Food and Chemical Toxicology 125 (2019) 614–620

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Isorhamnetin and its new derivatives isolated from sea buckthorn berries prevent H_2O_2/Fe – Induced oxidative stress and changes in hemostasis

Bartosz Skalski[a], Bernadetta Lis[a], Łukasz Pecio[b], Bogdan Kontek[a], Beata Olas[a,*], Jerzy Żuchowski[b], Anna Stochmal[b]

[a] Department of General Biochemistry, Faculty of Biology and Environmental Protection, University of Łódź, 90-236, Łódź, Poland
[b] Department of Biochemistry, Institute of Soil Science and Plant Cultivation, State Research Institute, 24-100, Puławy, Poland

ARTICLE INFO

Keywords:
Isorhamnetin
Oxidative stress
Elaeagnus rhamnoides (L.) A. Nelson
Sea buckthorn
Phenolic compounds
Hemostasis

ABSTRACT

The objective of this study is to investigate the biological effects of phenolic compounds extracted from the sea buckthorn berries on oxidative stress and hemostasis. The sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) berries are rich in flavonoids and non-polar compounds. In this study, the activity of the phenolic fraction from the sea buckthorn berries was evaluated *in vitro* in comparison with three phenolic compounds: isorhamnetin (compound 1) and its two new derivatives: compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) and compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3'''-isovaleryl)-rhamnoside). The impact of these phenolic compounds and the phenolic fraction against the effect of the donor of hydroxyl radicals - H_2O_2/Fe on proteins and lipids in human plasma was measured. Additionally, the aim of the study was to determine the effect of these phenolic compounds and the phenolic fraction on various typical hemostasis parameters. Our results show that the used derivatives of isorhamnetin possess different biological properties (e.g. antioxidant, anti-platelet and anticoagulant). The tested compounds can be seen as new natural beneficial compounds to be used in prevention and treatment of cardiovascular diseases.

1. Introduction

For a long time, herbal remedies have been used as therapeutics due to their relatively fewer side effects, however, the knowledge on their biological effects is yet limited. Various organs of *Elaeagnus rhamnoides* (L.) A. Nelson (sea buckthorn), including berries, are a rich source of bioactive compounds, including phenolic compounds, which are beneficial for human health (Malinowska and Olas, 2016; Olas, 2016 and 2018a and b). Nevertheless, the effect of different organs of sea buckthorn on components of hemostasis (e.g. plasma, blood platelets or other blood cells), is not yet clear. Moreover, most of the studies have been carried out using different kinds of extracts or fractions from the sea buckthorn. Their composition has not always been determined either, and their bioactive constituents have not been identified, including in the manuscripts by Michel et al. (2012) and Chen et al. (2013). Our earlier results demonstrated that the phenolic fraction (rich in non-acylated and acylated flavonols and non-polar compounds) from the sea buckthorn berries acts as an antioxidant and anticoagulant (Olas et al., 2018). In addition, using LC-MS analysis we were able to observe that various glycosides of isorhamnetin and quercetin, including compounds acylated with an unidentified aliphatic acid, were the dominant constituents of this fraction (Olas et al., 2018).

Various berries, including the sea buckthorn berries, are potent candidates for functional food products especially bearing in mind the fact that epidemiological studies indicate that diets rich in berry phenolic antioxidants may reduce the oxidative stress (Olas, 2018b). In addition, berries (in different forms: fresh, juice or medicinal product) may play an important role in the modulation of hemostasis, in particular the various steps of blood platelet activation, including blood platelet aggregation (Olas, 2017). In this study, we focused on biological actions of two isorhamnetin derivatives: compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) and compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3'''-isovaleryl)-rhamnoside), which were isolated from the phenolic fraction from the sea buckthorn berries (Zuchowski et al. (sub.)) (Fig. 1). The action of isorhamnetin derivatives was compared to biological effects of isorhamnetin - compound 1, which, among others, protects human retinal pigment epithelium cells from oxidative stress induced by H_2O_2 (Wang et al., 2018), and the phenolic fraction, which was used to isolate the tested phenolic compounds. Biological properties were studied *in vitro* using various tests (based on human plasma and human blood platelets) to observe its protective properties against oxidative damage to protein

* Corresponding author.
E-mail address: beata.olas@biol.uni.lodz.pl (B. Olas).

https://doi.org/10.1016/j.fct.2019.02.014
Received 11 December 2018; Received in revised form 18 January 2019; Accepted 5 February 2019
Available online 06 February 2019

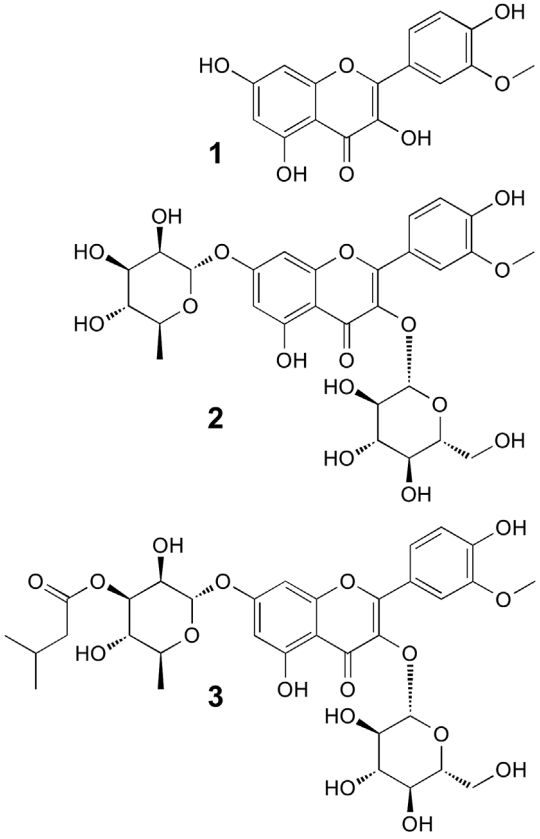

Fig. 1. Chemical structure of isorhamnetin (compound 1) and its derivatives: compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) and compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3‴-isovaleryl)-rhamnoside) isolated from the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries.

and lipid components of plasma, as well as their influence on the hemostasis: selected parameters of coagulation - the activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT); and one of the steps of blood platelet activation – platelet aggregation stimulated by various physiological agonists (ADP, collagen and thrombin), because oxidative stress is involved in pathogenesis of numerous diseases, including cardiovascular disorders, which, in addition, are very often associated with changes in hemostasis. Moreover, the objective was also to investigate the value of isorhamnetin, its derivatives and the examined phenolic fraction as potential functional food ingredients, since no undesired or toxic effects (including hematological or chemical effects) have been associated with the consumption of various berries (e.g. sea buckthorn berries) or their products (Olas, 2017 and 2018b).

2. Material and methods

2.1. Chemicals

Dimethylsulfoxide (DMSO), thiobarbituric acid (TBA), thrombin, formic acid (LC-MS grade), isorhamnetin, and H_2O_2 have been purchased from Sigma (St. Louis, MO., USA). Methanol (isocratic grade) and acetonitrile (LC-MS grade) have been acquired from Merck (Darmstadt, Germany). Other reagents represented analytical grade and were provided by commercial suppliers, including POCh, (Poland), Acros (Poland), and Chempur (Poland). Adenosine-5′-diphosphate (ADP) and collagen have been purchased from Chrono-Log (USA).

Derivatives of isorhamnetin: compound 2 and 3 were isolated from the phenolic fraction from sea buckthorn berries as described earlier (Zuchowski et al. (sub.)).

2.2. Plant material

The sea buckthorn (*E. rhamnoides* (L.) A. Nelson) berries have been obtained from a horticultural farm in Sokółka, Podlaskie Voivodeship, Poland (53°24′N, 23°30′E). The fruits were ground frozen in a meat grinder, lyophilized (Gamma 2–16 LSC, Christ, Osterode am Harz, Germany), and stored in a refrigerator.

2.3. Preparation and quantification of the phenolic fraction

The phenolic fraction of the sea buckthorn berries was prepared as described above (Olas et al., 2018). Various glycosides of isorhamnetin and quercetin, including compounds acylated with an unidentified aliphatic acid, were the dominant constituents of this fraction. Moreover, the preparation also contained small amounts of putative triterpenes and acylated triterpenes (Olas et al., 2018).

2.4. Stock solutions of tested compounds and plant fraction

Stock solutions of the *E. rhamnoides* (L.) A. Nelson phenolic fractions, isorhamnetin and its derivatives were made in 50% DMSO. The final concentration of DMSO in samples was lower than 0.05% and its effects were determined in all experiments.

2.5. Blood samples

Fresh human blood or plasma have been obtained from regular, medication-free donors from the Regional Center for Transfusion Medicine in Lodz (Poland) as well as peripheral blood from non-smoking men and women (collected into CPD solution (citrate/phosphate/dextrose; 9:1; v/v blood/CPD) or CPDA solution (citrate/phosphate/dextrose/adenine; 8:5:1; v/v; blood/CPDA)). All samples were drawn in the morning from fasting donors. Donors had not taken any medications or addictive substances (including alcohol, tobacco, antioxidant supplementation, aspirin or any other anti-platelet drugs) prior to the blood collection. Our analysis of the blood samples was performed under the guidelines of the Helsinki Declaration for Human Research. Moreover, the protocol was approved by the Committee for Research on Human Subjects of the University of Lodz number 3/KBBN-UŁ/II/2016.

2.6. Isolation of plasma and blood platelets

Human platelet-rich plasma (PRP) and blood platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustron (1992). The platelet pellet was washed with modified Tyrode's buffer (pH 7.4) twice; afterwards, the platelets were suspended in the same buffer. The concentration of platelets in suspensions (used in the experiments), estimated spectrophotometrically (Walkowiak et al., 1989), amounted to 2.5–3 × 10^8/mL. Suspensions of blood platelets or plasma were incubated (30 or 60 min, at 37 °C) with:

- *E. rhamnoides* (L.) A. Nelson fraction, isorhamnetin and its derivatives at the final concentrations of 5 and 10 μg/mL
- *E. rhamnoides* (L.) A. Nelson fraction, isorhamnetin and its derivatives at the final concentrations of 5 and 10 μg/mL plus 4.7 mM H_2O_2/3.8 mM Fe_2SO_4/2.5 mM EDTA.

The protein concentration was calculated according to the procedure devised by Whitaker and Granum (1980), on the basis of absorbance measurements at 280 nm (in tested samples).

2.7. Markers of oxidative stress

2.7.1. Lipid peroxidation measurement

Lipid peroxidation was quantified by measuring the concentration

of TBARS. Incubation of plasma (control, plant fraction/tested compound and H_2O_2/Fe-treated plasma) was stopped by cooling the samples in an ice bath. Samples of plasma were transferred to an equal volume of cold 15% (v/v) trichloroacetic acid in 0.25 M HCl and 0.37% thiobarbituric acid in 0.25 M HCl, immersed in a boiling water bath for 10 min, and then centrifugated at 10,000 × *g* for 15 min, 18 °C. Absorbance was measured at 535 nm (the SPECTROstar Nano Microplate Reader - BMG LABTECH Germany) (Wachowicz, 1984; Bartosz, 2008). The TBARS concentration was calculated using the molar extinction coefficient (ε = 156,000 $M^{-1}cm^{-1}$).

2.7.2. The carbonyl group measurement

The detection of carbonyl groups in proteins was carried out according to Levine et al. (1990) and Bartosz (2008). The carbonyl group concentration was calculated using a molar extinction coefficient (ε = 22,000 $M^{-1}cm^{-1}$), and the level of carbonyl groups was expressed as nmol carbonyl groups/mg of protein. Carbonyl content was determined using the SPECTROstar Nano Microplate Reader- BMG LABTECH Germany.

2.7.3. The thiol group determination

The thiol group content was measured spectrophotometrically (the SPECTROstar Nano Microplate Reader- BMG LABTECH Germany) by absorbance at 412 nm with Ellman's reagent: 5,5′-dithio-bis-(2-nitrobenzoic acid). The thiol group concentration was calculated using a molar extinction coefficient (ε = 13,600 $M^{-1}cm^{-1}$) (Ando and Steiner, 1973a and b; Bartosz, 2008). The level of thiol groups was expressed as nmol thiol groups/ml of plasma.

2.8. Parameters of hemostasis

2.8.1. The measurement of prothrombin time

Human plasma (50 μL) was added to measuring cuvette and incubated for 2 min at 37 °C on a block heater. The measuring cuvette was transferred to the measuring holes and 100 μL of Dia-PT liquid (commercial thromboplastin) was added. The PT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) (Malinowska et al., 2012).

2.8.2. The measurement of thrombin time

Human plasma (50 μL) was added to a measuring cuvette and incubated for 1 min at 37 °C on a block heater. The measuring cuvette was transferred to the measuring holes and 100 μL of thrombin was added (final concentration - 5 U/mL). The TT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) (Malinowska et al., 2012).

2.8.3. The measurement of APTT

Human plasma (50 μL) was added to a measuring cuvette and incubated with 50 μL of Dia-PTT liquid (commercial preparation) for 3 min at 37 °C on block heater. The measuring cuvette was transferred to the measuring holes and 50 μL of 25 mM $CaCl_2$ was added. The APTT was determined coagulometrically (Optic Coagulation Analyser model K-3002; Kselmed, Grudziadz, Poland) (Malinowska et al., 2012).

2.8.4. The measurement of blood platelet aggregation

Platelet aggregation was measured turbidimetrically in PRP or blood platelets in Tyrode's buffer using the optical Chrono-Log aggregometer (Chrono-Log, Havertown, PA, USA).

After the pre-incubation procedure for the PRP samples, ADP (10 μM) or collagen (2 μg/mL) were added and blood platelet aggregation measured for 10 min. The aggregometer was calibrated each time (100% aggregation) on blood platelet poor plasma (PPP).

After the pre-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 each time (100% aggregation) on Tyrode's buffer.

2.9. Data analysis

Statistical analysis was carried out using several tests. In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as a mean value ± SEM (n = 3–12 independent experiments). The statistically significant differences were assessed by applying the paired Student's *t*-test or by applying one-way ANOVA test followed by a multicomparison Tukey's test; and the significance level was $p < 0.05$.

3. Results

3.1. Effects of phenolic compounds/phenolic fraction from E. rhamnoides (L.) A. Nelson berries on oxidative stress markers in human plasma

In comparison to the control (control (neg) – untreated) samples, H_2O_2/Fe stimulated plasma exhibited a markedly raised concentration of TBARS (the marker of lipid peroxidation) and carbonyl groups in plasma proteins (the marker of protein oxidation), as well as a decrease in thiol groups in plasma proteins (the marker of protein oxidation) (Fig. 2). In the presence of isorhamnetin and its derivatives: compound 2 and 3 (at concentrations: 5 and 10 μg/mL), plasma lipid peroxidation induced by H_2O_2/Fe was significantly reduced (Fig. 2A). All used phenolic compounds effectively reduced plasma lipid peroxidation by about 30% at 10 μg/mL (for isorhamnetin and compound 3) and about 40% at 10 μg/mL (for compound 2) (Fig. 2A, Table 1). In this model, compound 2 (at the highest used concentration – 10 μg/mL) had stronger antioxidant properties than isorhamnetin and compound 3 (Fig. 2A, Table 1). However, the phenolic fraction from *E. rhamnoides* (L.) A. Nelson berries (at both tested concentrations: 5 and 10 μg/mL) did not change the level of plasma lipid peroxidation induced by H_2O_2/Fe (Fig. 2A).

Compound 2 and 3, and the phenolic fraction from *E. rhamnoides* (L.) A. Nelson berries (at both tested concentrations: 5 and 10 μg/mL) were found to protect the plasma against H_2O_2/Fe – induced thiol group oxidation in proteins, however, isorhamnetin (5 and 10 μg/mL) had no effect on this process (Fig. 2B, Table 1). In the other experiment, all tested compounds (isorhamnetin and its derivatives) and the phenolic fraction from *E. rhamnoides* (L.) A. Nelson berries reduced the protein carbonylation induced by H_2O_2/Fe by about 25% at 5 μg/mL and about 30% at 10 μg/mL (Fig. 2C, Table 1).

3.2. Effects of phenolic compounds/phenolic fraction from E. rhamnoides (L.) A. Nelson berries on hemostasis

Analysis of the effect of the tested phenolic compounds from *E. rhamnoides* (L.) A. Nelson berries (at concentrations: 5 and 10 μg/mL) on the coagulation activity of human plasma showed that only compound 3 significantly prolonged the thrombin time. Isorhamnetin and compound 2 did not change the thrombin time (Fig. 3A, Table 1). Moreover, in the presence of compound 3, the action was stronger than with isorhamnetin and compound 2 (Fig. 3A, Table 1). On the other hand, all tested phenolic compounds did not change the prothrombin time and the activated partial thromboplastin time (Fig. 3B and C). In addition, tested compound 3 (at concentrations – 5 and 10 μg/mL) did not prolong the thrombin time of human plasma, when the mixture of tested compound and thrombin (earlier pre-incubated thrombin with compound 3, and then added to the plasma) was used (Fig. 4).

In our measurements, we found that all the tested compounds (at the concentration – 10 μg/mL) did not change blood platelet aggregation stimulated by ADP or collagen (Fig. 5). On the other hand, isorhamnetin and compound 3 inhibit this process induced by thrombin (inhibition of blood platelet aggregation stimulated by thrombin was about 25%) (Fig. 5, Table 1).

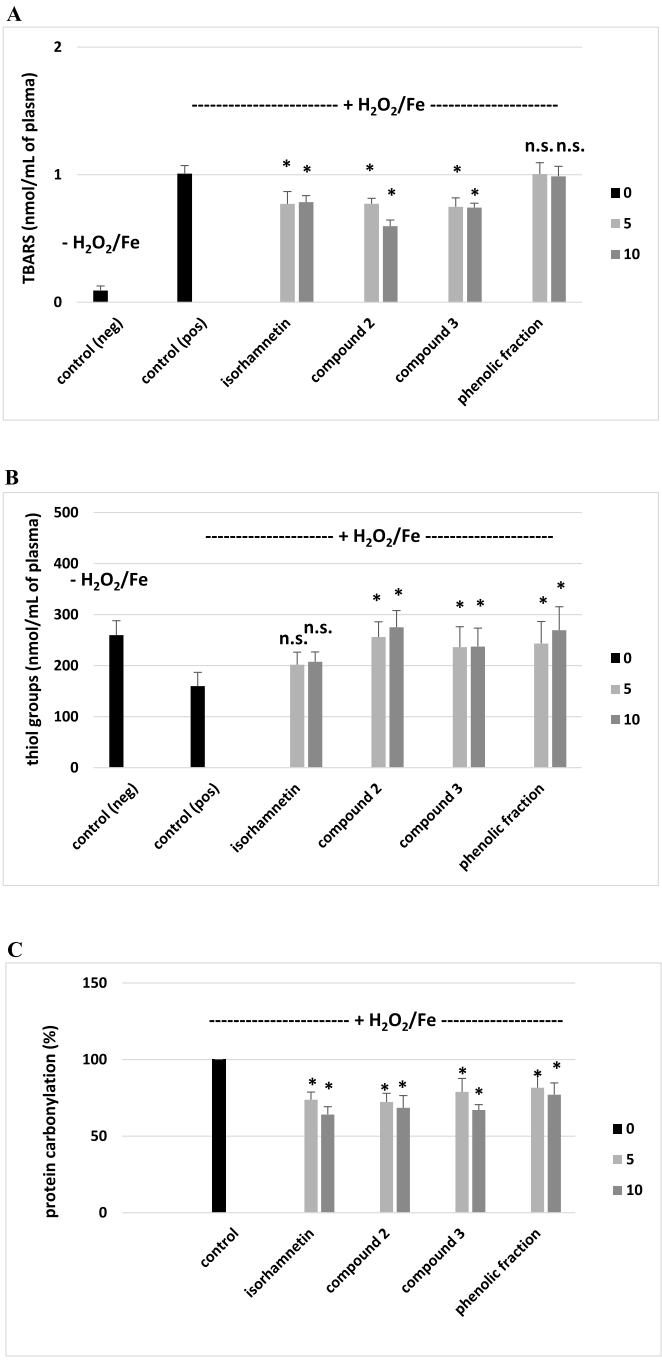

Fig. 2. Effects of isorhamnetin, its derivatives and the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries (5 and 10 μg/mL; 60 min) on plasma lipid peroxidation induced by H_2O_2/Fe (A), on the level of protein thiol groups in plasma treated with H_2O_2/Fe (B) and on plasma protein carbonylation induced by H_2O_2/Fe (C; in these experiments, the level of carbonyl groups in control samples: for plasma not treated with H_2O_2/Fe was 25.9 ± 4.9 nmol/mg of plasma proteins, and for plasma treated with H_2O_2/Fe was 61.4 ± 12.4 nmol/mg of plasma proteins and was expressed as 100% of protein carbonylation). Data represent means ± SEM of 5–12 independent experiments. Control negative (neg) refers to plasma not treated with H_2O_2/Fe, whereas control positive (pos) to plasma treated with H_2O_2/Fe ($p < 0.01$). *$p < 0.05$ (*vs.* control (pos)), n.s. – $p > 0.05$ (*vs.* control (pos)).

Table 1 demonstrated comparative effects of isorhamnetin and its derivatives (at the highest used concentration – 10 μg/mL) on selected parameters of oxidative stress and hemostasis. For example, compound 3 had stronger anti-coagulant and anti-aggregatory properties than

Table 1
Comparative effect of isorhamnetin and its derivatives (at the highest concentration - 10 μg/mL) on selected parameters of oxidative stress and hemostasis. Data represent means ± SE of 3–12 independent experiments.

Tested phenolic compound	Parameters of oxidative stress	Parameters of hemostasis			
	Inhibition of lipid peroxidation induced by H_2O_2/Fe (%)	Inhibition of protein carbonylation induced by H_2O_2/Fe (%)	The level of protein thiol groups (nmol/ml of plasma) in plasma treated with H_2O_2/Fe	Prolongation of TT (%) (Compound was incubated with plasma for 30 min and then thrombin was added)	Prolongation of TT (%) (The solution of thrombin was incubated for 30 min with tested fraction and then the mixture was added to the human plasma)	Inhibition of blood platelet aggregation induced by thrombin (%)
isorhamnetin (a)	29.7 ± 5.3 ($p > 0.05$; a *vs.* c; $p < 0.05$; a *vs.* b)	36.1 ± 5.1 ($p > 0.05$; a *vs.* b, c)	201.8 ± 24.6 ($p > 0.05$; a *vs.* c; $p < 0.05$; a *vs.* b)	3.5 ± 2.8 ($p < 0.05$; a *vs.* b; $p < 0.01$; a *vs.* c)	-	27.7 ± 4.9 ($p > 0.05$; a *vs.* b, c)
	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	No effect *vs.* control	No effect *vs.* control		Positive effect (anti-aggregatory action) *vs.* control
compound 2 (b)	40.6 ± 4.0 ($p < 0.05$; b *vs.* a, c)	37.6 ± 8.0 ($p > 0.05$; b *vs.* a. c)	266.1 ± 29.8 ($p < 0.05$; b *vs.* a, c)	10.3 ± 5.9 ($p < 0.05$; b *vs.* a, c)	-	17.1 ± 5.8 ($p > 0.05$; a *vs.* b, c)
	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	No effect *vs.* control		No effect *vs.* control
compound 3 (c)	29.6 ± 6.4 ($p > 0.05$; c *vs.* a; $p < 0.05$; c *vs.* b)	31.0 ± 3.5 ($p > 0.05$; c *vs.* a, b)	236.2 ± 40.0 ($p > 0.05$; c *vs.* a; $p < 0.05$; c *vs.* b)	24.2 ± 4.4 ($p < 0.01$; c *vs.* a; $p < 0.05$; c *vs.* b)	No effect *vs.* control	26.5 ± 5.6 ($p > 0.05$; a *vs.* b, c)
	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (antioxidant action) *vs.* control (plasma treated with H_2O_2/Fe)	Positive effect (anticoagulant action) *vs.* control		Positive effect (anti-aggregatory action) *vs.* control

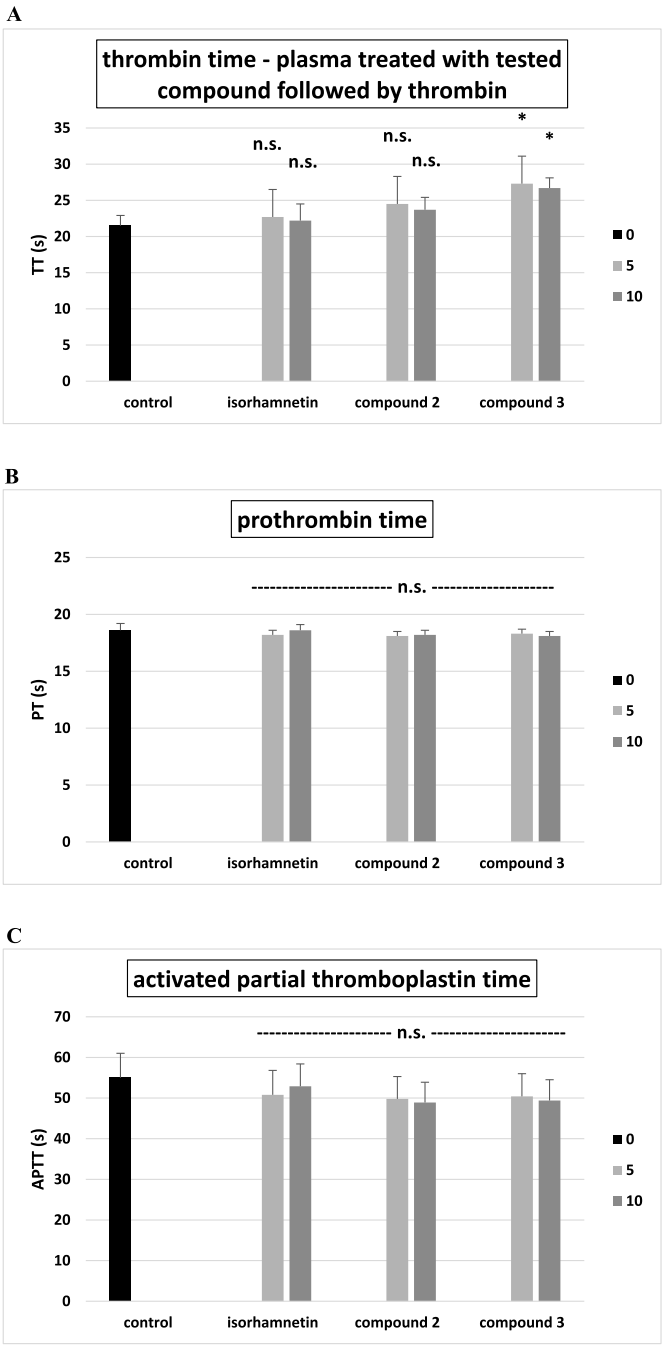

Fig. 3. Effects of isorhamnetin and its derivatives (5 and 10 μg/mL; 30 min) on the hemostatic parameters of human plasma (TT (A), PT (B), APPT (C)). Tested phenolic compounds were incubated with plasma for 30 min and then thrombin was added. Data represents means ± SEM of 4–6 independent experiments. *p < 0.05 *vs.* control, n.s. p > 0.05 *vs.* control.

compound 2. On the other hand, compound 2 had stronger antioxidant activity than compound 3 and isorhamnetin.

4. Discussion

It was demonstrated that the dietary intake of flavonoids from vegetables and fruits may reduce cardiovascular diseases (Eccleston et al., 2002; Giampieri et al., 2012). The sea buckthorn berries are a good source of various flavonoids, which show beneficial effects on health, including cardiovascular system (Cheng et al., 2003). Various flavonol glycosides from the sea buckthorn berries were identified using

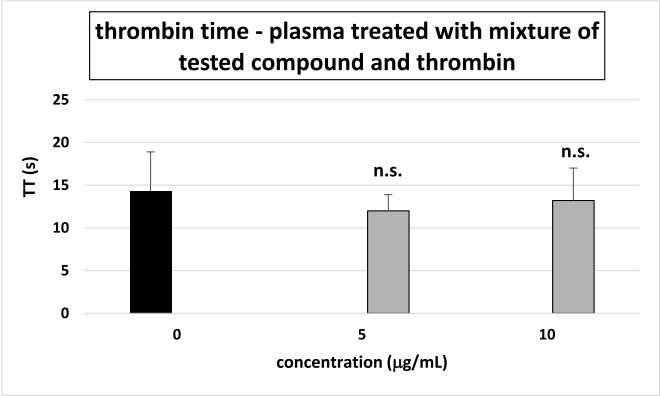

Fig. 4. Effects of compound 2 (5 and 10 μg/mL; 30 min) on the thrombin time of human plasma. The solution of thrombin was incubated for 30 min with tested compound 2 and then the mixture was added to the human plasma. Data represents means ± SEM of 4–6 independent experiments. n.s. p > 0.05 *vs.* control.

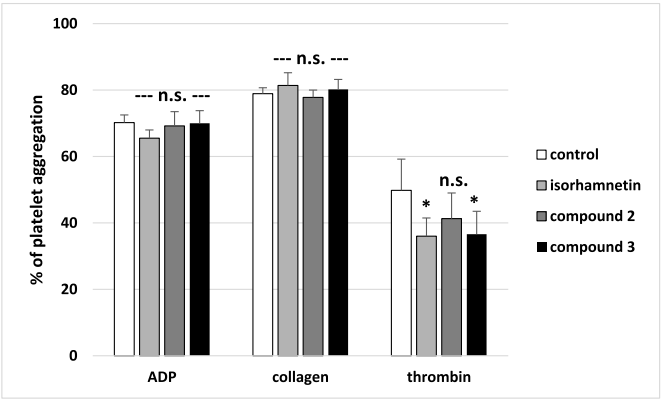

Fig. 5. Effects of isorhamnetin and its derivatives (10 μg/mL; 30 min) on blood platelet aggregation induced by ADP or collagen (in platelet-rich plasma) or by thrombin (in blood platelets in Tyrode's buffer). Data represents means ± SEM of 3–5 independent experiments. *p < 0.05 *vs.* control, n.s. p > 0.05 *vs.* control.

chromatographic methods by various authors (Gulieyev et al., 2004; Zheng et al., 2009; Korekar et al., 2011; Fang et al., 2013; Teleszko et al., 2015; Olas et al., 2016, 2017 and 2018; Guo et al., 2017). It is very important that the total flavonoids from the aqueous ethanol extract of sea buckthorn berries have been clinically used for the treatment of cardiovascular diseases in China since 1980 (Wang et al., 1993, 2000). Luo et al. (2015) observed that isorhamnetin inhibited atherosclerotic plaque development in apolipoprotein E knockout (ApoE−/−) mice by phosphatidylinositol 3-kinase/protein kinase B signal transduction and heme oxygenase-1 induction. Results of Guo et al. (2017) indicate that isorhamnetin protects against cardiac hypertrophy through blocking PI3K-AKT pathway. Various other experiments showed that isorhamnetin has anticancer activity (Teng et al., 2006; Li et al., 2014, 2015). In addition, isorhamnetin ameliorates lipopolysaccharide (the main component in Gram-negative bacteria) – induced inflammatory response through down-regulation of nuclear factor-kappa B signaling (Li et al., 2016a). Shi et al. (2018) have also observed that isorhamnetin is a potent immunesuppressive agent by inhibiting dendritic cells activation and trafficking. Another experiment of Li et al. (2016b) showed that isorhamnetin (especially at concentration 100 μM) has the protective role on human brain microvascular endothelial cells from cytotoxicity induced by methylglyoxal and oxygen-glucose deprivation.

Sanchez et al. (2007) observed that isorhamnetin inhibits the

production of reactive oxygen species (ROS). Sun et al. (2012) also demonstrated that this compound inhibits the H_2O_2 action by scavenging free ROS. Moreover, results of other authors (Sea et al., 2016; Wang et al., 2018; Zhao et al., 2018) indicate that isorhamnetin has antioxidant properties *in vitro*. Similar effects have been observed in our present experiments *in vitro*. In the present study, three various assays (lipid peroxidation, carbonyl and thiol groups determined) have been used to study the antioxidant properties of isorhamnetin and its two derivatives in human plasma treated with H_2O_2/Fe under *in vitro* conditions. Our results revealed differences in antioxidant properties between tested phenolic compounds in human plasma treated with H_2O_2/Fe. It seems possible that the difference in chemical structure of the tested compounds may justify this observation. For the first time, it is demonstrated by our three tests that compound 2 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-rhamnoside) had stronger antioxidant properties than compound 3 (isorhamnetin 3-*O*-beta-glucoside-7-*O*-alfa-(3'''-isovaleryl)-rhamnoside) and isorhamnetin.

Berries have been reported to exert the highest antioxidant activity among all kinds of fruits (Olas, 2018b). However, phenolic compounds isolated from berries are very often known to be less effective antioxidant or anti-platelet factors than berries and their food products (Chong et al., 2010; Olas, 2017 and 2018a and b). On the other hand, we report for the first time that isorhamnetin and its two derivatives have often stronger or similar antioxidant properties than used phenolic fraction. We suppose that the antioxidant potential of tested phenolic fraction from the sea buckthorn berries fraction may depend on the presence of flavonoids (especially isorhamnetin and its two used derivatives), which may act as hydroxyl radical scavengers.

Berry phenolic compounds not only possess antioxidant activities, but often demonstrate anti-platelet or anti-coagulant properties. A key novel finding of our experiment is a demonstration of the anti-platelet (anti-aggregatory) and the anti-coagulant properties, observed in the form of inhibited blood platelet aggregation stimulated by thrombin, and prolonged clotting time – the thrombin time, recorded for tested compound 3. However, we did not observe that compound 2 prolonged the TT of human plasma when this compound was preincubated with thrombin. We suppose that anti-coagulant activity of compound 3 is not associated with a modulation of thrombin activity. On the other hand, results of Choi et al. (2016) indicate that flavonoids may inhibit the enzymatic activity of thrombin. Thrombin as serine protease plays not only important functions in coagulation process, but it is also a blood platelet activator.

Blood platelets are the smallest un-nucleated blood cells, which play a significant function in hemostasis. Moreover, these cells have a fundamental role in acute coronary syndromes pathogenesis. Numerous endogenous agonists (named also stimuli, activator) induce blood platelet signal transduction *via* their receptors, including cascade of platelet activation, among them not only thrombin, but also ADP and collagen. Present *in vitro* study was designed to estimate the anti-platelet actions of isorhamnetin and its derivatives isolated from the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries. For the first time, it has been demonstrated through our test that isorhamnetin and its derivative – compound 3 exerted inhibitory action on thrombin – stimulated blood platelet aggregation. Moreover, we observed that none of the tested phenolic compounds blocked the inhibition of human blood platelet aggregation stimulated by other physiological agonists: ADP and collagen. It may suggest that isorhamnetin and compound 3 could modulate blood platelet activation by interfering with thrombin receptors on blood platelets. In addition, different action of tested compounds on platelet aggregation (measured in PRP and in blood platelets in Tyrode's buffer) may also depend on their binding ability to various components of plasma (Dangles et al., 2001).

The bioavailability and toxicity of phenolic compounds are important elements in the evaluation of their biological activities under different conditions, including *in vitro* and *in vivo* models (Manach et al., 2004, 2005). Flavonoids exhibit a low bioavailability, e.g. the oral bioavailability of quercetin aglycon is only about 1% in humans (Khaled et al., 2003). However, bioavailability of phenolic compounds differs from one berry to another. Recently, the results of Guo et al. (2017) have demonstrated that after enzymatic digestion, the phenolic compounds were quite different from the chemical extracts from the sea buckthorn berries, and more flavonoid aglycones were released, whereas less total phenolics, flavonoid glycosides and phenolic acids were detected. However, the cellular antioxidant property of berries was significantly enhanced by digestion. It is also important that there is information about the toxicity of berry phenolic compounds (Olas, 2017 and 2018b).

In our experiments, human plasma and blood platelets were preincubated with phenolic compounds or the phenolic fraction from the sea buckthorn berries in two concentrations (5 and 10 μg/mL). The concentration of phenolic substances (5 μg/mL) is likely to occur in plasma *in vivo* after oral supplementation can reach up to 5–7 μM (10 μM of isorhamnetin is around 3 μg/mL). However, they depend on the food, e.g. total plasma concentration of caffeoylquinic acids can reach about 5 μg/mL (Farah et al., 2008). Therefore, lower concentration of tested compounds or the phenolic fraction (5 μg/mL), used in our study model, appears to correspond to the physiological concentration of plant-derived phenolic compounds available after oral administration.

In conclusion, this is the first paper presenting a multi-method research study evaluating the antioxidant, anti-platelet and anti-coagulant properties of isorhamnetin and its two derivatives isolated from the phenolic fraction of *E. rhamnoides* (L.) A. Nelson berries. Our results demonstrate that there is a novel potential of these compounds in prevention and treatment of cardiovascular diseases. However, compound 3 is a better anti-coagulant and shows stronger anti-platelet activity than compound 2, yet compound 2 is a more efficient antioxidant than compound 3 and isorhamnetin.

Declaration of interest statement

None to declare.

Acknowledgements

This work was supported by National Science Centre, Poland (2015/19/B/NZ9/03164). The authors would also like to thank dr Mariusz Kowalczyk for performing the UHPLC-MS analyses.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.02.014.

References

Ando, Y., Steiner, M., 1973a. Sulphydryl and disulphide groups of platelet membranes: determination of sulphydryl groups. Biochim. Biophys. Acta 311, 38–44 (A).
Ando, Y., Steiner, M., 1973b. Sulphydryl and disulphide groups of platelet membranes: determination of disulphide groups. Biochim. Biophys. Acta 311, 26–37 (B).
Bartosz, G., 2008. Druga Twarz Tlenu, vol. 1.7. PWN, Warszawa, pp. 99–120.
Chen, C., Xu, X.M., Chen, Y., Yu, M.Y., Wen, F.Y., Zhang, H., 2013. Identification, quantification and antioxidant activity of acylated flavonol glycosides from sea buckthorn (*Hippophae rhamnoides ssp. sinensis*). Food Chem. 141, 1573–1579.
Cheng, J., Kondo, K., Suzuki, Y., Ikeda, Y., Meng, X., Umemura, K., 2003. Inhibitory effects of total flavones of *Hippophae Rhamnoides* L. on thrombosis in mouse femoral artery and in vitro platelet aggregation. Life Sci. 72, 2263–2271.
Choi, J.H., Kim, K.J., Kim, S., 2016. Comparative effect of quercetin and quercetin-3-O-β-d-glucoside on fibrin polymers, blood clots, and in rodent models. J. Biochem. Mol. Toxicol. 30, 548–558.
Chong, M.F.F., MacDonald, R., Lovegrove, J.A., 2010. Fruit polyphenols and CDV risk: a review of human intervention studies. Br. J. Nutr. 104, S28–S39.
Dangles, O., Dyfour, C., Manach, C., Morand, C., Remesy, C., 2001. Binding of flavonoids to plasma proteins. Methods Enzymol. 335, 319–333.
Eccleston, C., Baoru, Y., Tahvonem, R., Kallio, H., Rimbach, G.H., Minihane, A.M., 2002. Effects of an antioxidant-rich juice (sea buckthorn) on risk factors for coronary heart

disease in humans. J. Nutr. Biochem. 13, 346–354.
Fang, R., Veitch, N.C., Kite, G.C., Porter, E.A., Simmonds, M.S., 2013. Enhanced profiling of flavonol glycosides in the fruits of sea buckthorn (*Hippophae rhamnoides*). J. Agric. Food Chem. 61, 3868–3875.
Farah, A., Monteiro, M., Donangelo, C.M., Lafay, S., 2008. Chlorogenic acids from green coffee extract are highly bioavailable in humans. J. Nutr. 138, 2310–2315.
Giampieri, F., Tulipani, S., Alvarez-Suarez, J.M., Quiles, J.L., Mezzetti, B., Battino, M., 2012. The strawberry: composition, nutritional quality, and impact on human health. Nutrition 28, 9–19.
Gulieyev, V.B., Gul, M., Yildirim, A., 2004. *Hippophae rhamnoides* L.: chromatographic methods to determine chemical composition use in traditional medicine and pharmacological effects. J. Chromatogr. B 812, 291–307.
Guo, R., Guo, X., Li, T., Fu, X., Liu, R.H., 2017. Comparative assessment of phytochemical profiles, antioxidant and antiproliferative activities of sea buckthorn (*Hippophae rhamnoides* L.) berries. Food Chem. 221, 997–1003.
Khaled, K.A., El-Sayed, Y.M., Al-Hadiya, B.M., 2003. Disposition of the flavonoid quercetin in rats after single intravenous and oral doses. Drug Dev. Ind. Pharm. 29, 397–403.
Korekar, G., Stobdan, T., Singh, H., Chaurasia, O., Singh, S., 2011. Phenolic content and antioxidant capacity of various solvent extracts from seabuckthorn (*Hippophae rhamnoides* L.) fruit pulp, seeds, leaves and stem bark. Acta Aliment. 40, 449–458.
Levine, R.L., Garland, D., Oliver, C.N., Amici, A., Climent, I., Lenz, A.G., et al., 1990. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186, 464–478.
Li, C., Yang, X., Chen, C., Cai, S., Hu, J., 2014. Isorhamnetin suppresses colon cancer cell growth through the PI3K-akt-mTOR pathway. Mol. Med. Rep. 9, 935–940.
Li, Q., Ren, F.Q., Yang, C.L., Zhou, L.M., Liu, Y.Y., Xiao, J., Zhu, L., Wang, Z.G., 2015. Anti-proliferation effects of isorhamnetin on lung cancer cells *in vitro* and *in vivo*. Asian Pac. J. Cancer Prev. APJCP 16, 3035–3042.
Li, Y., Chi, G., Shen, B., Tian, Y., Feng, H., 2016a. Isorhamnetin ameliorates LPS-induced inflammatory response through downregulation of NF-κB signaling. Inflammation 39, 1291–1301.
Li, W., Chen, Z., Yan, M., He, P., Chen, Z., Dai, H., 2016b. The protective role of isorhamnetin on human brain microvascular endothelial cells from cytotoxicity induced by methylglyoxal and oxygen-glucose deprivation. J. Neurochem. 136, 651–659 (B).
Luo, Y., Sun, G., Ding, X., Wang, M., Qin, M., Yu, Y., Sun, X., 2015. Isorhamnetin attenuates atherosclerosis by inhibiting macrophage apoptosis via PI3K/akt activation and HO-1 induction. PLoS One 1–19.
Malinowska, P., Olas, B., 2016. Sea buckthorn – valuable plant for health. Kosmos 2, 288–292.
Malinowska, J., Kołodziejczyk-Czepas, J., Moniuszko-Szajwaj, B., Kowalska, I., Oleszek, W., Stochmal, A., Olas, B., 2012. Phenolic fractions from *Trifolium pallidum* and *Trifolium scabrum* aerial parts in human plasma protect against changes induced by hyperhomocysteinemia. Food Chem. Toxicol. 50, 4023–4027.
Manach, C., Scalbert, A., Morand, C., Remsey, C., Jimenez, L., 2004. Polyphenols: food sources and bioavailability. Am. J. Clin. Nutr. 79, 727–747.
Manach, C., Williamson, G., Morand, C., Scalbert, A., Remsey, C., 2005. Bioavailability and bioafficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am. J. Clin. Nutr. 81, 230–242.
Michel, T., Destandan, E., Le Floch, G., Lucchesi, M.E., Elfakir, C., 2012. Antimicrobial, antioxidant and phytochemical investigations of sea buckthorn (*Hippophae rhamnoides* L.) leaf, stem, root and seed. Food Chem. 131, 754–760.
Olas, B., 2016. Sea buckthorn as a source of important bioactive compounds in cardiovascular diseases. Food Chem. Toxicol. 97, 199–204.
Olas, B., 2017. The multifunctionality of berries toward blood platelets and the role of berry phenolics in cardiovascular disorders. Platelets 28, 540–549.
Olas, B., 2018a. The beneficial health aspects of sea buckthorn (*Elaeagnus rhamnoides* (L.) A.Nelson) oil. J. Ethnopharmacol. 213, 183–190 (A).
Olas, B., 2018b. Berry phenolic antioxidants – implications of human health? Front. Pharmacol. 9, 1–14 (B).
Olas, B., Kontek, B., Malinowska, P., Zuchowski, J., Stochmal, A., 2016. *Hippophae rhamnoides* L. fruits reduce the oxidative stress in human blood platelets and plasma. Oxid. Med. Cell. Longev. 1–8.
Olas, B., Kontek, B., Szczesna, M., Grabarczyk, L., Stochmal, A., Zuchowski, J., 2017. Inhibition of blood platelet adhesion by phenolics' rich fraction of *Hippophae rhamnoides* L. fruits. J. Physiol. Pharmacol. 2, 23–29.
Olas, B., Żuchowski, J., Lis, B., Skalski, B., Kontek, B., Grabarczyk, Ł., Stochmal, A., 2018. Comparative chemical composition, antioxidant and anticoagulant properties of phenolic fraction (a rich in non-acylated and acylated flavonoids and non-polar compounds) and non-polar fraction from *Elaeagnus rhamnoides* (L.) A.Nelson fruits. Food Chem. 247, 39–45.
Sanchez, M., Lodi, F., Vera, R., Villar, I.C., Cogolludo, A., Jimenez, R., Moreno, L., Romero, M., Tamargo, J., Perez-Vizcaino, F., Duarte, J., 2007. Quercetin and isorhamnetin prevent endothelial dysfunction, superoxide production, and overexpression f $p47^{phox}$ induced by angiotensin II in rat aorta. J. Nutr. 137 920-915.
Sea, S., Sea, K., Ki, S.H., Shin, S.M., 2016. Isorhamnetin inhibits reactive oxygen species-dependent hypoxia inducible factor (HIF)-1α accumulation. Biol. Pharm. Bull. 39, 1830–1838.
Shi, H., He, J., Li, X., Han, J., Wu, R., Wang, D., Yang, F., Sun, E., 2018. Isorhamnetin, the active constituent of a Chinese herb *Hippophae rhamnoides* L, is a potent suppressor of dendritic-cell maturation and trafficking. Int. Immunopharmacol. 55, 216–222.
Sun, B., Sun, G.B., Xiao, J., Chen, R.C., Wang, X., Wu, Y., Cao, L., Yang, Z., Sun, X.B., 2012. Isorhamnetin inhibits H_2O_2-induced activation of the intrinsic apoptotic pathway in H9c2 cardiomyocytes through scavenging reactive oxygen species and ERK inactivation. J. Cell. Biochem. 113, 473–485.
Teleszko, M., Wojdylo, A., Rudzinska, M., Oszmianski, J., Golis, T., 2015. Analysis of lipophilic and hydrophilic bioactive compounds content in sea buckthorn (*Hippophae rhamnoides* L.) berries. J. Agric. Food Chem. 63, 4120–4129.
Teng, B., Lu, Y.H., Wang, Z.T., Tao, X.Y., Wei, D.Z., 2006. In vitro anti-tumor activity of isorhamnetin isolated from *Hippophae rhamnoides* L. agonist BEL-7402 cells. Pharmacol. Res. 54, 186–194.
Wachowicz, B., 1984. Adenine nucleotides in thrombocytes of birds. Cell Biochem. Funct. 2, 167–170.
Wachowicz, B., Kustron, J., 1992. Effect of cisplatin on lipid peroxidation in pig blood platelets. Cytobios 70, 41–47.
Walkowiak, B., Michalak, E., Koziołkiewicz, W., Cierniewski, C.S., 1989. Rapid photometric method for estimation of platelet count in blood plasma or platelet suspension. Thromb. Res. 56, 763–766.
Wang, B.W., Feng, Y.Z., Yu, Y.M., Zhang, H.M., Zhu, R., 1993. The effect of total flavonoids of *Hippophae rhamnoides* (TFH) on cardiac performance and hemodynamics of normal people. J. Xi'an Med. Univ. 14, 137–140.
Wang, Z.R., Wang, L., Yin, H.H., Yang, F.J., Gao, Y.Q., Zhang, Z.J., 2000. Effect of total flavonoids of *Hippophae rhamnoides* on contractile mechanics and calcium transfer in stretched myocyte. Space Med. Med. Eng. 13, 6–9.
Wang, J., Gong, H.M., Zou, H.H., Liang, L., Wu, X.Y., 2018. Isorhamnetin prevents H_2O_2 – induced oxidative stress in human retinal pigment epithelial cells. Mol. Med. Rep. 17, 648–652.
Whitaker, J.R., Granum, P.E., 1980. An absolute method for protein determination based on difference in absorbance at 235 and 280 nm. Anal. Biochem. 109, 156–159.
Zhao, T.T., Yang, T.L., Gong, L., Wu, P., 2018. Isorhamnetin protects against hypoxia/reoxygenation-induced injure by attenuating apoptosis and oxidative stress in H9c2 cardiomyocytes. Gene 666, 92–99.
Zheng, R.X., Xu, X.D., Tian, Z., Yang, J.S., 2009. Chemical constituents from the fruits of *Hippophae rhamnoides*. Nat. Prod. Res. 23, 1451–1456.
Zuchowski, J., Pecio, L., Kontek, R., Stochmal, A. (sub.) Unusual isovalerylated flavonoids from fruit of the sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson). Phytochemistry.