ANTI-PLATELET FRACTION ISOLATED FROM *GALEGA OFFICINALIS*

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Summary. A fraction from crude extract of *Galega officinalis* has been purified by column chromatography on Sephadex G-25, Sepharose 4B, DEAE-Cellulose and Sephadex G-100. The final purification factor of the fraction is 120. The peak in elution profile after Sephadex G-150 shows a molecular weight of 100-140 kDa. The isolated fraction appears to have 74% polysaccharides and 23% of proteins. No loss of activity of the final fraction is observed after storage for several months at 4 °C and in lyophilized condition. The fraction compounds inhibit platelet aggregation induced by ADP, collagen and thrombin.

Key words: *Galega officinalis*, antiplatelet fraction

INTRODUCTION

G*alega officinalis* is a plant wide distributed in Yeast Europe. The plant is used in the traditional medicine in the treatment of *diabetes mellitus* [1]. About 20 biologically – active substances are isolated from *Galega officinalis*: alkaloids, flavonoids, glucosides, saponin and others. Phytochemical analyses gave positive results for lipids, protein and cellulose [2]. The biologically active alkaloid *galegine* (exhibiting a hypoglycaemic effect *in vivo*) was isolated from *Galega officinalis* [3]. The experimental results of Atanasov shown that the crude aqueous extracts [4, 5], and the fractions from gel-chromatography on Sephadex G-25, Sepharose 4b and ion-exchange chromatography on DEAE-Cellulose suppress platelet aggregation *in vitro* and *in vivo* induced by ADP, epinephrine, thrombin and collagen [6, 7]. In this paper we report the purification and characterization of all active fractions purified by 4 steps from crude extract of *Galega officinalis*.

MATERIAL AND METHODS

Plant material and extract preparation. The aerial parts of *Galega officinalis* at flowering stage were collected between May and August 2015 in different parts of Thrace, region of Bulgaria. Plant was verified by Department of Botany, Faculty of Pharmacy (University of Sofia, Bulgaria). Crude aqueous extracts were obtained by maceration of 200 g dry matter in 2000 ml distilled water (pH 8 adjusted with sodium hydrogen carbonate) for 20-24 hours at 18-20° C. Fresh extracts were filtered and concentrated at temperature below 35° C to about 165-170 mg/ml dry matter.

Isolation procedures. Concentrated crude extract (10 ml at 75 mg/ml dry matter) was applied onto a column with Sephadex G-25 (700 x 65 mm) equilibrated with $\text{NH}_3\text{-H}_2\text{O}$ solution, pH 7.3. The active compound was eluted with the starting buffer at 16 °C and flow-rate 720 ml/h. The active fraction (400 to 550 ml) was concentrated and lyophilized. Lyophilized column fraction (200 mg) obtained from Sephadex G-25 column was applied to a column with Sepharose-4B (550 x 24 mm) equilibrated with 0,01 M Tris-HCl, pH 7.3. The active compound was eluted with the same buffer at 16 °C and flow-rate 10 ml/h. The active fraction (120 to 150 ml) was collected, desalted on Sephadex G-25, concentrated and lyophilized. The lyophilized material (100 mg) from Sepharose 4B was applied onto a column with DEAE-cellulose equilibrated with 0.02 M potassium phosphate, pH 7,3. The active fraction was eluted with the same buffer, desalted on Sephadex G-25, concentrated and lyophilized. The lyophilized material (24 mg) from DEAE-cellulose column was applied onto a column with Sephadex G-100 (52 x 16 mm), equilibrated with 0,01 M Tris-HCl, pH 7.3. The active compound was eluted with the same buffer at 16 °C and flow-rate 16 ml/h. Active fractions from 25 to 55 ml were collected, desalted on Sephadex G-25, concentrated and lyophilized.

Determination of the molecular weight (M_w). The isolated fraction was applied onto a Sephadex G-150 column (100 x 1.5 cm) pre-equilibrated with 0.01M Tris-HCl, pH 7.3. β -amylase (M_w 200 kDa), alcohol dehydrogenase (M_w 150 kDa), albumin (M_w 66 kDa), ovalbumin (M_w 43 kDa), carbonic anhydrase (M_w 29 kDa) and cytochrome C (M_w 12.3 kDa) were used as protein standards.

Platelet aggregation-inhibition studies. Blood was taken from volunteers who did not receive medication for 15 days prior to blood collection. Blood was collected in disposable syringes and diluted at a ratio of 1 part 3,8% trisodium citrate and 9 parts venous blood. Platelet-rich plasma (PRP) was prepared by centrifugation (180 x g for 10 min) and diluted to 300×10^6 platelets per ml with autologous platelet-poor plasma (1800 x g for 15 min). The platelet aggregation was studied by a photometric method according to Born and Zucker [8]. The extinction change that takes place during the aggregation of 400 μl platelet-rich plasma compared with platelet-poor plasma (whose extinction was taken as zero) after adding aggregating agent at final concentration 25 μM ADP, 100 $\mu\text{g/ml}$ collagen or 0.8 U/ml thrombin at 37°C was the

basis of measurement of the aggregating effect. Theophylline, aspirin and heparin were used as a basis for comparison of the effect of the active fraction on the platelet aggregation.

Reagents and drugs. ADP from Reanal (Hungary), human thrombin and bovine collagen from Research Institute of Hematology and Blood Transfusion (Sofia, Bulgaria) were used as aggregating agents. Theophylline, aspirin and heparin from Pharmachim (Bulgaria) were used as a basis for comparison of the effect of the active fraction on the platelet aggregation.

Statistical analysis. The results were expressed as mean S.E.M.

RESULTS

Isolation of the active fractions. Column gel-filtration on Sephadex G-25 was used as first step for separation from the substances with low molecular mass (with M_w lower than 20 kDa) (Fig. 1a). Gel-filtration on Sepharose 4B was used for elimination of fraction with high molecular mass (with M_w higher than 200 kDa) and colored substances (Fig. 1b). Column ion-exchange chromatography on DEAE-cellulose was applied for total elimination of the colored substances. The active fraction was eluted with 20 mM potassium phosphate buffer, pH 7.3. The colored substances were eluted with gradient of 0.1–3.0% NaCl in 20 mM potassium phosphate buffer, pH 7.3. Gel-filtration on Sephadex G-100 was used as final step for obtaining of a separate active fraction (Fig. 1c). This fraction was pooled, dialyzed and concentrated at temperature below 35°C. The all purification steps are presented in Table 1.

Table 1. Purification steps of antiplatelet fractions from *Galega officinalis*

Purification step	Total matter (mg)	Specific activity (U/mg)*	Total activity (U)	Purification fold	Yield (%)
Crude extract	1000.10 ³	0.9	900.10 ³	1	100
Sephadex G-25	24.10 ³	32	770.10 ³	36	85
Sepharose 4B	6.10 ³	83	500.10 ³	92	55
DEAE-Cellulose	2.5.10 ³	90	225.10 ³	100	25
Sephadex G-100	2.10 ³	108	216.10 ³	120	24

*One unit of specific activity for extract or fraction was taken to be equal to IC₅₀ – this is the quantity of extract or fraction after each step of purification that inhibits with 50% the platelet aggregation of 1ml platelet-rich plasma initiated by 25 μM ADP. For crude extract IC₅₀ was 1.1 mg; after Sephadex G-25 IC₅₀ was 0.030 mg; after Sepharose 4B IC₅₀ was 0.012mg; after DEAE-cellulose IC₅₀ was 0.011 mg; after Sephadex G-100 IC₅₀ was 0.0093 mg.

All five steps resulted in 120-fold purification. From 1000 g dry matter was obtained 2 g leophylized active compound with 216.10³ units of total activity. The obtained 24% yield is sufficient, for example 6.10³ units activity inhibit with 50% the platelet aggregation in vitro of 6L platelet-rich plasma.

Determination of the molecular weight (M_w). The molecular weight of the main fraction was estimated to be 100-140 kDa by gel-filtration on Sephadex G-150. Fractions with lower M_w manifesting activity were also identified – Fig. 2.

a. Gel-filtration on Sephadex G-25;
b. Gel-filtration on Sepharose 4B;
c. Ion-exchange chromatography on DEAE-cellulose; d. Gel-filtration on Sephadex G-100

Fig.1. Purification of an active fraction from *G. officinalis* crude extract

Legend: The solid lines correspond to the eluted fractions. The active fraction is indicated by the shaded area.

Fig. 2. Determination of molecular weight of the final fraction from *G. officinalis*

Platelet aggregation-inhibition studies. The 50% (IC₅₀) and 100% (IC₁₀₀) inhibiting effect of the crude extract and the final fraction (after Sephadex G-100) on platelet aggregation, initiated by ADP, collagen and thrombin in 1 ml PRP was investigated. Data are presented in Table 2.

Table 2. Inhibiting effect on the platelet aggregation of the crude extract, final fraction from *G. officinalis* and drugs (IC₅₀ and IC₁₀₀ are given as µg substance in 1 ml PRP)

Aggregating agents	Crude extract (µg/ml)	Final fraction (µg/ml)	Drugs (µg/ml)
25 µM ADP	(IC ₅₀) 1100.0 ± 30.6	9.3 ± 0.4 5	Theophylline 250 ± 0.10
0.1 mg/ml collagen	(IC ₁₀₀) 1870.0 ± 74.0	12.8 ± 0.63	Aspirin 1800 ± 63
0.8 U/ml thrombin	(IC ₁₀₀) 3125.0 ± 98.0	15.1 ± 0.58	Heparin** 0.7 ± 0.10 U/ml

*Mean ± SEM for 8 experiments

** U/ml

In the case of aggregation induced by 25 µM ADP the activity of the final fraction was found to be 118 times higher than that of the crude extract and 27 times higher than that of theophylline (IC₅₀ data are shown in Table 2). The fraction possess 100% anti-aggregating activity on the platelet aggregation, initiated by 100 µg/ml PRP collagen and 0,8 U/ml thrombin. In a dose, containing 12-16 µg fraction in 1 ml platelet-rich plasma, the fraction inhibited fully the initiation of the platelet aggregation. For comparison 1.8 mg/ml aspirin and 0.7 U/ml heparin inhibited fully the platelet aggregation initiated by 100 µg/ml collagen and 0,8 U/ml thrombin respectively (IC₁₀₀ data are presented in Table 2).

DISCUSSION

The observed activity of the fraction was higher than those of theophylline, aspirin, heparin and other clinically used drugs and substances. For comparison, IC₅₀ of tea catechins (tannins), an active component of the green tea was 200 µg/ml PRP for collagen-induced platelet aggregation [9]. It was found that the anti-platelet snake venom peptide gabonin (M_w 21100 Da) has IC₅₀ between 8.5 and 33.6 µg/ml PRP for human platelet aggregation, stimulated by ADP, thrombin and collagen [10]. The activity of the fraction from *Galega officinalis* was 9.3 to 15.1 µg/ml for the same aggregating agent, a value that is near to the activity of the snake venom peptide. The disaggregating effect of the fraction on previously – aggregated PRP shown that the fraction's active compounds disrupted the fibrinogen bridges between the aggregating platelets, responsible for the irreversible aggregation [11]. It is well established that the platelet and the thrombus inhibition activities is possible because of some amino acids sequence as RGD, KRDS, RGDW and others [12]. The peptides with same sequences appear as antagonists of fibrinogen receptors on platelet surface [13, 14].

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