The Changes of Blood Platelet Reactivity in the Presence of Crude Extracts Isolated from Leaves and Twigs of *Elaeagnus Rhamnoides* (L.) A. Nelson Measuring in Whole Blood

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Research Article

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Abstract

Uncontrolled blood platelet activation is an important risk factor of cardiovascular disease (CVDs). Various studies on phenolic compounds indicate that they have a protective effect on the cardiovascular system through different mechanisms, including the reduction of blood platelet activation. One of the plants that is particularly rich in phenolic compounds is sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson). The aim of the present study *in vitro* was to determine the anti-platelet properties of crude extracts isolated from leaves and twigs of *E. rhamnoides* (L.) A. Nelson in whole blood using flow cytometric and total thrombus-formation analysis system (T-TAS). The aim of our study was also to analyze of blood platelet proteomes in the presence of different sea buckthorn extracts. A significant new finding is a decrease surface expression of P-selectin on blood platelets stimulated by 10 µM ADP and 10 µg/mL collagen, and a decrease surface expression of GPIIb/IIIa active complex on non-activated platelets and platelets stimulated by 10 µM ADP and 10 µg/mL collagen in the presence of sea buckthorn leaf extract (especially at the concentration 50 µg/mL). The twig extract also displayed antiplatelet potential. However, this activity was higher in the leaf extract than in the twig extract in whole blood. In addition, our present findings clearly demonstrate that investigated plant extracts have anticoagulant properties (measured by T-TAS). Therefore, the two tested extracts may be promising candidates for the natural anti-platelet and anticoagulant supplements.

Introduction

Cardiovascular disease (CVD) is a general term that includes disorders in the heart and/or blood vessel system. These diseases are the main cause of men's and women's death and has been hailed as civilization diseases. CVDs usually include a heart attack, stroke, pressure or atherosclerosis. Cardiovascular diseases are closely related to the hemostasis system, including hyperactivation of blood platelets. The treatment of cardiovascular diseases is extremely difficult, and the drugs (for example, anti-platelet drugs, including aspirin) currently available on the market, have many side effects [1].

Scientists from around the world are trying to find new anti-platelet drugs among plants that are a rich source of phenolic compounds. The mechanisms of anti-platelet activity of different compounds are mainly based on inhibition of cyclooxygenase activity, and thus, impairment of thromboxane A2 synthesis and blocking surface receptors for adhesion proteins, such as collagen or fibrinogen. Phenolic compounds can be used as a supplement or substitute for anti-platelet agents without causing side effects [2, 3]. One of the plants that is particularly rich in phenolic compounds is sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson). This plant is a shrub, but it can reach the size of a small tree. Its characteristic feature is the presence of thorns. It has gray-green lanceolate leaves and hedge-orange fruit-berries. Naturally, sea buckthorn is found in coastal environments. It has low soil requirements. Numerous *in vitro* and *in vivo* studies confirm the anti-tumor effect, anti-ulcer activity, hepatoprotective properties, as well as antibacterial and antiviral properties of this plant [4, 5]. In addition, sea buckthorn can be consumed in various products. Zhuo et al. [6] have observed that the powdered leaves have a positive effect on the atherosclerosis risk factors. Research performed by the team of Skalski et al. [7–9] shows that various organs of this plant have also antioxidant and anti-platelet effects (using washed blood platelets). For example, our earlier results demonstrated that phenolic fraction from sea buckthorn fruits has anti-platelet potential in whole blood. This fraction had also anticoagulant potential [10]. Moreover, this fraction had stronger anti-platelet potential than phenolic fraction from sea buckthorn leaves and twigs [10]. Its action may be associated with the presence of flavonol glycosides. Leaf and twig fractions did not have these compounds [10].

The aim of the present study *in vitro* was to determine the anti-platelet properties of crude extracts isolated from leaves and twigs of *Elaeagnus rhamnoides* (L.) A. Nelson in whole blood using flow cytometric and total thrombus-formation analysis system (T-TAS). The aim of our study was also to analyze of blood platelet proteomes (using gel electrophoresis) in the presence of different sea buckthorn extracts.

Materials And Methods

Chemicals

The PL microchips and other equipment needed for T-TAS device were acquired from Bionicum (Poland). All reagents necessary for flow cytometry methods were provided by commercial suppliers including Becton Dickinson (USA), Sigma-Aldrich (USA), Chrono-Log Corporation (USA) and BioCytex (France). The reagents needed for electrophoresis were purchased from BIO-RAD (Poland).

Plant material

Sea buckthorn was bred in horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (53°24'N, 23°30'E), the biggest Polish plantation of sea buckthorn. Sea buckthorn organs were transported to the Institute of Soil Science and Plant Cultivation – State Research Institute in Pulawy, Poland (IUNG/HRH/2015/2). All plant studies involved in the research were carried out in accordance with relevant institutional, national or international guideline.

Chemical characteristics of sea buckthorn extracts (twigs and leaves)

A stock of tested extracts was prepared using 50% DMSO. The final DMSO concentration in the tested samples was 0.05%. Two butanol extracts of leaves and twigs of sea buckthorn were tested. The chemical composition of the tested extracts was determined using ACQUITY UPLC™ system.
Both investigated extracts are a rich source of phenolic compounds. The total phenolic compound in leaf extracts was 341.5 mg/g of extract, in twig extracts 621.2 mg/g of extract [8, 11].

**Blood and blood platelets samples**

Fresh human blood was collected from healthy volunteers (non-smokers; not taking medication). Blood was drawn into a tube with CPDA₁ anticoagulant. Flow cytometry and T-TAS methods were performed on whole blood. Platelets were used for the electrophoretic method. The platelets were obtained by differential centrifugation, and suspended in Barber buffer. The final concentration of the tested extracts in the samples was 5 and 50 µg/mL.

Confirmation by human participants

All experiments were approved by the University of Lodz Committee for Research on Human Subjects and carried out under permission number 3/KBBN-UL/-II/2016.

We confirm that all experiments were performed in accordance with relevant guidelines and regulations. All donors were informed about the purpose of the study and gave their informed consent to participate.

**Flow cytometry analysis**

Whole blood was incubated with extracts and with platelet agonists: ADP at final concentration 10 and 20 µM and collagen final concentration 10 µg/mL. The samples were diluted in PBS. Antibodies (CD61/PerCP, CD62/PE and PAC-1/FITC) were added to the tests. Cells were fixed in cell-fix. The obtained results were analyzed using the FACSDiva program [10, 12, 13].

**Platelet VASP phosphorylation**

PLT VASP/P2Y12 test was performed according to the instructions provided by the manufacturer (BioCytex). Platelet response was expressed as platelet reactivity index (PRI).

**Polyacrylamide gel electrophoresis analysis**

Platelet lysis was performed using lysis buffer (0.12 M Tris/HCl; 4% SDS; 20% glycerol). Electrophoresis was carried out in two systems: under reducing conditions (+ β-mercaptoethanol) and under non-reducing conditions (- β-mercaptoethanol). Electrophoresis was carried out at 500 mA and 120V. Proteins were separated using 4-20% Tris-HCl gel [14].

**Total Thrombus-formation Analysis System (T-TAS)**

The analysis of plate plug formation was performed using the Total Thrombus-formation Analysis System (T-TAS). The measurement was carried out through a real-time hydrodynamic model under blood flow conditions. Whole blood was incubated with the tested extracts (30 min, 37°C). Subsequently, blood was applied to the PL chip and the time of clot appearance was measured. Area Under the Curve (AUC₁₀) was used as the measurement parameter [15].

**Data analysis**

The results were presented as the mean +/- SD. Statistical analysis was performed with one-way ANOVA for repeated measurements using Statistica program (Statistica 13.1).

**Results**

Based on flow cytometry technique, our results demonstrated changes both basal and agonist (collagen and ADP) – treated platelet activation states in samples treated with tested sea buckthorn extracts, compared to control samples (without plant extracts) (Fig. 1 and 2), but these changes were not always statistically significant. For example, the extract from sea buckthorn leaves (at two used concentrations: 5 and 50 µg/mL) statistically significantly reduced the expression of CD62P on platelets activated by 10 µM ADP and 10 µg/mL collagen (Fig. 1). We observed the same process for only 50 µg/mL extract from sea buckthorn twigs (Fig. 1). The used extracts from sea buckthorn leaves and twigs also changed PAC-1 binding in non-stimulated platelets and platelets activated by agonist (Fig. 2). For example, the extracts from sea buckthorn leaves and twigs (at two used concentrations: 5 and 50 µg/mL) statistically significant reduced PAC-1 binding in platelets activated by 10 µg/mL collagen (Fig. 2).

Two of the tested plant extracts markedly decreased AUC₁₀ relative to control when administered at 50 µM/mL (for example, AUC₁₀ for control = 295.9, for leaf extract = 173.6, for twig extract = 205.6) (Fig. 3). On the other hand, no differences were observed between the PRI values of the samples treated with 50 µg/mL of the plant extracts and the control samples (Fig. 4). Nor were any differences observed between the electropherograms of blood platelets treated with plant extracts and the control samples (data not presented).

A comparison of the effects of the two tested plant extracts at the highest used concentration (50 µg/mL) on selected parameters of blood platelet activation, as measured by cytometric analysis, is demonstrated in Table 1. We observed that the extract from leaves had stronger anti-platelet
potential than the extract from twigs. For example, the extract from leaves inhibited PAC-1 expression in three used models: (1) non-activated platelets, (2) platelets activated by 10 µM ADP, (3) platelets activated by 10 µg/mL collagen. Moreover, this extract reduced the expression of CD62P on platelets in two used models: (1) platelets activated by 10 µM ADP and (2) platelets activated by 10 µg/mL collagen. In addition, the two tested extracts demonstrated anti-coagulant potential (measured by T-TAS) (Table 1).

A comparison of the effects of the extracts isolated from various organs of sea buckthorn (tested at 50 µg/mL) on biomarkers of platelet activation measured by cytometric analysis and T-TAS.

<table>
<thead>
<tr>
<th>Extract</th>
<th>CD62P expression</th>
<th>PAC-1 expression</th>
<th>T-TAS</th>
<th>VASP phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-activated platelets</td>
<td>Platelets activated by 10 µM ADP</td>
<td>Platelets activated by 20 µg/mL collagen</td>
<td>Non-activated platelets</td>
</tr>
<tr>
<td>Leaves</td>
<td>No effect</td>
<td>Decrease (anti-platelet potential)</td>
<td>No effect</td>
<td>Decrease (anti-platelet potential)</td>
</tr>
<tr>
<td>Twigs</td>
<td>No effect</td>
<td>No effect</td>
<td>Decrease (anti-platelet potential)</td>
<td>No effect</td>
</tr>
</tbody>
</table>

**Discussion**

Uncontrolled blood platelet activation is an important risk factor of CVDs. Various studies on phenolic compounds indicate that they have a protective effect on the cardiovascular system through different mechanisms, including the reduction of oxidative stress, inflammation, and blood platelet activation [7, 8]. Our earlier results show that the preparations (including extracts and fractions with various chemical contents) obtained from sea buckthorn organs have a high content of phenolic compounds (for example, total phenolic compounds: 341.5 mg/g (for leaf extract) and 621.2 mg/g (for twig extract) [7, 11]). In contrast to the crude extracts from sea buckthorn leaves and twigs, the phenolic fractions obtained from these extracts did not contain oleanolic and ursolic acid, and had a lower content of other triterpenoids (compounds containing 4, 5, and 6 hydroxyl groups), and the remaining less polar compounds. For this reason, e.g., the phenolic fraction of sea buckthorn leaves had a lower content of saponins than the original extract.

Moreover, our earlier results [7] demonstrated that sea buckthorn leaf and twig extracts have antioxidant properties. They inhibited lipid peroxidation and protein carbonylation in human plasma treated with strong oxidants *in vitro*. In addition, these extracts had anticoagulant potential (measured by coagulometry method using human plasma *in vitro*). However, anti-platelet properties were only observed in washed blood platelets and platelet-rich plasma (*in vitro* models) [8]. As mechanisms of anti-platelet action of sea buckthorn extracts (isolated from leaves and twigs) in whole blood are not known, this is the first study to investigate their anti-platelet effect in this experimental setting. In the present work, for the first time, we studied the effect of sea buckthorn extracts on blood platelet activation in their natural environment, which is the whole blood collection. Changes in blood platelet activation in the presence of the two tested plant extracts were analyzed by flow cytometry (measuring expression of two markers on the surface of blood platelets: P-selectin and GP IIb/IIIa (the main receptor for fibrinogen)). After platelet activation the number of GP IIb/IIIa copies grows and the receptors change their conformation. P-selectin is a glycoprotein present in alpha-granules of resting blood platelets. However, during platelet activation, P-selectin appears on their surface and it is the main biomarker of platelet activation. A significant new finding is a decrease in surface expression of P-selectin on platelets stimulated by 10 µM ADP and 10 µg/mL collagen, and a decrease in surface expression of GP IIb/IIIa active complex on non-activated platelets and platelets stimulated by 10 µM ADP and 10 µg/mL collagen in the presence of sea buckthorn leaf extract (especially at the concentration 50 µg/mL) (Tab. 1). The twig extract also displayed antiplatelet potential. However, the leaf extract showed a stronger such activity than the twig extract in whole blood. For example, the extract from leaves inhibited platelet activation (measured by flow cytometry) in five used models.

Blood clot formation treated with tested crude extracts was analyzed in a real-time hydrodynamic blood flow model (by T-TAS). T-TAS is new technique for visualizing blood platelet thrombus formation on surfaces coated with collagen (PL-chips) under the high shear. Analysis of thrombus formation using T-TAS showed that the two tested plant extracts significantly depress thrombus formation. In addition, our present findings clearly demonstrate that the investigated plant extracts have anticoagulant properties (measured by T-TAS) and these properties may be helpful in reducing prothrombotic states (Tab. 1). We demonstrated that the AUC10 with the PL-chip was especially depressed by the extract from leaves compared to control.

Different antithrombotic agents are clinically used to inhibit the cascade of venous or arterial thrombosis in cardiovascular diseases. For example, anti-platelet therapy with aspirin or P2Y12 inhibitors often use in prevention and treatment of CVDs. However, our present results show that
incubation of whole blood with tested plant extracts do not change VASP phosphorylation index. Therefore, we suppose that the mechanism of anti-platelet action of sea buckthorn leaf extract and twig extract, is not associated with the platelet surface membrane P2Y12 receptor.

The differences in inhibition of platelet activation induced by the two tested plant extracts may be associated with differences in their phenolic compound content. The UHPLC-MS analysis showed that ellagitannins were identified as principal phenolic constituents of the sea buckthorn leaf extract. In this extract, flavonoids (including glycosides of isorhamnetin, kaempferol and quercetin) were also present. In contrast, the sea buckthorn twig extract consisted mainly of proanthocyanidins and catechin [7]. Possibly, the greater potency of leaf extract may be associated with its high concentration of flavonoids compared to the twig extract.

The bioavailability of phenolic compounds is an important factor in the evaluation of their biological activities, including anti-platelet potential in vivo. For example, the concentration of phenolic compounds, including flavonoids present in sea buckthorn leaf extract (5 and 50 µg/mL) can be achieved in whole blood during supplementation with this extract. An important aspect of the use of natural compounds is not only their bioavailability, but also their toxicity. However, our earlier results showed that the concentrations (0.5 – 50 µg/mL) tested in this experiment demonstrate non-cytotoxic effect, and they did not induce damage to human platelets [8]. Results of Gupta et al. [16] also indicate no cytotoxicity.

Our present experiments shed a new light on the mechanisms of anti-platelet action of crude extracts from sea buckthorn leaves and twigs in whole blood. For example, three-color flow cytometry have demonstrated an altered level of GPIIb/IIIa receptors and P-selectin on blood platelets treated with these extracts. Changes of GPIIb/IIIa and P-selectin may be associated with the inhibition of platelet activation and anti-platelet potential of these crude extracts. Moreover, we suggest that especially sea buckthorn leaf extract may be a promising source of bioactive substances that can be used in the prevention and treatment CVDs associated with hyperactivity of blood platelets. In addition, the two tested extracts may be promising candidates for the natural anticoagulant supplements.

**Declarations**

**Acknowledgements**

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**Conflict of Interest**

The authors declare that there are no conflicts of interest.

**References**


**Figures**

**Figure 1**

Effects of different plant extracts (5 and 50 µg/mL; 30 min) on expression of the active form of GPIIb/IIIa on resting or agonist – stimulated blood platelets: 10 µM ADP, 20 µM ADP and 10 µg/mL collagen in whole blood samples. The blood platelets were distinguished based on the expression of CD61. For each samples, 5000 CD61-positive objects (blood platelets) were acquired. For the assessment of GPIIb/IIIa expression, samples were labeled with fluorescently conjugated monoclonal antibody PAC-1/FITC. Results are shown as the percentage of platelets binding PAC-1/FITC. Data represent mean ± SE of 6 healthy volunteers (each experiment performed in triplicate). *p<0.05 (vs. control platelets)

![Figure 1](image1.png)

**Figure 2**

Effects of different plant extracts (5 and 50 µg/mL; 30 min) on expression of P-selectin on resting or agonist – stimulated blood platelets: 10 µM ADP, 20 µM ADP and 10 µg/mL collagen in whole blood samples. The blood platelets were distinguished based on the expression of CD61/PerCP. For each samples, 5000 CD61-positive objects (blood platelets) were acquired. For the assessment of P-selectin expression, samples were labeled with fluorescently conjugated monoclonal antibody CD62P. Results are shown as the percentage of platelets expressing CD62P. Data represent mean ± SE of 6 healthy volunteers (each experiment performed in triplicate). *p<0.05 (vs. control platelets)

![Figure 2](image2.png)
Figure 3

Effects of different plant extracts (50 µg/mL; 30 min; 37°C) on the T-TAS using the PL-chip in whole blood samples. Whole blood samples were analyzed by the T-TAS at the shear rates of 1000 s⁻¹ on the PL-chips. Area under the curve (AUC₁₀) in PL are shown as closed circles. Figure 3 demonstrates selected diagram for the two tested plant extracts.

Figure 4

Effects of different plant extracts (50 µg/mL; 30 min; 37°C) on VASP phosphorylation in ADP – activated blood platelets. Data represent mean ± SD of 6 healthy volunteers (each experiment performed in triplicate).