Biomedicine & Pharmacotherapy 137 (2021) 111301

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

In vitro antiplatelet activity of extract and its fractions of Paulownia Clone in Vitro 112 leaves

Weronika Adach [a], Jerzy Żuchowski [b], Barbara Moniuszko-Szajwaj [b], Małgorzata Szumacher-Strabel [c], Anna Stochmal [b], Beata Olas [a,*], Adam Cieslak [c,*]

[a] University of Łódź, Department of General Biochemistry, Faculty of Biology and Environmental Protection, 90-236, Łódź, Poland
[b] Department of Biochemistry, Institute of Soil Science and Plant Cultivation, State Research Institute, 24-100, Puławy, Poland
[c] Department of Animal Nutrition, Poznań University of Life Sciences, Wołyńska 33, 60-637, Poznań, Poland

ARTICLE INFO

Keywords:
Adhesion
Aggregation
Platelets
Platelet activation
Paulownia

ABSTRACT

Background: Paulownia Clone in Vitro 112, also known as Oxytree is a hybrid of *Paulownia elongata* and *Paulownia fortunei*, developed under laboratory conditions. Its seeds are sterile, making it a noninvasive variety that can only be propagated in the laboratory. In China, species from the *Paulownia* genus (Paulowniaceae) are widely used in traditional medicine for the treatment of infectious diseases, such as gonorrhea and erysipelas. It has a broad spectrum of bioactivity, including neuroprotective, antioxidant, antibacterial, antiphlogistic, antiviral, and cytotoxic actions. However, the antiplatelet potential of Paulownia Clone in Vitro 112 has not yet been described.

Study design: The aim of our study was thus to examine the effect of an extract and four fractions from leaves of Paulownia Clone in Vitro 112 on various parameters of platelet activation in an in vitro model.

Methods: Composition of the investigated extract and fractions was determined by UHPLC-UV-MS. The following parameters of platelet activation were investigated: nonenzymatic lipid peroxidation in resting platelets; enzymatic lipid peroxidation (AA metabolism) in platelets activated by thrombin; superoxide anion ($O_2^{-\cdot}$) generation in the resting and activated platelets; platelet adhesion to collagen type I and fibrinogen; platelet aggregation stimulated by various physiological agonists, such as ADP, collagen, and thrombin. The effect of the extract and fractions on extracellular LDH activity, a marker of cell damage, was also determined.

Results: Verbascoside a phenylethnanoid glycoside, was the main secondary metabolite of the extract from leaves of oxytree (constituting approximately 45 % of all compounds). There were also iridoids, such as catalpol, aucubin, and 7-hydroxytomentoside, as well as flavonoids, such as luteolin and apigenin glycosides. Moreover, the extract had stronger antiplatelet properties than the fractions. For example, the extract at 10 μg/mL inhibited five parameters of platelet activation.

Conclusions: Our results show that Paulownia Clone in Vitro 112 leaves are a new valuable source of compounds with antiplatelet potential.

1. Introduction

The activation of blood platelet plays a crucial role in hemostasis, taking part in many biochemical processes, including inflammation and the formation and course of cancer. In the process of blood coagulation, platelets are activated at the site of vascular damage by the action of numerous substances called agonists or activators/stimulators, including ADP, collagen, and thrombin. A range of compounds are then released from intracellular platelet granules, including adhesive glycoproteins, such as P-selectin (CD62 P), fibrinogen, von Willebrand factor, as well as coagulation factors, responsible for initiating coagulation and vasoconstriction [1–3].

During platelet activation, arachidonic acid (AA) cascade is activated. AA is involved in many key processes, including the formation

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; CVD, cardiovascular disease; LOX, lipoxygenase; LDH, lactate dehydrogenase; $O_2^{-\cdot}$, superoxide anion; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substance; TX, thromboxane.

* Corresponding authors.
E-mail addresses: beata.olas@biol.uni.lodz.pl (B. Olas), adam.cieslak@up.poznan.pl (A. Cieslak).

https://doi.org/10.1016/j.biopha.2021.111301
Received 20 November 2020; Received in revised form 15 January 2021; Accepted 18 January 2021
Available online 6 February 2021

and development of cardiovascular disease (CVD) and metabolic diseases [4]. The concentration of AA in platelets is negligible, but when it is activated, it is released from membrane phospholipids, such as phosphatidylcholine and phosphatidylethanolamine. AA can be released by the action of phospholipase A_2 or by the action of phospholipase C, followed by diacylglycerol lipase. Phospholipase A_2 has the ability to cleave an acid molecule from a membrane phospholipid. Free AA acid can be transformed by enzymatic reactions, cyclooxygenase (COX), lipoxygenase (LOX), cytochrome p450 (CYP 450), or the anandamide pathway. The main AA pathway is initiated by cyclooxygenase, which converts the acid into prostanoids, including in prostaglandins (PG) prostacyclin (PGI_2), and thromboxanes (TXA_2, TXB_2). Prostaglandins (PGG_2, PGH_2) are formed during primary AA transformations, while PGD_2, PGE_2, and PGF_2 show the highest proaggregation activity. Both TXA_2 and PGG_2 and PGH_2 regulate the contractility of blood vessels, participate in inflammatory reactions, and regulate platelet aggregation [1,5–7]. The LOX pathway involves such enzymes as LOX-5, LOX-8, LOX-12, and LOX-15, depending on the position of attachment of oxygen to a carbon atom in the AA acid molecule. Leukotriene and lipoxin production is the responsibility of derivative LOX products from hydroperoxyeicosatetraenoic acid (5, 8, 12, 15 HPETE); more precisely, 5-HPETE and 15-HPETE acid are necessary to produce the leukotrienes and lipoxins, respectively. These arise in leukocytes, causing blood vessel spasm and increasing vascular permeability [4,8]. Another mechanism is the cytochrome p450 (CYP450) pathway, whose enzymes are rich in heme iron. The enzymes are restricted mainly to the liver and are responsible for the elimination of toxins. Epoxygenase comediated with CYP 450 leads to the conversion of AA into EET the epoxyeicosatrienic acids (5, 6, 8, 9, 11, 12, 14, 15-EET) and hydroxyeicosatetraenoic (HETE) [1,4,7,9–12]. The final enzymatic mechanism is the anandamide pathway, which includes fatty acid amide hydrolase (FAAH), and which produces AA and ethanolamine. This is a reversible reaction: when tissue damage occurs, the levels of free AA and ethanolamine increases, and the action of amide hydrolase is reversed and anandamide is produced [4,7,13–17].

AA is also processed in nonenzymatic reactions. In platelets, AA can be converted to isoprostanes and nitroeicosatetraenoic acid by a nonenzymatic route. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can contribute to the production of isoprostanes, causing AA oxidation [4,18]. The eicosanoids are produced in small amounts in the resting phase of cells, increasing with inflammatory stimulation. Isoprostanes have effects on vasoconstriction, platelet aggregation, and smooth muscle proliferation [1,4,7,19,20].

Platelet activation may be inhibited by natural and synthetic compounds using a range of mechanisms; such compounds may be used in the prevention and treatment of CVD associated with the hyperactivation of platelets. However, many antiplatelet drugs—including aspirin and its derivatives—may induce serious adverse effects. The aim of our study was thus to investigate the effect of an extract and of four fractions of different chemical composition from Paulownia Clone in Vitro 112 leaves on various parameters of platelet activation, including nonenzymatic lipid peroxidation in resting platelets; enzymatic lipid peroxidation (AA metabolism) in platelets activated by thrombin; superoxide anion ($O_2^{\cdot-}$) generation in the resting and activated platelets; platelet adhesion to collagen type I and fibrinogen; and platelet aggregation stimulated by various physiological agonists, such as ADP, collagen, and thrombin). The effect of the extract and the fractions on extracellular lactate dehydrogenase (LDH), a marker of cell damage.

2. Materials and methods

2.1. Chemicals

Acetonitrile (LC–MS grade) and formic acid (LC–MS grade) were obtained from Merck (Darmstadt, Germany). Verbascoside was obtained from HWI Analytik (Ruelzheim, Germany), rutin from PhytoLab (Vestenbergsgreuth, Germany). Catalpol, maslinic acid and dimethyl sulfoxide (DMSO) from Sigma Aldrich. Tubes of (citrate/phosphate/dextrose/adenine) were obtained from Mar-Four (Poland).

2.2. Plant material

Leaves of six-month Paulownia Clone in Vitro 112 trees were collected from a local plantation at Łęka, Lubelskie Voivodeship, Poland (21°54′N, 51°27′E). A voucher specimen (IUNG/PCIV112/2017/1) was deposited at the Department of Biochemistry and Crop Quality, Institute of Soil Science and Plant Cultivation, State Research Institute, Puławy, Poland.

2.3. Preparation of the extract and fractions (A–D)

Freshly picked Paulownia Clone in Vitro 112 leaves were chopped, frozen, and lyophilized (Martin Christ Gamma 2–16 LSC, Germany). The extract of Paulownia Clone in Vitro 112 leaves was obtained through sequential extraction. Active metabolites of this extract were eluated with 80 % methanol (v/v) t give fraction A. Next, fraction A gived three subfractions: B–D. A more detailed description of preparation and the extract and four fractions (A–D) can be found in the paper of Adach et al. [21].

2.4. LC–MS analyses

Sample composition was determined by UHPLC-DAD-ESI-MS. Chromatographic separations were performed using an Acquity UPLC system (Waters, Milford, MA, USA) coupled with an Acquity TQD (Waters) mass detector on an Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm; Waters) column. The mobile phase was composed of mixtures of solvent A (0.1 % FA in Milli-Q water) and solvent B (0.1 % FA in acetonitrile). The injection volume was 2.5 μL. The sample constituents were identified on the basis of their MS and UV spectra, and identification was supported by previously performed LC-HRMS/MS analysis of a Paulownia Clone in Vitro 112 extract (data not shown); authentic standards (verbascoside, catalpol, maslinic acid), and literature data were also employed for identification. Three different UHPLC-MS methods were applied for semiquantization of phenolic compounds, iridoids, and triterpenoids, as described in the work of Adach et al. [21]. UV detection (λ =330 nm) was used for semiquantization of phenolics. The content of individual compounds was determined on the basis of calibration curves of verbascoside (HWI Analytik, Ruelzheim, Germany) and rutin (PhytoLab, Vestenbergsgreuth, Germany). The content of phenylethanoids, other derivatives of phenolic acids, and all minor and unidentified compounds was expressed as equivalent of verbascoside, while the concentrations of the major flavonoids were expressed as rutin equivalents.

Iridoids were semiquantified by MS, using a negative-ion SIM method. Two ions were monitored, at *m/z* 407 (FA adduct of catalpol) and *m/z* 391 (FA adduct of aucubin/7-hydroxytomentoside). The iridoid level was determined on the basis of the calibration curve of catalpol and expressed as equivalents of catalpol.

Triterpenoids were semiquantified using another negative ion SIM method. Four ions were monitored, at *m/z* 503, *m/z* 487, *m/z* 471, and *m/z* 455. The triterpenoid content was determined on the basis of a calibration curve of maslinic acid (Sigma), and expressed as equivalents of maslinic acid. A detailed description of the UHPLC separation methods and MS settings used can be found in the work of Adach et al. [21].

2.5. Platelet isolation

Platelets were isolated from fresh human blood collected at Rydygier Medical Center in Łódź (Poland) from healthy, medication-free, nonsmoking donors (male and female). None of the subjects had taken

Table 1
MS (negative ionization) data, UV maxima, and the content of different secondary metabolites in the extract and fractions from leaves of Paulownia Clone in Vitro 112.

Compound	m/z	UV	Concentration [mg/g DM]				
			Extract	Fraction A	Fraction B	Fraction C	Fraction D
catalpol	(407)		0.7 ± 0.0	1.5 ± 0.0	traces		
7-hydroxytomentoside/aucubin	(391)	248	14.5 ± 0.3[#]	25.4 ± 0.4[#]	19.8 ± 0.4[#]		
caffeic acid-Hex-dHex	487	245, 327	2.7 ± 0.1[*]	6.9 ± 0.1[*]	26.8 ± 1.9[*]		
luteolin-HexA-HexA	637	255, 345	6.9 ± 0.5[$]	17.0 ± 0.8[$]	28.4 ± 1.7[$]	38.1 ± 1.4[$]	traces
hydroxyverbascoside I	639	220, 330	7.2 ± 0.2[*]	16.2 ± 0.2[*]	31.8 ± 2.0[*]	28.0 ± 1.0[*]	1.1 ± 0.0[*]
hydroxyverbascoside II	639	220, 330	7.9 ± 0.3[*]	17.9 ± 0.3[*]	34.3 ± 2.1[*]	31.3 ± 1.2[*]	1.3 ± 0.0[*]
apigenin-HexA-HexA	621	266, 335	14.2 ± 0.5[$]	30.7 ± 0.3[$]	31.0 ± 1.9[$]	80.5 ± 3.2[$]	traces
methoxyverbascoside	653	220, 330	17.4 ± 0.7[*]	36.4 ± 0.4[*]	45.6 ± 2.8[*]	84.3 ± 2.9[*]	1.5 ± 0.1[*]
verbascoside	623	220, 330	74.6 ± 4.9	164.9 ± 1.8	154.1 ± 9.9	424.7 ± 14.2	4.5 ± 0.1
maslinic acid	471		0.3 ± 0.0	traces			traces

(formic acid adduct).
[#] - catalpol equivalent.
[$] - rutin equivalent.
[*] - verbascoside equivalent.

any medication or addictive substances, such as tobacco, alcohol, antioxidant supplementation, aspirin, or any other antiplatelet drug, for at least a few days prior to blood sampling. Blood donors were healthy people aged 25–35, without diagnosed diseases. The blood was collected into CPDA-1 solution (citrate/phosphate/dextrose/adenine; 8.5:1; v/v; blood/CPDA-1). Composition of the solution: adenine ($C_5H_5N_5$) - 0.275 g/L; glucose ($C_6H_{12}O_6$) - 31.90 g/L; trisodium citrate ($Na_3C_6H_5O_7$) - 26.30 g/L; sodium dihydrogen phosphate (NaH_2PO_4) - 2.22 g/L; and citric acid ($C_6H_8O_7$) – 3,27 g/L.

Platelet-rich plasma (PRP) was prepared by centrifugation of fresh blood at 1200 × g for 15 min (at room temperature). Blood platelets were then sedimented by centrifugation at 2300 × g for 15 min (at room temperature). Platelet poor plasma (PPP) was obtained as the supernatant, and platelets constituted a precipitate. The platelet pellet was suspended in the Barber's buffer (pH 7.4). The concentration of platelets in suspensions was estimated spectrophotometrically at 800 nm [22–24] and amounted to 2 × 10^8 cells/mL. Suspensions of blood platelets or plasma were incubated at 37 °C for 30 min with the extract and the four tested fractions (concentration range 1–50 μg/mL).

The experiments were conducted with the consent of the Bioethics Committee at the University of Łódź (number 11/KBBN-UŁ/I/2019).

2.6. Preparation of stock solutions for bioassay

The extract and the four fractions were dissolved in 1 mL 50 % DMSO, giving final concentrations of 1 μl/mL, 5 μl/mL, 10 μl/mL, and 50 μl/mL (in blood platelet samples). DMSO, a universal solvent for many different plant substances. The final concentration of DMSO in the tested blood platelet samples was below 0.05 % (v/v). In addition, the addition of a low concentration of DMSO to human plasma or platelets has no effect on oxidative stress or coagulation parameters [22–24].

2.7. Determination of lipid peroxidation level in platelets by measuring TBARS

The samples—consisting of the extract and fractions plus platelets—were incubated for 30 min at 37 °C. We then added 500 μL trichloroacetic acid and 500 μL 2-thiobarbituric acid. The samples were heated in a water bath at 100 °C for 10 min. They were cooled to 4 °C and centrifuged at 10,000 x g at 18 °C for 15 min. The absorbance at λ =535 nm was measured on a SpectroStar Nano BMG Labtech spectrophotometer [25,26].

2.8. Determination of superoxide anion content in platelets by cytochrome C reduction

The release of the anion radical from the cell is triggered by the reduction of cytochrome C, which is in turn caused by the superoxide anion. The amount of superoxide anion was measured spectrophotometrically at a wavelength λ =550 nm, with the changes in the light absorption spectra described previously by Olas et al. [27].

2.9. Measurement of platelet aggregation by the turbidimetric method

The method measures platelet aggregation stimulated by platelet agonists (ADP, thrombin, and collagen) and observes changes in light transmission. Platelet aggregates are formed, leading to increased light transmission, as described earlier by Born [28].

The samples—consisting of the extract and fractions plus platelets—were incubated for 30 min at 37 °C, and then measurements were made in an aggregometer (Chrono-Log 490-2D). After the incubation procedure for the PRP samples, ADP (10 μM) or collagen (2 μg/mL) were added and blood platelet aggregation measured for 10 min. After the incubation procedure for the blood platelet samples, thrombin (1 U/mL) was added and blood platelet aggregation measured for 10 min. The aggregometer was calibrated against platelet-poor plasma (PPP, with 100 % aggregation for collagen and ADP) or against Barber's buffer with 100 % aggregation for thrombin [28].

2.10. Platelet adhesion to fibrinogen and collagen (static method based on acid phosphatase determination)

Targeted to the adhesive protein, the cells rested at the bottom of the microtiter plate well. Acid phosphatase activity was measured spectrophotometrically at wavelength λ =405 nm, as described previously by Bellavite et al. [29].

2.11. The measurement of LDH activity

The level of LDH was determined by observation of the changes in absorbance, which indicates the rate of reaction and is proportional to the concentration of LDH. Absorbance was measured spectrophotometrically at wavelength λ =340 nm, as described previously by Wroblewski et al. [30].

2.12. Data analysis

Statistical analysis was performed using Statistica 10 (StatSoft). Normal distribution of data was checked by normal probability plots and the homogeneity of variance by the Brown–Forsythe test. Differences within and between groups were assessed using one-way ANOVA followed by a multicomparison Duncan's test; for clarity, only the differences between the tested preparations and the control/control positive were noted. Results are presented as means ± SD. Significance was

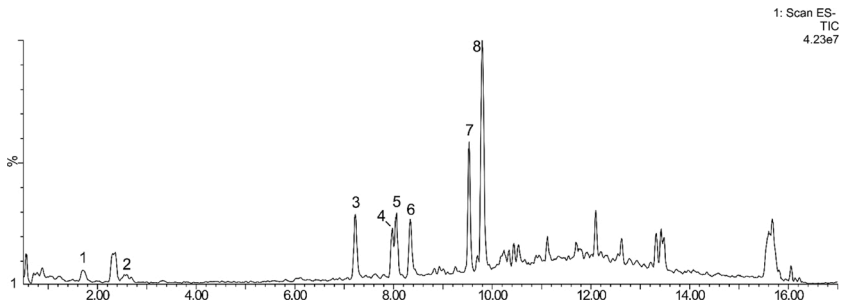


Fig. 1. LC–MS TIC chromatogram (negative ion mode) of Fraction A from leaves of Paulownia Clone in Vitro 112. 1 – 7-hydroxytomentoside / aucubin; 2 - caffeic acid-Hex-dHex; 3 - luteolin-HexA-HexA; 4 & 5 – hydroxyverbascoside; 6 – apigenin-HexA-HexA; 7 – methoxyverbascoside; 8 – verbascoside.

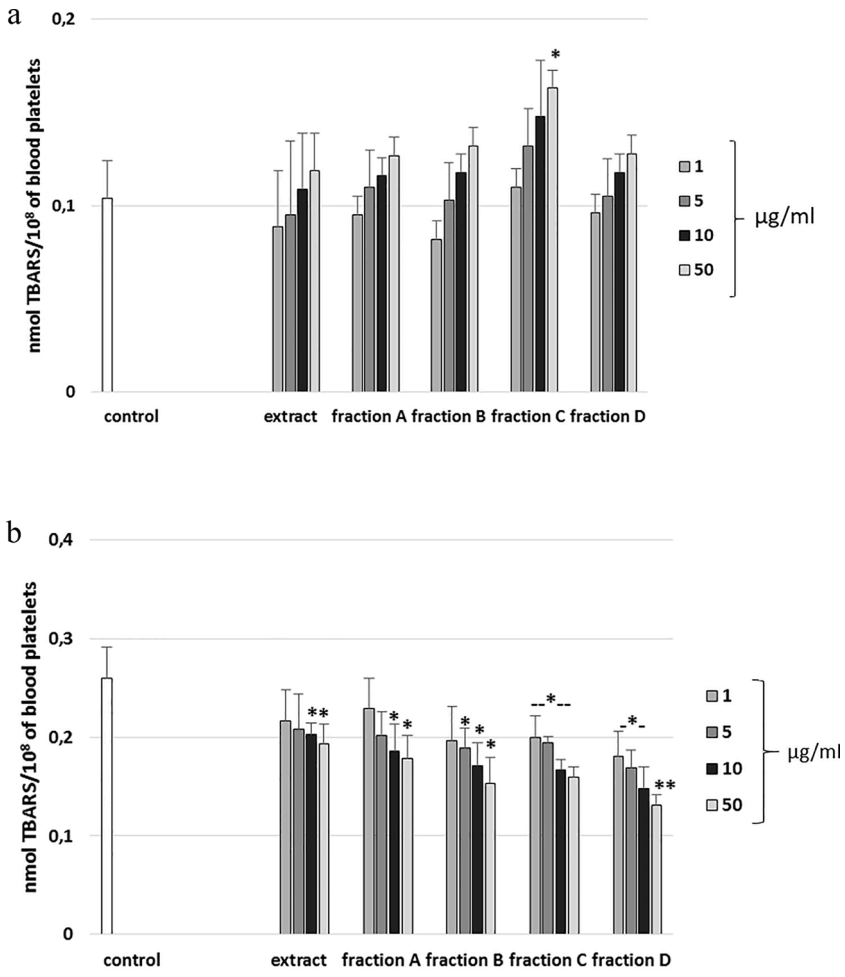


Fig. 2. Effects of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration range 1–50 µg/mL, incubation time - 30 min.) on lipid peroxidation in resting blood platelets (a), and platelets activated by 5 U/mL thrombin (pre-incubation time with plant extract/fraction - 25 min; incubation time with thrombin – 5 min.) (b). Results are given as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, compared with control.

considered at $p < 0.05$. Dixon's *Q*-test was used to eliminate uncertain data.

3. Results

Verbascoside was the dominant phenolic compound in the extract and in fractions A, B, and C (Table 1; Fig. 1). In contrast, fraction D contained only small amounts (4.5 ± 0.1 mg g^{-1}) of this compound. Putative hydroxy and methoxy derivatives of verbascoside, as well as dihexuronides of apigenin and luteolin, were other major constituents of the extract and of fractions A, B, and C. Fraction D contained a complex mixture of phenolic compounds less polar than verbascoside, poorly separated during the chromatographic analysis. These were mainly putative phenylethanoid glycosides or other derivatives of hydroxycinnamic acids (as indicated by their UV and MS spectra); luteolin and apigenin were also present. The preparations in order of increasing total phenolic content:the extract (205.50 ± 6.41 mg g^{-1}), fraction B (379.00 ± 24.25 mg g^{-1}), fraction A (453.70 ± 5.22 mg g^{-1}), fraction D (700.00 ± 5.76 g^{-1}), and fraction C (743.10 ± 25.96 mg g^{-1}). The extract, and fractions A and B also contained iridoids, mainly 7-hydroxytometoside or aucubin, and small amounts of catalpol (Table 1). Total iridoid content was 15.160 ± 0.274 mg g^{-1} of the extract, 26.930 ± 0.484 mg g^{-1} of

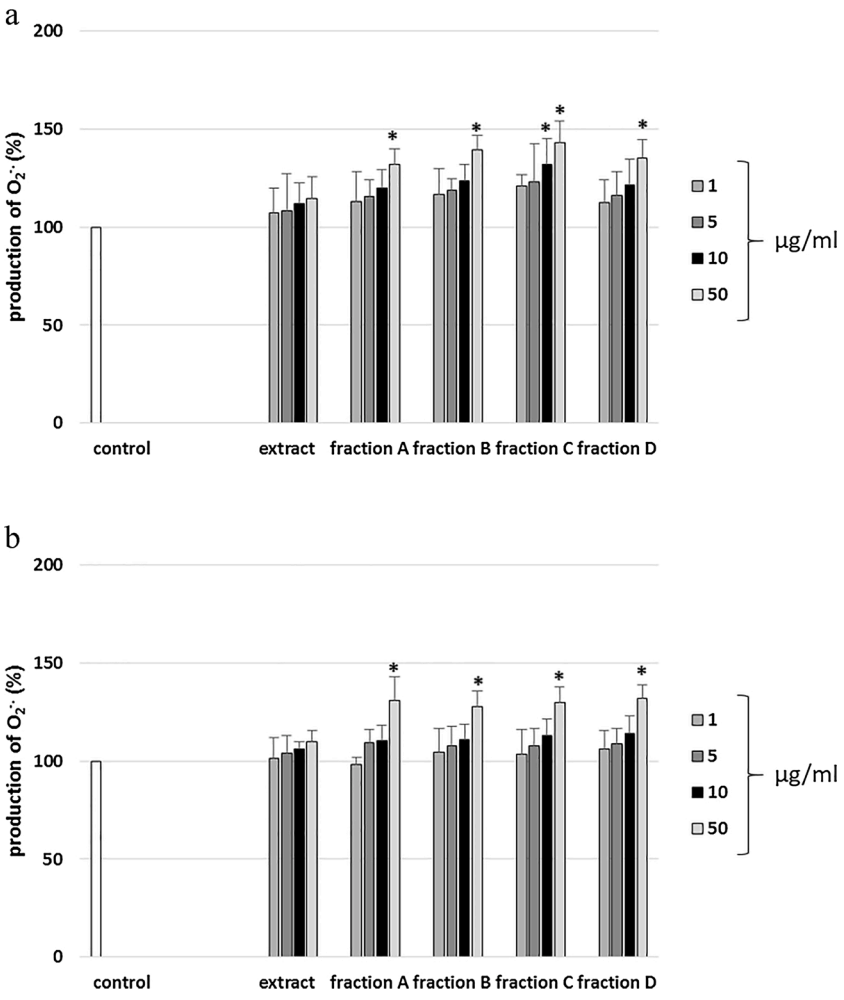


Fig. 3. Effects of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration range 1–50 µg/mL, incubation time - 30 min.) on $O_2^{\cdot-}$ production in resting blood platelets (a), and platelets activated by 5 U/mL thrombin (pre-incubation time with plant extract/fraction - 25 min; incubation time with thrombin – 5 min.) (b). In the graph, $O_2^{\cdot-}$ production is expressed as a percentage of the control sample (blood platelets without extract or fraction). Results are given as mean ± SD (n = 6). * $p < 0.05$, compared with control.

fraction A, and 19.770 ± 0.415 mg g^{-1} of fraction B. The extract and fractions A and D also contained small amounts of triterpenoids. Maslinic acid (Table 1) and different $C_{30}H_{48}O_6$, $C_{30}H_{48}O_5$, $C_{30}H_{48}O_4$, and $C_{30}H_{48}O_3$ compounds, as well as $C_{30}H_{48}O_6$-hexosides, were present in the extract. Fractions A and D mostly contained $C_{30}H_{48}O_6$, $C_{30}H_{48}O_5$, and $C_{30}H_{48}O_6$-hexosides. The total triterpenoid content was 3.650 ± 0.278 mg g^{-1} in the extract, 4.230 ± 0.121 mg g^{-1} in fraction A, and 9.360 ± 0.010 mg g^{-1} in fraction D.

As demonstrated in Fig. 1a, no change in TBARS concentration was observed in the resting platelets after incubation with extract and three fractions (A, B, and D) at any tested concentration (1–50 µg/mL). Fraction C increased lipid peroxidation in resting platelets only at the highest concentration of 50 µg/mL (Fig. 2a). On the other hand, all concentrations of the extract and the four fractions reduced lipid peroxidation in thrombin-activated platelets (Fig. 1b). However, the inhibitory action was not always statistically significant (Fig. 2b). The strongest inhibition of this process was observed for fraction D at 50 µg/mL; inhibition of lipid peroxidation was then found to be about 60 % (Fig. 2b). All concentrations of tested extract and four fractions increased the level of $O_2^{\cdot-}$ in resting platelets and in thrombin-activated blood platelets. These incrases were dose-dependent. However, only the fractions at the highest concentration of 50 µg/mL were seen to increase statistically significant the process of $O_2^{\cdot-}$ generation in resting platelets and platelets activated by thrombin (Fig. 3).

The extract and fractions were not found to display any anti-aggregatory activities when collagen or thrombin was used as an agonist (Fig. 4b and c). However, at the highest concentration of 50 µg/mL, the extract and fractions statistically significantly inhibited ADP-induced platelet aggregation (Fig. 4a). For example, the percentage inhibition of ADP-induced platelet aggregation was about 22 % for fraction A (50 µg/mL) (Fig. 4a). Our results demonstrate lower adhesion to collagen or fibrinogen for platelets following incubation with the extract and four fractions (Figs. 5 and 6). For example, inhibition of adhesion of thrombin-activated platelets to collagen or fibrinogen was dose-dependent (Figs. 5b and 6 b). Fraction A (50 µg/mL) demonstrated about 70 % inhibition of adhesion to fibrinogen when thrombin was used as an agonist (Fig. 6b). Neither the extract nor any of the fractions caused lysis of platelets at any dose (1–50 µg/mL) (Fig. 7).

Table 2 compares the effects of the extract and the four fractions on platelet activation in vitro. It can be seen that extract demonstrated stronger antiplatelet properties than did the other fractions (Table 1). The extract (10 µg/mL) inhibited five of the examined parameters (Table 2).

4. Discussion

The UHPLC-MS analysis showed that phenolic compounds, mainly phenylethanoid glycosides, were the main specific metabolites in the Paulownia Clone in Vitro 112 leaf extract and in fractions A–D. Of these, verbascoside was the dominant constituent of the extract and of fractions A–C; putative hydroxy and methoxy derivatives of verbascoside were also present, but in much lower amounts. The preparations also contained flavone glycosides, mainly diglucuronides of apigenin and luteolin; small amounts of other glycosides of apigenin and luteolin, as well as free aglycones were also present. In addition, the preparations contained small amounts of diverse putative phenylethanoid glycosides

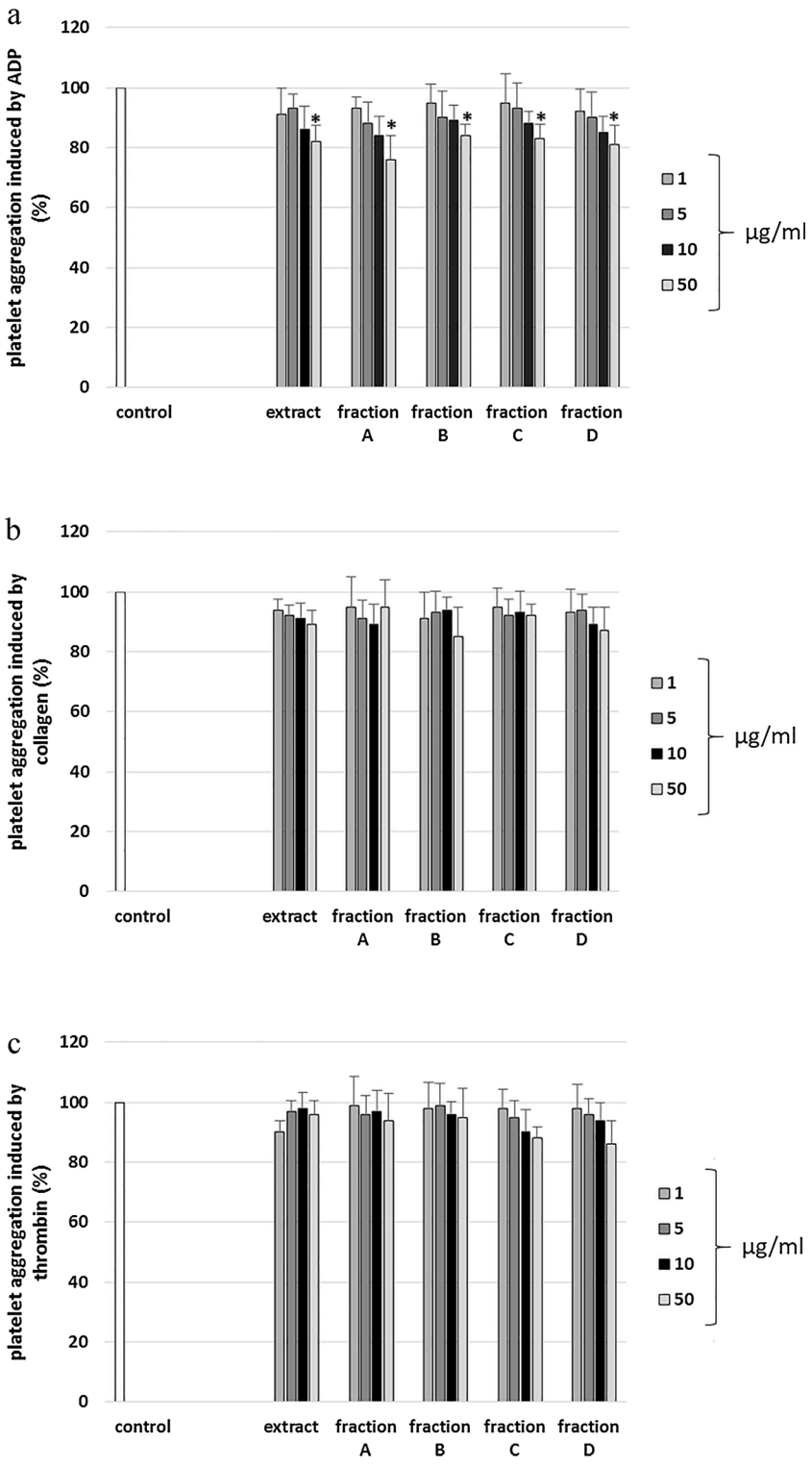


Fig. 4. Effects of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration range 1–50 µg/mL, incubation time - 30 min.) on blood platelet aggregation stimulated by 10 µM ADP (a), 2 µg/mL collagen (b), and 1 Units/mL thrombin (c). In the graph, the aggregation is expressed as a percentage of the control sample (PRP without extract or fraction, and washed blood platelets without extract or fraction). Results are given as mean ± SD (n = 6). * $p < 0.05$, compared with control.

(mostly less polar than verbascoside) and other derivatives of hydroxycinnamic acids. Verbascoside, its derivatives, apigenin and its glycosides were previously found in the bark of *Paulownia tomentosa* [31]. Apart from phenolics, the extract and fractions A and C contained iridoids, mainly 7-hydroxytometoside or aucubin (the compounds are isomers), and small amounts of catalpol. Also these compounds were earlier isolated from leaves of *P. tomentosa* [31]. Moreover, small amounts of triterpenoids were found in the crude extract and in fractions A and D. Diverse $C_{30}H_{48}O_6$, $C_{30}H_{48}O_5$, $C_{30}H_{48}O_4$ (including maslinic acid), and $C_{30}H_{48}O_3$ compounds were detected. Several $C_{30}H_{48}O_6$-hexosides were also present. Similar triterpenoids were earlier isolated from *P. tomentosa* and *P. coreana* [31,32]. Verbascoside is a chemical compound quite commonly found in plants. Its chemical structure consists of phenylethanite and caffeic acid, bound by an ether bond and an ester bond, respectively, to the glucose residue of α-rhamnopyranosyl-(1→3)-β-glucopyranose. Due to its potential clinical use, verbascoside has gained some attention from researchers and has been shown to exhibit anti-inflammatory and antibacterial activity [33].

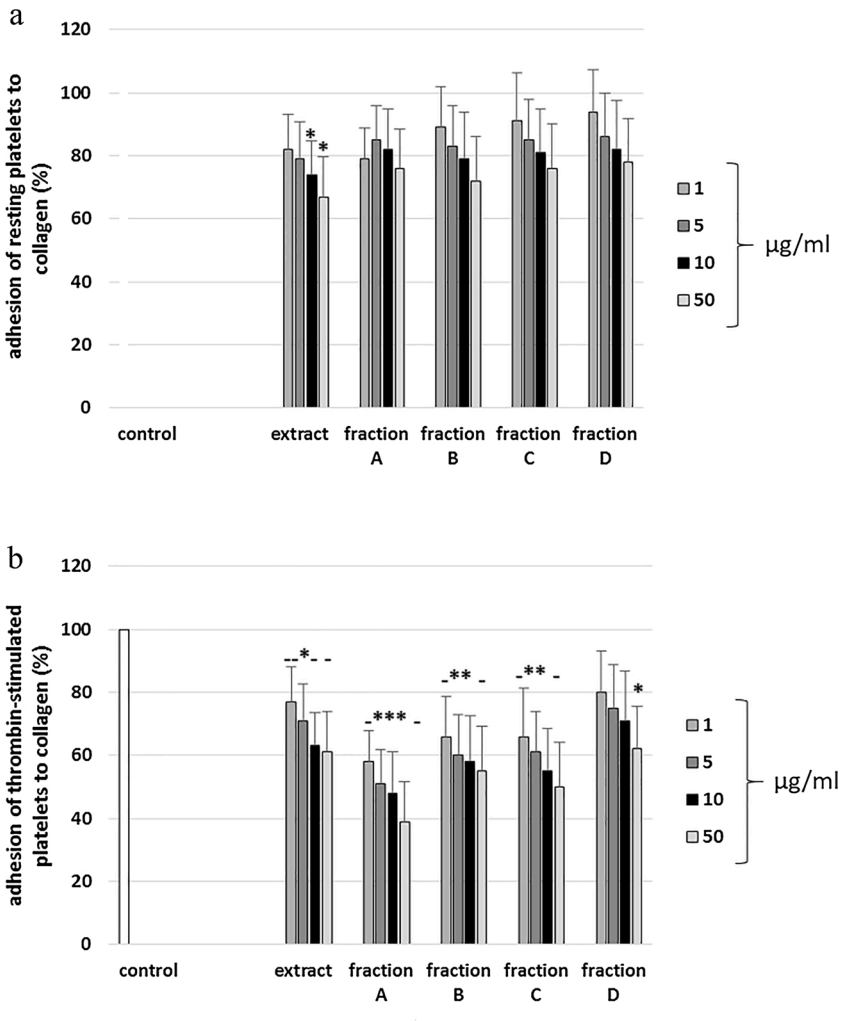


Fig. 5. Effects of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration range 1–50 µg/mL, incubation time - 30 min.) on adhesion resting platelets to collagen (a) and adhesion to collagen and thrombin (final concentration 0.2 U/mL) - activated platelets (b). In the graphs, the adhesion is expressed as a percentage of the control sample (platelets without extract or fraction). Results are given as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control.

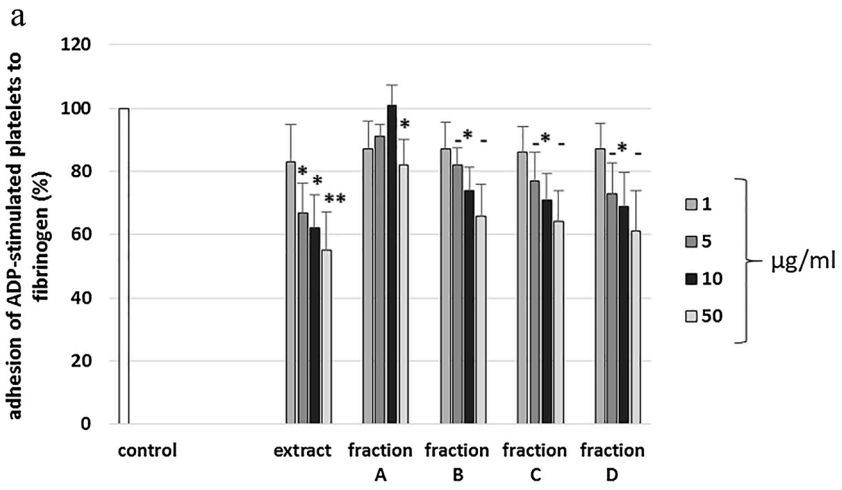
Pennacchio et al. [34] demonstrated the effect of verbascoside on the cardiovascular system. The experiment was carried out on isolated rat hearts. Verbascoside increased the heart rate by 37 %, increased coronary blood flow by 68 %, and increased myocardial contraction by 9 %. The compound was obtained from Verbascum and used at a dose of 1 µM [34]. Akdemir et al. [35] showed that verbascoside accelerates wound healing and when taken orally shows high anti-inflammatory activity. They experimentally demonstrated that verbascoside has no toxicity and does not damage the stomach walls [35].

Iridoids are a large group of chemical compounds, belonging to the group of monoterpenoids, which usually occur as glycosides. In plants, they have a protective function against herbivores and pathogens, and when extracted they may have a therapeutic effect. These compounds include catalpol and aucubin, which exhibit anti-inflammatory, expectorant, antibacterial, antioxidant, and immunostimulant activity [36]. Recio et al. [37] demonstrated the anti-inflammatory effects of aucubin: an inflammation was induced in rats by the administration of carrageenan; aucubin was given orally at 100 mg/kg body weight, suppressing the inflammation by 20.7 % within five hours. For comparison, indomethacin was used as a control at a dose of 7 mg/kg body weight, showing a 12.2 % reduction in inflammation [37].

Flavonoids are chemical compounds that occur in plants and have strong antioxidant and antiradical properties. They are commonly found in vegetables, fruits, and herbs. In addition to their affinity to estrogen receptors, flavonoids have a great impact on the metabolism of AA, a prostaglandin precursor. They inhibit cyclooxygenase and lipoxygenase, resulting in the formation of prostaglandins from AA. They thus show anti-inflammatory and antiedemic effects. It is well known that flavonoids and raw flavonoid materials have a sealing and strengthening effect on the walls of small blood vessels, and also affect myocardial circulation. Flavonoids inhibit hyaluronidase, an enzyme found in vascular walls is responsible for increasing the permeability of intercellular spaces. Flavonoids have additionally been shown to exert anti-aggregatory effects on platelets [38–43]. Our preliminary results indicate that two fractions C and D (from Paulownia Clone in Vitro 112 leaves), which are rich in phenolic compounds have an inhibitory effect on oxidative stress in human plasma treated with hydroxyl radicals [21].

The observed inhibitory activity of investigated preparations against lipid pexoridation in thrombin –activated platelets was most probably caused by the presence of phenylethanoids, which were dominant constituents of the Paulownia Clone in Vitro 112 extract and fractions; like many other phenolic compounds, phenylethanoids are known for their antioxidant properties. The highest antioxidant activity of the Fraction D may be attributted to the presence of less polar phenolics, which could potentially have stronger interactions with platelet membranes. However, a true identification of active constituents of the extract and fractions will not be possible without tests on purified compounds.

The present work is the first to characterize the effect of the Paulownia Clone in Vitro 112 leaf extract and its four fractions (A–D) on platelet properties in vitro. Our results indicate that the extract and fractions have different effects on platelet activation, which may be attributed to differences in their chemical content. Of the five preparations we tested, the most promise as an antiplatelet preparation was shown by the Paulownia Clone in Vitro 112 extract, which was rich in



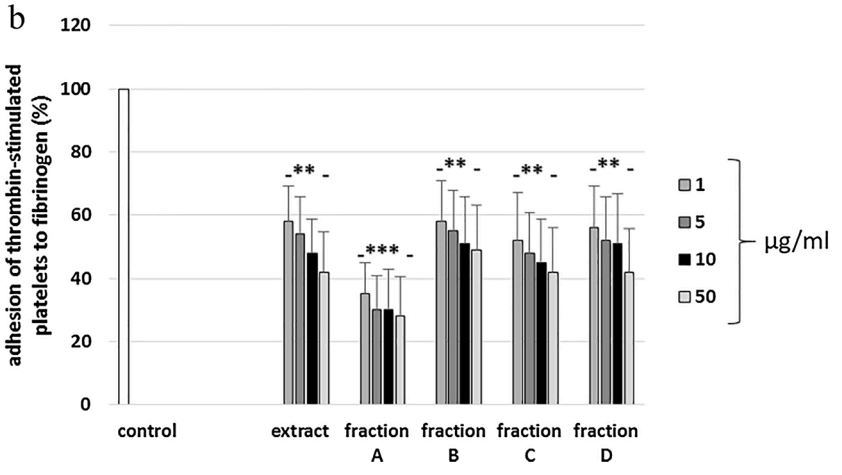


Fig. 6. Effects of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration range 1–50 µg/mL, incubation time - 30 min.) on adhesion to fibrinogen and ADP (final concentration 30 µM) - activated platelets (a), and thrombin (final concentration 0.2 U/mL) - activated platelets (b). In the graphs, the adhesion is expressed as a percentage of the control sample (platelets without extract or fraction). Results are given as mean ± SD (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control.

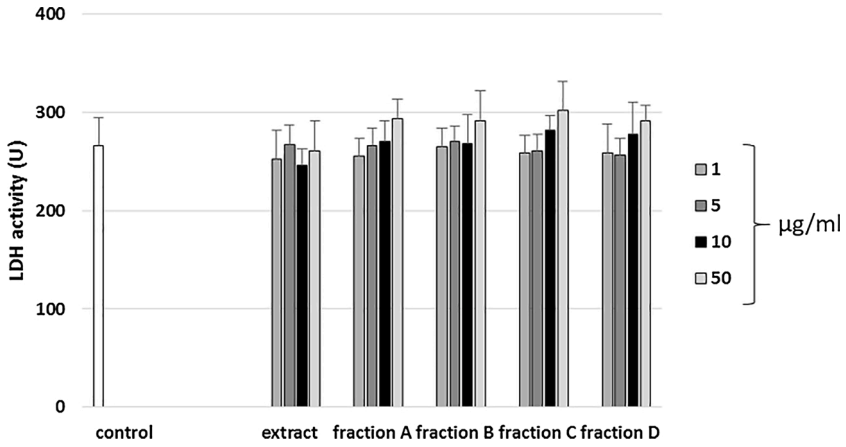


Fig. 7. Cytotoxic effects of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration range 1–50 µg/mL incubation time - 30 min.) on human blood platelets. Results are given as mean ± SD (n = 6).

various phenolic compounds; this potential may also be associated with the extract's triterpenoid content. For example, fraction D (10 µg/mL) lacked these compounds, and they only inhibited platelet activation in the case of three parameters: (1) enzymatic lipid peroxidation in platelets stimulated by thrombin; (2) adhesion of ADP-activated platelets to fibrinogen; and (3) adhesion of thrombin-activated platelets to fibrinogen. On the other hand, the extract at 10 µg/mL inhibited five parameters: (1) enzymatic lipid peroxidation in platelets stimulated by thrombin; (2) adhesion of ADP-activated platelets to fibrinogen; (3) adhesion of thrombin-activated platelets to fibrinogen; (4) adhesion of resting platelets to collagen; and (5) adhesion of thrombin-activated platelets to collagen. The observed anti-adhesion potential of the Paulownia Clone in Vitro 112 extract and fractions is in agreement with the inhibition of AA metabolism in platelets. However, the explanation

Table 2
Comparison of the anti-platelet activity of extract and four fractions from Paulownia CLON IN VITRO 112 leaves (A-D, concentration 10 μg/mL). positive effect – "+"; negative effect – "-".

	Extract	Fraction A	Fraction B	Fraction C	Fraction D
Enzymatic lipid peroxidation	+	+	+	+	+
Resting platelet adhesion to collagen	+	-	-	-	-
Adhesion of thrombin-activated platelets to collagen	+	+	+	+	-
Adhesion of thrombin-activated platelets to fibrinogen	+	+	+	+	+
Adhesion of ADP-activated platelets to fibrinogen	+	-	+	+	+
Platelet aggregation stimulated by thrombin	-	-	-	-	-
Platelet aggregation stimulated by ADP	-	-	-	-	-
Platelet aggregation stimulated by collagen	-	-	-	-	-

of the other mechanisms responsible for the inhibition of platelet activation seems to need further investigation.

The most important risk factor for CVD is uncontrolled platelet aggregation. However, the observed action of the Paulownia Clone in Vitro 112 extract and fractions on platelet aggregation, stimulated by various used agonists (thrombin, ADP, and collagen), was not statistically significant. It is possible that the extract and fractions, and the compounds they contain, do not interact with agonist receptors on the platelet membrane. Crucially, Paulownia Clone in Vitro 112 leaves are safe plant material, as neither the extract nor any of the four fractions isolated from them caused the lysis of platelets (as measured by LDH activity) at any of the tested doses (1−50 μg/mL).

In conclusion, our studies shows that leaves of Paulownia Clone in Vitro 112 indicate lack of cytotoxicity and are a new valuable source of compounds with antiplatelet potential. However, further experiments should be carried out to determine the in vitro effect of Paulownia Clon on the activity of various haemostatic elements, including platelets. Precise and detailed research is needed to gain a fuller understanding of these compounds, especially regarding their antiplatelet potential.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

This study was supported by a grant from the National Science Center, Poland (Grant No. 2016/23/B/NZ9/03427) and by the framework of the Ministry of Science and Higher Education, Poland, programme "Regional Initiative Excellence" in years 2019-2022, Project No. 005/RID/2018/19.

References

[1] B. Olas, Znaczenie erytrocytów w aktywacji płytek krwi, Vol. 14, Acta Universitatis Lodziensis Folia Biochemica Et Biophysica, 1999.
[2] S.R. Patel, J.H. Hartwig, J.E. Italiano Jr., The biogenesis of platelets from megakaryocyte proplatelets, J. Clin. Invest. 115 (2005) 3348–3354.
[3] G. Stachowiak, A. Zając, T. Pertyński, Hemostaza płytkowa w okresie menopauzy, Przegląd Menopauzalny 4 (2008) 205–209.
[4] T. Sonnweber, A. Pizzini, M. Nairz, G. Weiss, I. Tancevski, Arachidonic acid metabolites in cardiovascular and metabolic diseases, Int. J. Mol. Sci. 19 (2018) 3285.
[5] K.K. Wu, Platelet activation mechanisms and markers in arterial thrombosis, J. Intern. Med. 239 (1996) 17–34.
[6] A.R. Brash, Arachidonic acid as a bioactive molecule, J. Clin. Invest. 107 (2001) 1339–1345.
[7] V.S. Hanna, E.A.A. Hafez, Synopsis of arachidonic acid metabolism: a review, J. Adv. Res. 11 (2018) 23–32.
[8] L. Sautebin, D. Caruso, G. Galli, R. Paoletti, Preferential utilization of endogenous arachidonate by cyclooxygenase in incubations of human platelets, FEBS Lett. 157 (1983) 173–178.
[9] Y. Liu, Y. Zhang, K. Schmelzer, T.S. Lee, X. Fang, Y. Zhu, A.A. Spector, S. Gill, C. Morisseau, B.D. Hammock, J.Y.-J. Shyy, The antiinflammatory effect of laminar flow: the role of PPARgamma, epoxyeicosatrienoic acids, and soluble epoxide hydrolase, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 16747–16752.
[10] V.Y. Ng, Y. Huang, L.M. Reddy, J.R. Falck, E.T. Lin, D.L. Kroetz, Cytochrome P450 eicosanoids are activators of peroxisome proliferator-activated receptor alpha, Drug Metab. Dispos. 35 (2007) 1126–1134.
[11] B. Inceoglu, K.R. Schmelzer, C. Morisseau, S.L. Jinks, B.D. Hammock, Soluble epoxide hydrolase inhibition reveals novel biological functions of epoxyeicosatrienoic acids (EETs), Prostaglandins Other Lipid Mediat. 82 (2007) 42–49.
[12] F. Fan, Y. Muroya, R.J. Roman, Cytochrome P450 eicosanoids in hypertension and renal disease, Curr. Opin. Nephrol. Hypertens. 24 (2015) 37–46.
[13] A.A. Izzo, D.G. Deutsch, Unique pathway for anandamide synthesis and liver regeneration, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 6339–6340.
[14] B. Mukhopadhyay, R. Cinar, S. Yin, J. Liu, J. Tam, G. Godlewski, J. Harvey-White, I. Mordi, B.F. Cravatt, S. Lotersztajn, B. Gao, Q. Yuan, K. Schuebel, D. Goldman, G. Kunos, Hyperactivation of anandamide synthesis and regulation of cell-cycle progression via cannabinoid type 1 (CB1) receptors in the regenerating liver, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 6323–6328.
[15] N. Ueda, K. Tsuboi, T. Uyama, Metabolism of endocannabinoids and related N-acylethanolamines: canonical and alternative pathways, FEBS J. 280 (2013) 1874–1894.
[16] Y.K. Denisenko, E.G. Lobanova, T.P. Novgorodtseva, T.A. Gvozdenko, A. V. Nazarenko, The role of arachidonic acid metabolites (Endocannabinoids and eicosanoids) in the immune processes: a review, Int. J. Chem. Biomed. Sci. 1 (2015) 70–78.
[17] I. Drenjančević, I. Jukić, Z. Mihaljević, A. Ćosić, A. Kibel, The Metabolites of Arachidonic Acid in Microvascular Function. Microcirculation Revisited - From Molecules to Clinical Practice, IntechOpen, Rijeka, 2016.
[18] M. Balazy, C.D. Poff, Biological nitration of arachidonic acid, Curr. Vasc. Pharmacol. 2 (2004) 81–93.
[19] P. Kunapuli, J.A. Lawson, J.A. Rokach, J.L. Meinkoth, G.A. FitzGerald, Prostaglandin F2alpha (PGF2alpha) and the isoprostane, 8, 12-iso-isoprostane F2alpha-III, induce cardiomyocyte hypertrophy. Differential activation of downstream signaling pathways, J. Biol. Chem. 273 (1998) 22442–22452.
[20] A. Trostchansky, L. Bonilla, C.P. Thomas, V.B. O'Donnell, L.J. Marnett, R. Radi, H. Rubbo, Nitroarachidonic acid, a novel peroxidase inhibitor of prostaglandin endoperoxide H synthases 1 and 2, J. Biol. Chem. 286 (2011) 12891–12900.
[21] W. Adach, J. Zuchowski, B. Moniuszko-Szajwaj, M. Szumacher-Strabel, A. Stochmal, B. Olas, A. Cieslak, Comparative phytochemical, antioxidant, and hemostatic studies of extract and four fractions from Paulownia clone in Vitro 112 leaves in human plasma, Molecules 25 (2020) 1–14.
[22] D.C. Wood, J. Wood, Pharmacologic and biochemical considerations of dimethyl sulfoxide, Ann. N. Y. Acad. Sci. 243 (1975) 7–19.
[23] B. Walkowiak, A. Kesy, L. Michalec, Microplate leader – a conventient tool in studies of blood coagulation, Thromb. Res. 87 (1997) 95–103.
[24] B. Walkowiak, E. Michalak, W. Koziolkiewicz, C.S. Cierniewski, Rapid photometric method for estimation of platelet count in blood plasma or platelet suspension, Thromb. Res. 56 (1989) 763–766.
[25] B. Wachowicz, Adenine nucleotides in thrombocytes of birds, Cell Biochem. Funct. 2 (1984) 167–170.
[26] G. Bartosz, Druga Twarz Tlenu, Warszawa PWN, 2008, pp. 99–120.
[27] B. Olas, H.M. Żbikowska, B. Wachowicz, T. Krajewski, A. Buczynski, A. Magnuszewska, Inhibitory effect on resveratrol on free radical generation in blood platelets, Acta Biochim. Pol. 46 (1999) 991–996.
[28] G.V.R. Born, Aggregation of blood platelets by adenosine diphosphate and its reversal, Nature 194 (1962) 927–929.
[29] P. Bellavite, G. Andrioli, P. Guzzo, P. Arigliano, S. Chirumbolo, F. Manzato, C. Santonastaso, A colorimetric method for the measurement of platelet adhesion in microtiter plates, Anal. Bioch. 216 (1994) 444–450.
[30] F. Wroblewski, J.S. Ladue, Lactic dehydrogenase activity in blood, Proc. Soc. Exp. Biol. Med. 90 (1955) 210–213.
[31] K. Schneiderová, K. Šmejkal, Phytochemical profile of *Paulownia tomentosa* (Thunb), Steud. Phytochem Rev. 14 (2015) 799–833.

[32] T.W. Kim, K.M. Min, S.J. Yu, M.J. Lee, H.M. Jung, W.J. Cho, M.J. Kim, W. Chun, Y. Kwon, Chemical constituents of the twigs of *Paulownia coreana*, Kor. J. Pharmacogn. 46 (2015) 99–104.
[33] K. Alipieva, L. Korkina, I.E. Orhan, M.I. Georgiev, Verbascoside — a review of its occurrence, (bio)synthesis and pharmacological significance, Biotechnol. Adv. 32 (2014) 1065–1076.
[34] M. Pennacchio, Y.M. Syah, E. Alexander, E.L. Ghisalberti, Mechanism of action of verbascoside on the isolated rat heart: in-creases in level of prostacyclin, Phytother. Res. 13 (1999) 254–255.
[35] Z. Akdemir, C. Kahraman, I. Tatli, E.K. Akkol, I. Suntar, H. Keles, Bioassay-guided isolation of anti-inflammatory, antinociceptive and wound healer glycosides from the flowers of Verbascum mucronatum Lam, J. Ethnopharmacol. 14 (2011) 436–443.
[36] A. Ludwiczuk, K. Skalicka-Woźniak, M.I. Georgiev, Terpenoids, Pharmacogn. J. (2017) 233–266.
[37] M.C. Recio, R.M. Giner, S. Máñez, J.L. Ríos, Structural considerations on the iridoids as anti-inflammatory agents, Planta Med. 60 (1994) 232–234.
[38] M. Saleem, M. Nazir, M.S. Ali, H. Hussain, Y.S. Lee, N. Riaz, A. Jabbar, Antimicrobial natural products: an update on future antibioticdrug candidates, Nat. Prod. Rep. 27 (2010) 238–254.
[39] B.M. Tincusi, I.A. Jiménez, I.L. Bazzocchi, L.M. Moujir, Z.A. Mamani, J.P. Barroso, A.G. Ravelo, B.V. Hernández, Antimicrobial terpenoids from the oleoresin of the peruvian medicinal plant Copaifera paupera, Planta Med. 68 (2002) 808–812.
[40] T.P.T. Cushnie, A.J. Lamb, Antimicrobial activity of flavonoids, Int. J. Antimicrob. Agents 26 (2005) 343–356.
[41] W. Byłka, I. Matławska, N.A. Pilewski, Natural flavonoids as antimicrobial agents, JANA 7 (2) (2004) 24–31.
[42] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, Biological effects of essential oils – a review, Food Chem. Toxicol. 46 (2008) 446–475.
[43] A. Braca, I. Morelli, J. Mendez, L. Battinelli, L. Braghiroli, G. Mazzanti, Antimicrobial triterpenoids from Licania heteromorpha, Planta Med. 66 (2000) 768–769.