

The antiplatelet-antithrombotic effect and antioxidant activity of the *Phlojodicarpus sibiricus* extract

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Abstract

Introduction: Cardiovascular disease and ischemic stroke remain the leading causes of death and disability worldwide. *Phlojodicarpus sibiricus* has a wide range of pharmacological properties due to its content of coumarins. **The aim of this study** was to determine the antiplatelet effect and antioxidant activity of *Phlojodicarpus sibiricus* extract (*P. sibiricus*).

Conclusion: *P. sibiricus* exerts an antiplatelet effect at doses of 50–200 mg/kg due to the combined antiplatelet and antioxidant effects of coumarins.

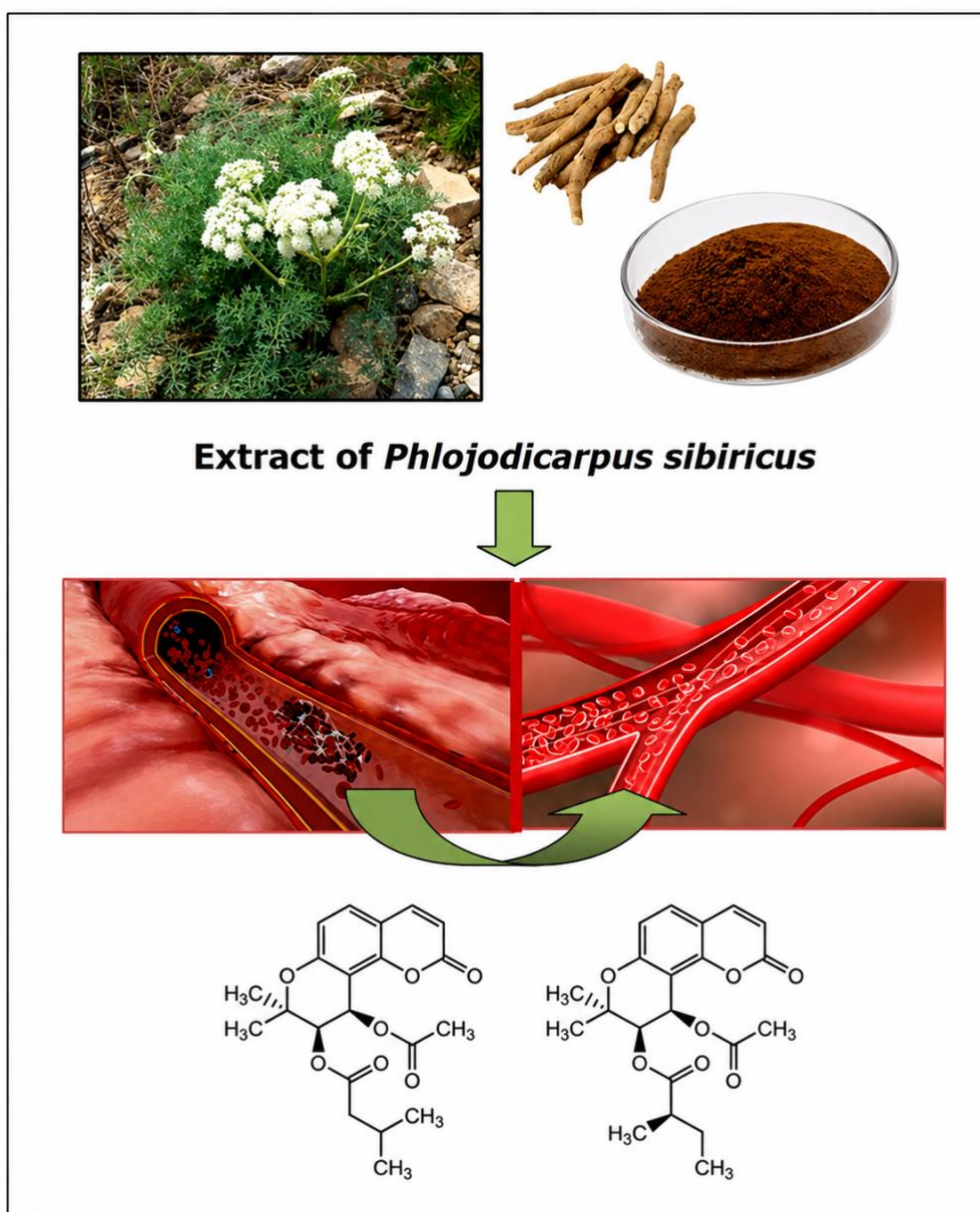
Material and Methods: A dry ethanol extract of *P. sibiricus* was used in the experiments. The antiplatelet activity of *P. sibiricus* was assessed using *in vitro* test. The effect of the extract on haemostasis was evaluated in models of blood coagulation and chemically induced vascular thrombosis. The experiments were conducted on 86 Wistar rats weighing 180–220 g, divided into groups: Group 1 – Vehicle, groups 2–4 – rats receiving *P. sibiricus* at doses of 50, 100, and 200 mg/kg, respectively; groups 5–6 – rats treated with aspirin (30 mg/kg) and heparin (200 U/kg), respectively. The antioxidant activity of the extract was assessed *in vitro* using the DPPH assay and the Fe²⁺ binding model.

Results: *P. sibiricus* demonstrated antiplatelet activity (in concentration 50–200 µg/mL) *in vitro*. Administration of *P. sibiricus* to rats at doses of 50, 100 and 200 mg/kg increased the duration of bleeding by 2 times and suppressed thrombus formation by 52.6% compared to the control.



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Graphical Abstract



Keywords

Phlojodicarpus sibiricus; coumarins; dihydrosamidine; visnadin; antiplatelet activity; antioxidant activity

Introduction

Thrombotic disorders including acute coronary syndrome, ischemic stroke and other peripheral arteries diseases remain the main causes of mortality and disability all over the world (Jackson 2011; Benjamin et al. 2018; Chen and Ju 2020). When atherosclerotic changes in a vessel wall occur, clotting starts with the processes of adhesion, activation and aggregation of thrombocytes (Chen and Ju 2020). Hyper-production of reactive oxygen species and bio-radicals in the endothelium, erythrocytes and platelets in the case of pathological change of the blood flow is considered to be one of the leading mechanisms of the thrombus formation (Pernow et al. 2019; Masselli et al. 2020). In this connection, a complex impact on the leading factors and trigger mechanisms of clotting is of great importance for thrombosis prevention.

In the clinical practice, to prevent thrombosis in many vascular malformations and thrombophilic states, it is customary to use antiplatelet agents and antithrombotic synthetic preparations. However, their antiplatelet effect is achieved through a certain mechanism, i.e. influence on a particular pharmacological target. The developments of side-effects and/or complications such as hemorrhages are often noted. These conditions exclude a long-term use of preparations in chronic pathology, which, on the whole, reduces the efficacy of the preventive therapy. In this connection, it is worth to use plant remedies which are known to have a wide spectrum of pharmacological properties, polymodal mild effect and low toxicity (Sokolov 2000).

Phlojodicarpus sibiricus Koso-Pol. is a plant which belongs to Apiaceae family and grows on the vast territory of eastern Siberia, Trans-Baikal and in particular areas of Yakutia, Mongolia, and Amur River region (Fig. 1). Its roots contain a wide range of coumarin substances among which dihydrosamidin and visnadin dominate (Fig. 2), as well as a complex of fatty acids, ether oils, amino acids, and microelements (Gantimur 1985; Taraskin 2011).

In the practice of Tibetan, Buryat and Mongolian traditional medicine, the local healers ('emchi-lamas') used the roots of this plant under the name 'ru-rta' (Tibet) for the treatment of 'rlung' (Tibet) diseases – pathologies of the central nervous system (memory impairment, epilepsy, neurotic disorders) and the blood congestion in the body of aged individuals (Khaidav et al. 1985; Batorova et al. 2013).

Coumarins from the roots of this plant were earlier used for the production of the preparation 'floverin' (combination of dihydrosamidin and visnadin), which has a vasodilating property and is indicated for the treatment of cardiovascular diseases (Mashkovsky 2025). However, many coumarin compounds are known to have a wide spectrum of pharmacological properties: antiplatelet, spasmolytic, antioxidant, neuromodulating and neuroprotective ones (Hoult and Paya 1996; Skalicka-Wozniak et al. 2016; Zaragozá et al. 2016; Gao et al. 2021).

In spite of established pharmacological properties of *P. sibiricus* and a rich content of coumarin compounds, the scientific information on its antiplatelet effect and its use in thrombotic disorders, particularly, in vascular diseases of the brain is not available. In this connection, the present study was aimed at the estimation of the antiplatelet effect and antioxidant activity of the extract from the *P. sibiricus* root.

Materials and Methods

Preparation of the plant remedy

The *P. sibiricus* roots with rhizomes were gathered in the Aginsky district of Trans-Baikal region in September 2022, 51°00'N, 114°47'E. The plant specimens were identified by Prof. G.G. Nikolaeva (Institute of General and Experiments Biology of the Siberian Branch of the Russian Academy of Sciences (IGEB SB RAS)). The plant material was air-dried and then it was comminuted to 3 mm-particle size. The portion of the plant material (500 g) was triply extracted with 5.0 L of 60% ethyl alcohol with constant stirring and ultrasound use. The obtained extracts were mixed and filtered; the solvent was removed with the use of rotary evaporator at 60°C. The obtained dry extract was the brown powder, and the yield value was 26.3%. Total content of coumarins determined by UV spectrophotometry method was 3.63%.



Figure 1. Photography of *Phlojodicarpus sibiricus* (Fisch.) Koso-Pol. in natural habitat, Eastern Siberia (a), the roots of this plant (b).

Chromatographic condition and identification of coumarins

The studies were carried out on a microcolumn liquid chromatograph Milichrom A-02 (Econova; Novosibirsk, Russia), equipped with an autosampler, a UV detector and a column with a reversed-phase sorbent ProntoSIL-120-5-C18 AQ (2×75 mm, $\text{\AA}$ 5 μm ; Metrohm AG; Herisau, Switzerland).

Mobile phase: eluent A – 0.2 M LiClO_4 in 0.006 M HClO_4 , eluent B – acetonitrile; elution mode – gradient; Gradient program (%B): 5–100% (0–29 min); mobile phase speed 150 $\mu\text{L}/\text{min}$; column temperature 35 $^\circ\text{C}$; detector wavelengths – 230, 290 nm. The standard samples used in this work were dihydrosamidine and visnadin (Fig. 2) produced by Extrasynthese (France) and SigmaAldrich (USA).

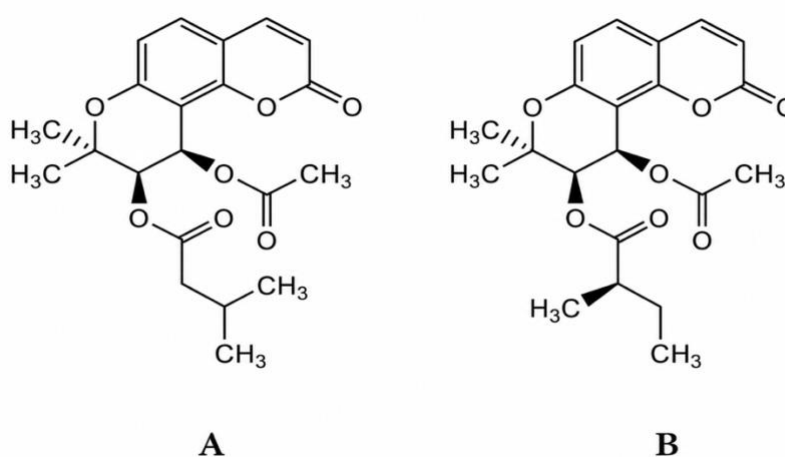


Figure 2. Chemical structures of dihydrosamidine (A) and visnadin (B).

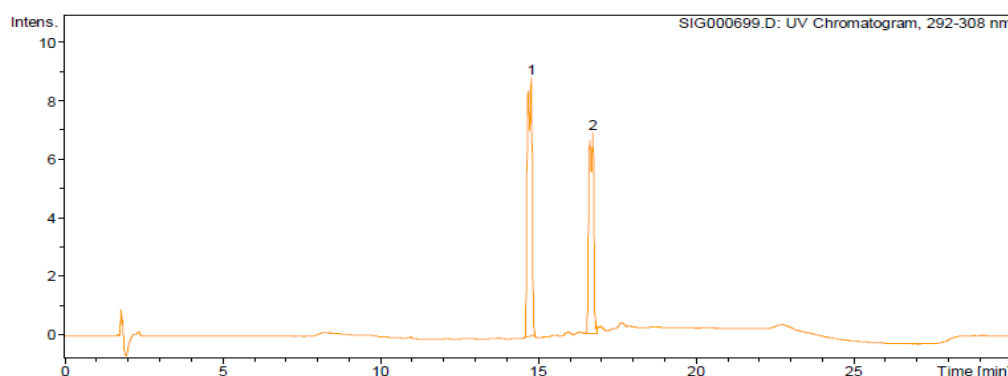


Figure 3. HPLC-UV chromatographic profile of the ethanol extract from roots of *Phlojodicarpus sibiricus*, the number indicates the marker compound of coumarins: 1 – dihydrosamidine, 2 - visnadin.

Animals

The experiments were carried out on 86 male Wistar rats weighing 180-220 g. The animals were maintained in standard laboratory conditions of the certified vivarium at the Institute of General and Experimental Biology SB RAS ($t = 20\text{--}22^\circ\text{C}$, humidity – no more than 50%, air exchange (air intake/outlet) – 8:10, light conditions (day/night) - 1:1) with free access to water. The experimental work followed the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes (Strasbourg, 2010); GOST 33044-2014 “Principles of Good Laboratory Practice” approved by Order of the Federal Agency for Technical Regulation and Metrology No. 1700-st of November 20, 2014 (Russia).

The animals were removed from experiments by instantaneous decapitation under brief ether anesthesia. The design and protocol of research works were approved by the Ethics Committee at the Institute of General and Experimental Biology SB RAS (Minutes No. 1 dated 20.01.2024).

Influence of *P. sibiricus* on the aggregation of platelets *in vitro*

Influence of *P. sibiricus* on the aggregation of platelets was determined with the use of “Agreskreen-test”, Technology-standard LLC (Barnaul, Russia) and the donated blood (Barkagan and Momot 2008). The blood was centrifuged at $200 \times g$ for 10 min to obtain the platelet rich plasma (PRP). The platelet-poor plasma (PPP) was obtained by centrifugation at $1200 \times g$ for 15 min and it was used as a control.

P. sibiricus extract in 25, 50, 100 and 200 $\mu\text{g/mL}$ concentrations in physiologic solution was introduced into PRP ($250 \times 10^9/\text{l}$) and then it was incubated at 37°C for 5 min. After that, the aggregation of platelets was determined: time interval between the moment of adding universal aggregation inductor (UAI) to the tested sample and appearance of thrombocyte aggregates which are visualized under high power in transmitted light.

Influence of *P. sibiricus* extract on the blood coagulation

Influence of *P. sibiricus* extract on the blood coagulation was estimated in the experiments with the use of 6 groups of rats ($n=10$): the 1st group was used as a control, the 2nd, 3rd and 4th groups were experimental ones; the rats of these groups received the distilled water extract of *P. sibiricus* per os in the dose of 50, 100 and 200 mg/kg respectively for 7 days. The rats of the 5th group received aspirin (ASA) in the dose of 30 mg/kg according to the same scheme. To the rats of the 6th group, a single dose (200 U/kg) of heparin solution was administered intravenously 10 minutes before the starting of the experiment. Bleeding was simulated by transverse excision of the tail tip (5 mm) by a razor blade (Wang 2004). The blood in the wound was mopped with the blotting paper every 30 seconds. The time interval from the beginning up to the end of bleeding was considered as the duration of bleeding.

Influence of *P. sibiricus* extract on FeCl_3 -induced thrombosis in the carotid artery

To estimate the antithrombotic activity of *P. sibiricus* extract, the model of FeCl_3 -induced thrombosis in the carotid artery adapted to our research was used (Wang and Xu 2005; Surin 2010).

The rats were divided into 6 groups ($n=6$) where the 1st group was the control one (thrombosis without treatment, water); the 2nd, 3rd and 4th groups received the *P. sibiricus* extract per os in the dose of 50, 100 and 200 mg/kg respectively for 7 days. The rats of the 5th group received aspirin in the dose of 30 mg/kg according to the same scheme. The standard method was as follows: the midline incision was made on the anterior surface of the anesthetized (thiopental sodium, 45 mg/kg) rats' neck; the left common carotid artery was isolated; the piece of the filter paper (0.5×0.5 mm) soaked in 10% FeCl_3 solution was tightly set to the vessel surface for 3 min and then it was removed. Then, the outer surface of the artery was immediately flushed and wiped with the filter paper. After that, the rats were sacrificed under ether anesthesia; the parts of the affected arteries (0.8 cm) were cut up and dried for 24 hours at room temperature. Then, the mass of thrombi in the affected vessel was determined by weighing and subtracting it from the whole mass of the analogous part of the intact artery.

DPPH-radical-binding activity of the *P. sibiricus* extract

The activity of the extract as radical scavenger was estimated spectrophotometrically at $\lambda_{\text{max}} = 517$ nm, using the stable radical 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich) (DPPH) (Brand-Williams et al. 1995). The *P. sibiricus* extract was introduced into the incubating medium at the 0.5, 1.0, 1.5 and 2.0 mg/mL concentrations. The measurements were carried out 30 min after the adding of the tested remedy. The antiradical activity was expressed in the index – IC50 – concentration of the tested remedy when 50% inhibition of DPPH radicals was noted.

Iron chelation assay of *P. sibiricus* extract

The study of the Fe^{2+} -binding activity of *P. sibiricus* extract was carried out by the method based on the ability of the tested remedy to bind Fe^{2+} . The *P. sibiricus* extract was added to the incubating medium at the ascending concentrations: 0.008; 0.04; 0.24; 1.21; 4.88 and 6.66 mg/mL. The Fe^{2+} -binding activity of the tested remedy was expressed in percentage relative to the control and the index of the half-value inhibition IC50 was calculated (Li et al. 2013).

Statistical analysis

The normality of the distribution was assessed by the Shapiro-Wilk test. Results were expressed as means $\pm$ SEM. The standard Student's t-test was used. Values were considered statistically significant at $p < 0.05$. The statistical analysis between groups was carried out by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests.

Results

Influence of the *P. sibiricus* extract on the aggregation of platelets *in vitro*

The *P. sibiricus* extract in 50, 100 and 200 µg/mL concentrations increased the time of the platelet aggregation by 4, 3 and 2 times respectively as compared to the control (Fig. 4). The index of the platelet aggregation in the *P. sibiricus* extract in the 25 mg/mL concentration had much in common with the control that was indicative of the poor effect of the active substances on the platelets.

Thus, it has been established that the antiplatelet activity of the *P. sibiricus* extract *in vitro* reaches a maximum at the 50 µg/mL concentration with its gradual decreasing in the inverse relation.

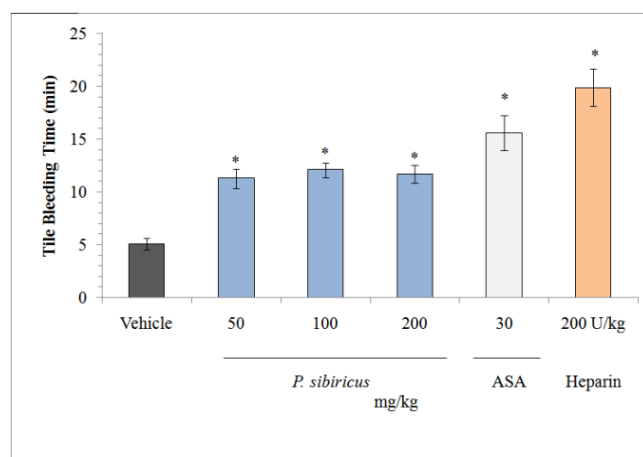


Figure 4. Influence of the *P. sibiricus* extract on the aggregation of platelets *in vitro*. **Note:** Values are presented as mean $\pm$ S.E.M.; significance of the difference in comparison with the control: * – $p < 0.05$; ** – $p < 0.01$.

Influence of the *P. sibiricus* extract on the blood coagulation *in vivo*

Introduction of the extract of *P. sibiricus* in the doses of 50, 100 and 200 mg/kg increased the bleeding duration by 2 times as compared to the control. However, these indices were 25% and 41% lower than in rats administered with aspirin and heparin respectively and the bleeding was 3 and 4 times more prolonged respectively as compared to such in the control (Fig. 5).

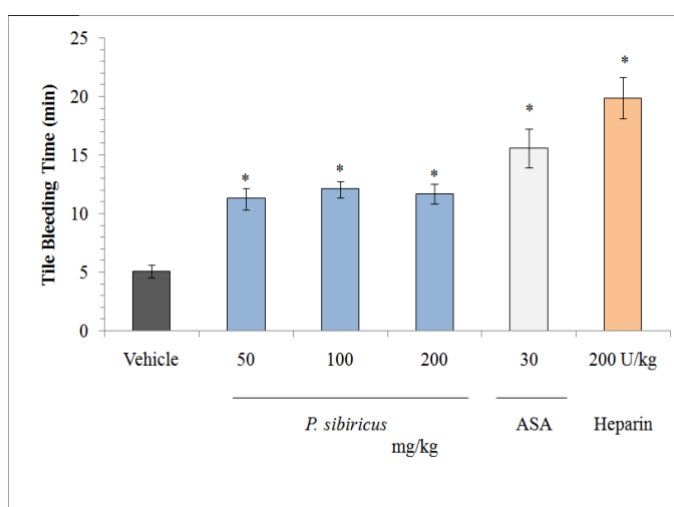


Figure 5. Effect of *P. sibiricus* extract, ASA p.o. and heparin i.v. on tile bleeding time in rats. **Note:** Vehicle (H₂O) and 50, 100 and 200 mg/kg p.o. of extract of *P. sibiricus* was administrated. Values are presented as mean $\pm$ S.E.M.; significance of the difference in comparison with the Vehicle group: * – $p < 0.05$.

Influence of *P. sibiricus* extract on FeCl₃-induced thrombus formation in the carotid artery

Introduction of the extract of *P. sibiricus* in the doses of 50, 100 and 200 mg/kg inhibited the thrombi formation: thrombotic masses were 52.6% less than in the control and comparable with these indices in rats treated with aspirin (Fig. 6).

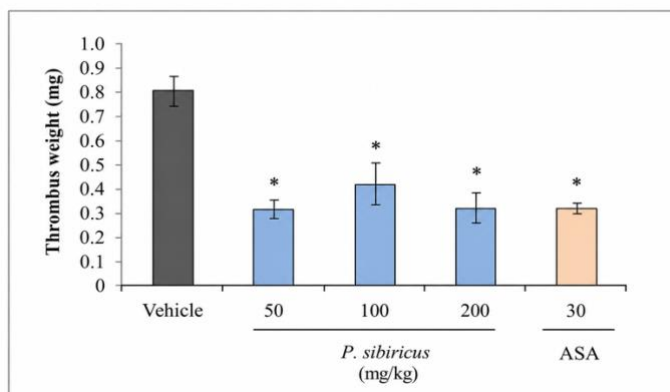


Figure 6. Effect of *P. sibiricus* and ASA on thrombus formation in FeCl₃-induced arterial thrombosis model in rats. **Note:** Vehicle (H₂O), *P. sibiricus* (50, 100, 200 mg/kg), ASA (30 mg/kg). Values are presented as mean ± S.E.M.; significance of the difference in comparison with the Vehicle group: * – $p < 0.05$.

DPPH-radical-binding activity of the *P. sibiricus* extract

It was established that the extract of *P. sibiricus* demonstrated the marked antiradical activity to DPPH-radicals (Fig. 7): the stronger the concentration, the higher was its antiradical activity.

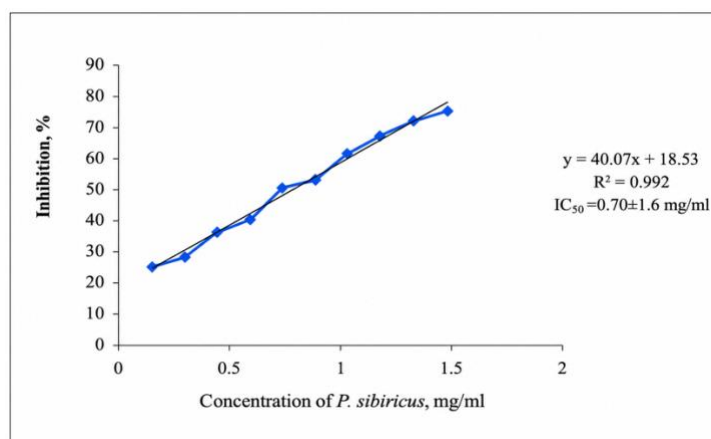


Figure 7. Antiradical activity of *P. sibiricus* extract in DPPH test.

Iron chelation assay of *P. sibiricus* extract

It was established that the addition of *P. sibiricus* extract to the reaction stock decreased the concentration of iron ions that was indicative of its chelating activity. The higher concentration of the extract was, the higher iron chelating activity was noted. IC₅₀ Fe²⁺-chelating activity of the *P. sibiricus* extract was 0.39 mg/mL. The data obtained are presented in Fig. 8.

Thus, the *P. sibiricus* extract demonstrated the marked antiradical activity to DPPH-radicals and Fe²⁺-chelating effect.

Discussion

The *in vitro* experiments have shown that the *P. sibiricus* extract has the effect on the platelet functions, i.e. manifests the maximum antiplatelet activity in 50 µg/mL concentration with the gradual decrease in activity in the inverse relation. The activity decrease with dose escalation of the *P. sibiricus* extract *in vitro* conditions can be deemed due to the background effect of ballast substances on the functional activity of platelets or interference of pharmacological agents with related substances contained in the extract.

The model of the bleeding duration is a simple method for estimation of the primary hemostasis *in vivo* and for testing the remedies which effect adhesion and aggregation of

platelets. It has been found experimentally that the *P. sibiricus* extract has inhibitory influence on the primary hemostasis: the bleeding was significantly longer as compared to such in the control. The duration of the bleeding was obviously increased under the influence of the *P. sibiricus* extract due to the inhibition of platelets adhesion to the injured endothelial layer and their activation that retards the formation of the platelet plug.

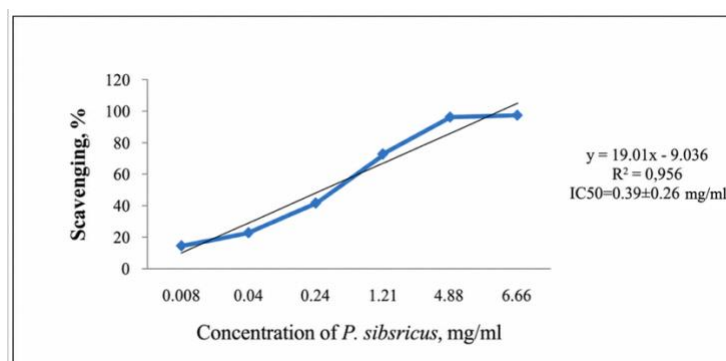


Figure 8. Iron chelation activity of *P. sibiricus* extract.

The carotid artery thrombosis simulation in rats showed that the *P. sibiricus* extract inhibited the thrombus formation in the artery wall. This model is based on the local injury of endothelium due to diffusion of iron ions through the vessel wall. The iron ions are known to cause excessive production of free radicals and irreversible platelet aggregation, which, in turn, leads to thrombus formation (Wang et al. 2004; Li et al. 2013). The antioxidant activity is in the spectrum of antithrombotic effect of the *P. sibiricus* extract. In the experiments which were carried out, namely *in vitro*, DPPH-test and Fe^{2+} -chelating model, the *P. sibiricus* extract demonstrated the marked antioxidant effect. Antioxidant activity of the *P. sibiricus* extract, particularly its Fe^{2+} -chelating property prevented from the formation and growth of the thrombus in the artery wall. The antioxidant activity of the *P. sibiricus* extract is due to the content of coumarins and the presence of ether oils (Kassim et al. 2013). Besides, many coumarin compounds reportedly have antiplatelet effect inhibiting phosphodiesterase and increasing cAMP in platelets (Thastrup et al. 1985). In this connection, it is postulated that the *P. sibiricus* extract has the antithrombotic effect due to the antiplatelet and antioxidant effects of the coumarin and ether oils complex.

Conclusion

The results of our study shows that ethanol extract of *P. sibiricus* has the polymodal antithrombotic property due to the harmonic combination of the antioxidant and anti-platelet effects. The findings have shown that the remedies from this plant may be used for prevention of thrombosis in vascular diseases.

Additional Information

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement

The design and protocol of research works were approved by the Ethics Committee at the Institute of General and Experimental Biology SB RAS (Minutes No. 1 dated 20.01.2024).

Acknowledgement

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Data availability

All relevant data generated and analyzed during this study are included in this article.

Abbreviations

ASA, acetylsalicylic acid; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

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