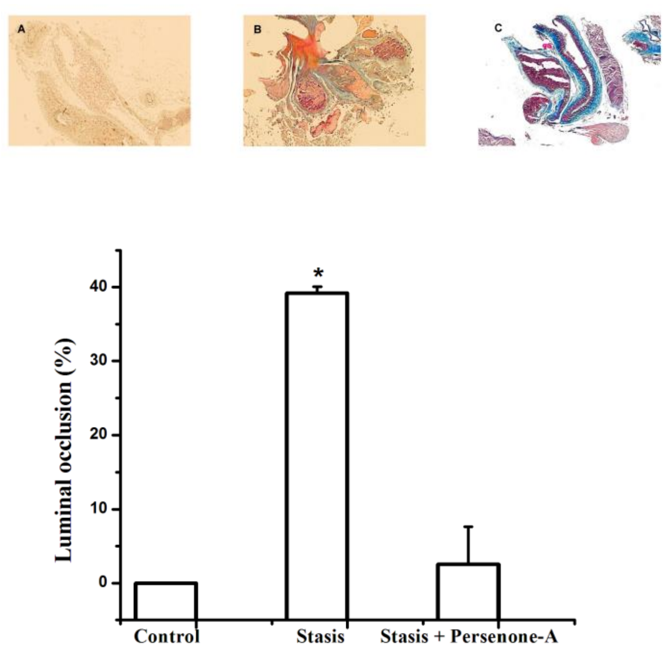
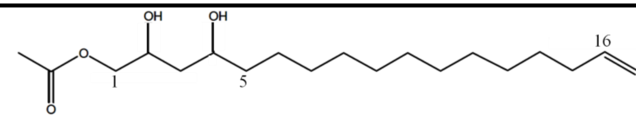
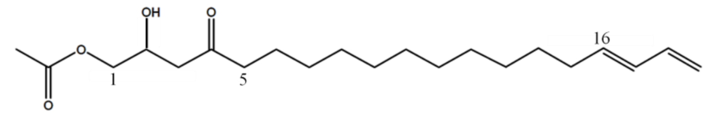
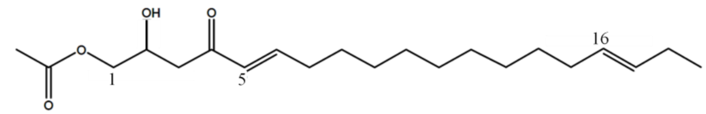
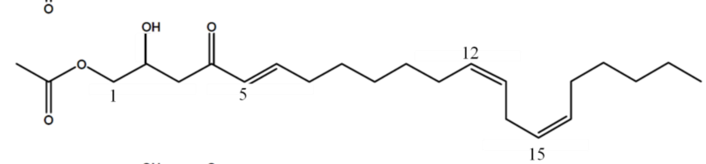
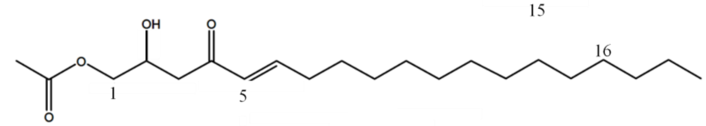
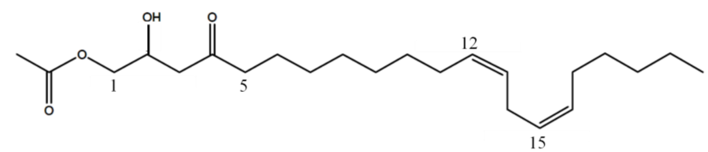
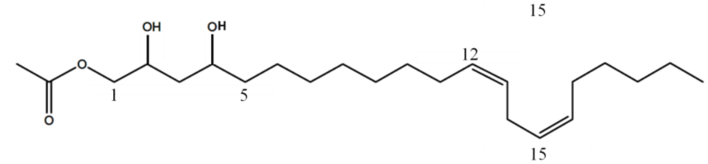


Table 1. Chemical identity of the common acetogenins present in the groups of fractions with highest antiplatelet activity (**GF04** to **GF07**).

Compound	**[M+H]$^+$ (m/z)^c**	**Ions Pattern (m/z)^c**	**Structure**	**References**
(2S,4S)-1-acetoxy-2,4-di-hydroxy-n-heptadeca-16-ene **(1)**	329	351, 311, 269, 251		[18]
Persediene (**2**)	353	375, 335, 293		[13]
Persenone-C (**3**)	353	375, 335, 293		[13]
Persenone-A (**4**)	379	401, 361, 319, 301		[18]
Persenone-B (**5**)	355	377, 337, 295		[20]
Persin (**6**)	381	403, 363, 321, 303		[21]
(12Z,15Z)-1-acetoxy-2,4-dihydroxy-heneicosa-12,15-diene (**7**)	383	365, 323, 305		[19]

^{c}MS/TOF detection using electrospray ionization interface in positive-ion mode of analysis.

Table 2. Half-maximal inhibitory concentrations (IC_{50}) presented by purified acetogenins in a platelet aggregation assay, induced by different agonists.

Evaluated Sample	**Half-maximal inhibition concentrations (IC_{50}, mM)**		
	Collagen (5 µg/mL)	**ADP (20 µM)**	**Arachidonic Acid (500 µM)**
(2S,4S)-1-acetoxy-2,4-dihy-droxy-n-heptadeca-16-ene **(1)**	8.18 ± 1.48 bc	7.29 ± 1.21 b	13.42 ± 1.26 c
Persediene (**2**)	11.99 ± 1.24 c	> 15	> 15
Persenone-C (**3**)	5.23 ± 1.19 b	3.42 ± 1.56 a	7.40 ± 1.20 b
Persenone-A (**4**)	8.73 ± 1.25 bc	13.48 ± 1.41 c	> 15
Persin (**6**)	> 15	> 15	> 15
Aspirin (Acetylsalicylic acid)	0.38 ± 0.07 a	3.65 ± 0.07 a	0.07 ± 0.01 a

[1] Values represent mean ± standard deviation (n=4). [2] Different letters in the same column indicate that values are significantly different ($p < 0.05$) by the LSMean Student's t- test. [3] Less than 50% inhibition at 15 mM.

Table 1. Chemical identity of the common acetogenins present in the groups of fractions with highest antiplatelet activity (**GF04** to **GF07**).

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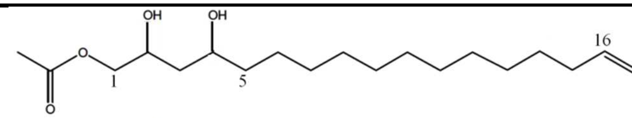
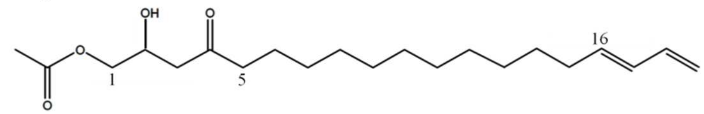
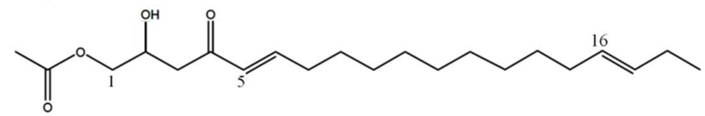
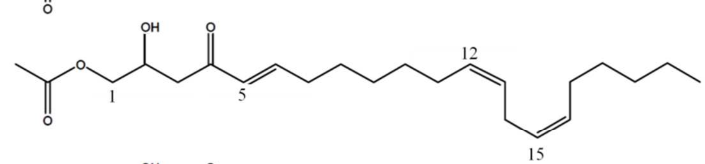
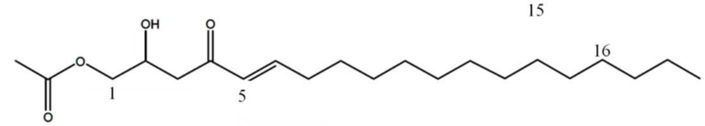
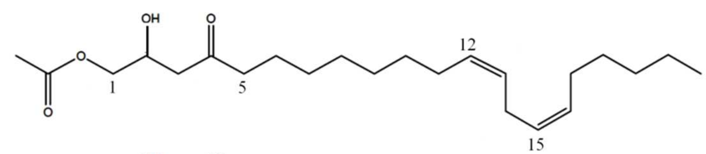
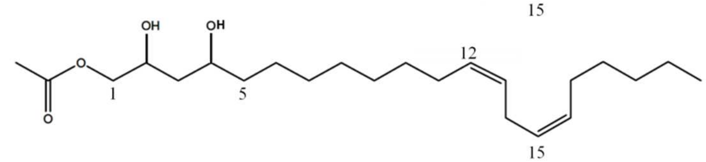
(2S,4S)-1-acetoxy-2,4-di-hydroxy-n-heptadeca-16-ene **(1)**	329	351, 311, 269, 251		[18]
Persediene (**2**)	353	375, 335, 293		[13]
Persenone-C (**3**)	353	375, 335, 293		[13]
Persenone-A (**4**)	379	401, 361, 319, 301		[18]
Persenone-B (**5**)	355	377, 337, 295		[20]
Persin (**6**)	381	403, 363, 321, 303		[21]
(12Z,15Z)-1-acetoxy-2,4-dihydroxy-heneicosa-12,15-diene (**7**)	383	365, 323, 305		[19]

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Evaluated Sample	Half-maximal inhibition concentrations (IC_{50}, mM)		
	Collagen (5 µg/mL)	**ADP (20 µM)**	**Arachidonic Acid (500 µM)**
(2S,4S)-1-acetoxy-2,4-dihy-droxy-n-heptadeca-16-ene (**1**)	8.18 ± 1.48 bc	7.29 ± 1.21 b	13.42 ± 1.26 c
Persediene (**2**)	11.99 ± 1.24 c	> 15	> 15
Persenone-C (**3**)	5.23 ± 1.19 b	3.42 ± 1.56 a	7.40 ± 1.20 b
Persenone-A (**4**)	8.73 ± 1.25 bc	13.48 ± 1.41 c	> 15
Persin (**6**)	> 15	> 15	> 15
Aspirin (Acetylsalicylic acid)	0.38 ± 0.07 a	3.65 ± 0.07 a	0.07 ± 0.01 a

[1] Values represent mean ± standard deviation (n=4). [2] Different letters in the same column indicate that values are significantly different ($p < 0.05$) by the LSMean Student's t- test. [3] Less than 50% inhibition at 15 mM.