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Oral treatment with a chemically characterized parsley (*Petroselinum crispum* var. *neapolitanum* Danert) aqueous extract reduces thrombi formation in rats

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ABSTRACT

Petroselinum crispum var. *neapolitanum* Danert (Apiaceae) (PC), popularly known as parsley, is an herb native to the Mediterranean region widely cultivated around the world for culinary and ethnomedicinal purposes. The herb is traditionally used in various parts of the world to treat arterial hypertension, hemorrhoid, nose bleeding, hyperlipidemia, and pain, among other indications. The aim of this study was to evaluate the antithrombotic activity of an aqueous extract PC in rats. Aerial parts of a flat-leaf variety of parsley were extracted by decoction. *In vivo* thrombosis in rat models as well as *ex vivo* assays were used in the evaluation of PC antithrombotic effects. Intravenous administration of PC (25 mg/kg.b.w), 5 min before thrombosis induction, reduced the venous thrombus formation by 98.2%, while oral administration (125 mg/kg.b.w) impaired it by 76.2%. In the arterial thrombosis model, the oral administration of PC at 15 or 25 mg/kg.b.w, 60 min before thrombosis induction, increased the carotid artery occlusion time by 150% (37.0 ± 6.44 min) and 240% (more than 60 min), respectively. A HPLC-DAD-MS/MS profile of PC extract used in this study was provided. Apiin showed to be the most abundant phenolic compound in the extract. It also revealed the presence of many coumaric acid derivatives. Our results indicate that PC is a potential candidate for the development of a phytotherapeutic drug in the treatment of thromboembolic diseases and provide a detailed chemical profile useful for controlling PC extract production in view of phytotherapy.

1. Introduction

Parsley (*Petroselinum crispum* (Mill.) Nym. ex A.W. Hill - Apiaceae) is an herb native to the Mediterranean region and widely cultivated for food and medicinal purposes. People use parsley to combat gastritis, nasal bleeding as well as to treat menstrual disorders, diabetes and cardiovascular diseases, among others. The popular use of the plant to treat cardiovascular diseases has encouraged the investigation of the antiplatelet potential of its phenolic substances.[1–3]

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Abbreviations

CVD	Cardiovascular diseases
DVT	Deep vein thrombosis
HPLC-DAD	High Performance Liquid Chromatography with Diode Array Detection
HPLC-DAD-MS/MS	High Performance Liquid Chromatography with a Diode Array Detector coupled to a Tandem Mass Spectrometry
I.M.	Intramuscular
I.V.	Intravenous
mg/kg.b.w	milligram per kilogram body weight
PBS	Phosphate buffer saline
PC	*Petroselinum crispum* var. *neapolitanum* Danert
PPP	Platelet poor plasma
PRP	Platelet rich plasma
TXA2	Thromboxane A2
w/v	weight per volume
WHO	Word Health Organization

Cardiovascular diseases (CVDs) are the main cause of premature death and are growing worldwide, with high costs for the health system. According to Word Health Organization, 17.9 million people die annually worldwide from cardiovascular diseases, which represent 31% of all global deaths.[4] Atherosclerosis is predominantly associated with CVDs such as coronary artery disease, cerebrovascular disease, peripheral vascular disease and venous thromboembolism.[5]

Although the number of deaths due to CVDs is significant worldwide, investment in drugs for CVDs therapy and prevention have stagnated, mainly due to the high costs of clinical trials. Strategies to search new candidate drugs have been encouraged.[6] Medicinal plants have been used empirically since antiquity and play an important role in primary health care in the less developed nations.[7,8] Natural compounds are the first historical source of antithrombotic compounds (heparin, vitamin K antagonists); molecules obtained from plants provided some of the most original and promising approaches for the discovery of new drugs in this class.[9–12]

In a previous study, we demonstrated that an aqueous leaf extract of *Petroselinum crispum* exhibited a significant antiplatelet activity, with no anticoagulant activity.[3] Gadi et al.[1] evidenced similar effect *ex vivo*. These results incited us to evaluate the antithrombotic effect of PC by means of *in vivo* arterial and venous thrombosis models and *ex vivo* platelet function. We also take in account in this study the correlation between the chemical profile of PC extract and the pharmacological effects observed. The availability of the chemical profile is important since components of plant extracts can vary depending on several biotic and abiotic factors. For this reason, the standardization of the chemical profile is needed to guarantee comparable therapeutic effects.[13]

2. Results and discussion

2.1. Effect of PC on venous thrombosis model by intravenous injection and by oral administration

The thrombus formation was evaluated after venous or oral administration of PC extract. Thrombi weighing 9.3 ± 0.6 mg were formed in control group animals with the administration of the vehicle (PBS buffer). The intravenous administration of 25 mg/kg.b.w, 5 min before thrombosis induction reduced the thrombus formation in about 98.2% (0.2 ± 0.2 mg), compared to the control group (Fig. 1A). The bleeding effect of PC was measured 5 min after the intravenous injection of 25 mg/kg.b.w and compared to control and heparin. The loss of blood was evaluated (Fig. S1A) leading to a minimal loss, especially when compared to an antithrombotic dose of heparin.

In order to evaluate if this extract could impair thrombus formation by oral route, PC at doses of 75 and 125 mg/kg.b.w were administered orally 60 min before thrombosis induction. Only the higher dose was able to reduce the thrombus formation by 76.2% (2.2 ± 1.9 mg) when compared to the control group (Fig. 1B). The anticoagulant activity of PC was assessed in an *ex vivo* model by means of PT and aPTT tests (Fig. S1B), using the same PC doses, administration route and time before blood collection. Our results show that the extract was not able to prolong the coagulation time in any of the assays used, confirming a previous study where PC did not exhibit any *in vitro* anticoagulant effect.[3] We used the same doses that where effective to inhibit thrombus formation. Neither of the two tested doses significantly increased bleeding when compared to heparin (Fig. S1C).

Previous study of Gadi et al.[1] showed that a parsley extract at the dose of 3 g/kg.b.w. doubled the bleeding time; nevertheless, the antithrombotic activity was not determined. Moreover, the chemical composition of their extract as well as the parsley variety used was not presented, which did not allow comparing the results obtained by those authors with ours. In the present study, we show that, at least in terms of weight, doses 20 times lower of PC are capable to block thrombus formation without inducing augmentation of blood loss.

Although the participation of platelets in venous thrombosis is less important when compared to arterial thrombosis, the antiplatelet effect of compounds previously identified in PC may be contributing to the effect on venous thrombosis model.[14]

2.2. Effect of PC on arterial thrombosis model

Rats were administered with PC (10, 15 and 25 mg/kg.b.w) orally 60 min before being submitted to $FeCl_3$-induced carotid artery injury. Fig. 1C shows that the blood flows of control animals (vehicle administration) stopped in about 15 min. Time of occlusion was not significantly different between control group and rats previously treated with PC at 10 mg/kg.b.w. In contrast, at 15 or 25 mg/kg.b.w, it was observed a delayed thrombus formation, increasing the carotid artery occlusion time by 150% (37.0 ± 6.44 min) and 240% (more than 60.0 min), respectively, when compared to the control. The maximum occlusion time was monitored up to 60 min. Note that doses to impair arterial thrombus are lower than those necessary to impair venous thrombus formation. In fact, we needed a 5 times smaller dose in order to lead to an augmentation of the occlusion time as observed for the arterial thrombosis.

Anyway, in these conditions neither doses increased recalcification time tested on platelet poor plasma (PPP). The recalcification time were T = 163.6 ± 5.1 s and T = 164.6 ± 7.5 s for the control group, treated with PBS buffer, and that of the group treated with PC, in the interval of 60 min, respectively. On the other hand, when recalcification time assay was evaluated using platelet rich plasma (PRP), PC increased the recalcification time by 58.2% (T = 123.2 ± 18.2s) as shown in Fig. 1D.

These data corroborate previous findings showing that PC presented significant antiplatelet activity in ADP-induced platelet aggregation without *in vitro* anticoagulant activity.[1,3]

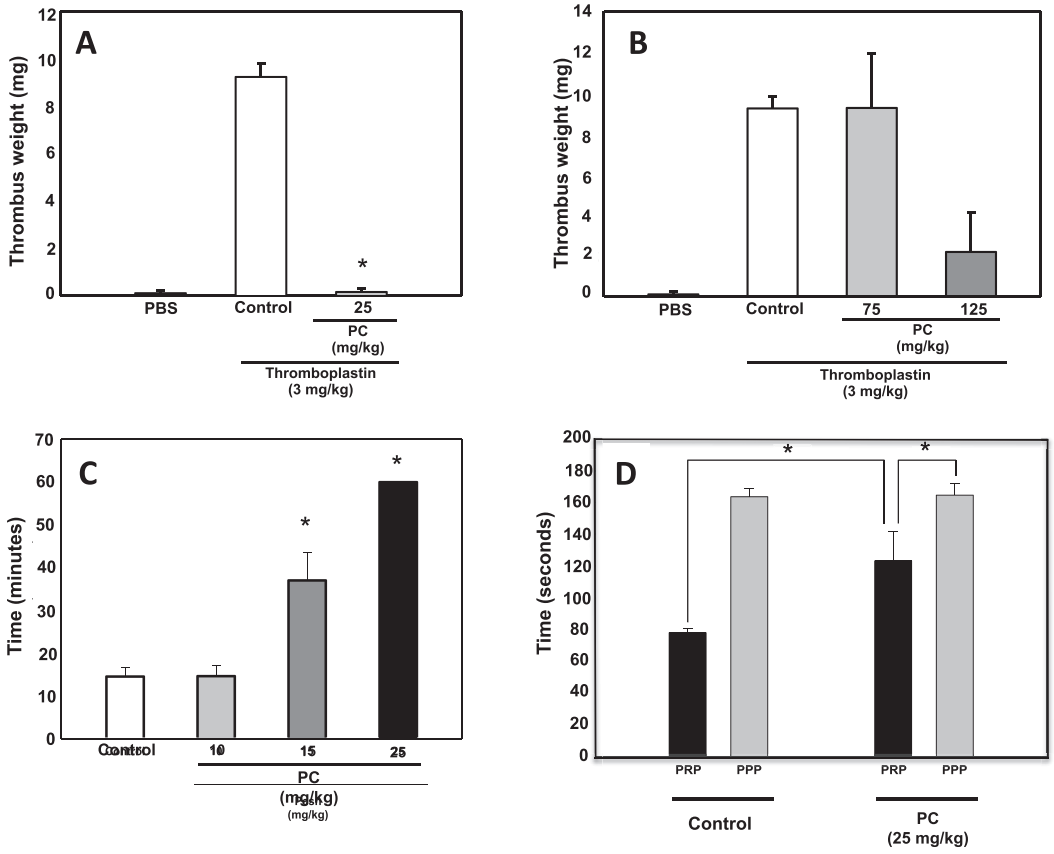

Fig. 1. *In vivo* antithrombotic effect of PC extract.
(A) Effect of PC administered *i.v.* in a stasis-induced venous thrombosis model. PC was given intravenously (25 mg/kg.b.w) as a suspension in saline (vehicle) 5 min before *i.v.* injection of the thrombogenic stimulus. Heparin (5 mg/kg.b.w) was administered *i.v.* Rats (200–250 g body weight, both sexes) were anesthetized with an *i.m.* injection of xylazine (16 mg/kg.b.w) and ketamine (100 mg/kg.b.w). The cava vein was exposed and the thrombotic event was induced by thromboplastin (3 mg/kg.b.w) and stasis. After 20 min, the thrombus was dried and weighed (B) **Effect of PC orally administered in a stasis-induced venous thrombosis model.** PC was given orally (75 and 125 mg/kg.b.w) as a suspension in saline (vehicle) 60 min before *i.v.* injection of the thrombogenic stimulus. Experiment proceeded as described above. (C) **Oral antithrombotic effect of PC in an arterial thrombosis model induced in carotid artery by $FeCl_3$.** PC was given orally (10–25 mg/kg.b.w) as a suspension in saline (vehicle) 60 min before of the thrombogenic stimulus, blood collection and induction of bleeding. Heparin (5 mg/kg.b.w) was administered *i.v.*. Rats were anesthetized as previously described. Thrombus formation was induced by applying a piece of filter paper saturated with 25% $FeCl_3$ solution to the adventitial surface of the artery for 3 min. The flow in the vessel was monitored by an ultrasonic flow probe until complete occlusion occurred. (D) **Effect of PC orally administered in Recalcification Time *ex-vivo*.** Blood citrated was collected, through the carotid, and evaluated for recalcification time tests with PRP and PPP. The results are presented as the mean ± SD of five animals for each time interval tested. * $P < 0.001$.

2.3. Chemical profile of PC

We evaluated the chemical profile of PC extract to ensure the quality control of the herbal preparation used in this study. PC extract had its chemical composition assessed by HPLC-DAD-MS/MS in the negative ion mode. The molecular formula of the major constituents detected was inferred with low mass errors, as the TOF analyzer enables high-resolution mass measurements (Fig. 2, Table S1).

Peaks corresponding to Rt 1.9 and 2.2 min were assigned to common primary metabolites: sucrose and citric acid, respectively. The peaks at Rt 7.4 and 9.9 min were tentatively identified as isomeric glycosidic forms of coumaric acid, for instance the glucose esters of *o*-coumaric acid (melilotoside) and *p*-coumaric acid.[15,16] The former was reported as a putative metabolite in parsley.[17]

The calculated molecular formula for the four peaks at Rt 17.1 min, 17.5 min, 19.1 min and 19.8 min was $C_{24}H_{24}O_{10}$. All of them showed fragments compatible with coumaric acid derivatives. Thus, we hypothesize these compounds could correspond to isomeric dicoumaroylhexosides such as 1,6-di-*O*-*p*-coumaroyl-*β*-D-glycopyranoside.[18] To the best of our knowledge, these dicoumaroyl-hexosides have ever been reported in parsley.

Substances at Rt 8.9 min, 13.2 min and 14.2 min showed UV spectrum and mass fragmentation pattern compatible with flavonoids (Table S1). Their properties are compatible with flavonoids previously described in parsley: isorhamnetin dihexoside (3′-methoxyquercetin dihexoside, peak 8.9 min),[17] luteolin 4′-methyl ether apiosylglucoside (methylated luteolin glycoside, peak 14.2 min),[17,19] and, as expected, apiin (13.2 min), whose structure was unambiguously assigned based on literature data[20] and comparison with the spectrometric and chromatographic properties of this flavonoid previously isolated by our group.[3]

Citric acid, as well as some other organic acids, are known to quelate calcium ions, thus exerting *in vitro* anticoagulant activity. This activity is not observed *in vivo*, as citrate ions are rapidly metabolized into bicarbonates.[21]

Our aqueous extract of parsley aerial parts revealed a prominent content of coumaric acid derivatives (Table S1). Similar substances were detected as minor compounds of PC extracts.[17] There are no reports on hemostasis-related bioactivities for glycosidic forms of coumaric acid, as those tentatively identified in PC. However, these substances probably undergo hydrolysis by *in vivo* metabolism, resulting in free coumaric acid, as often observed for similar cinnamic acid conjugates.[22] *p*-Coumaric acid in its free form has

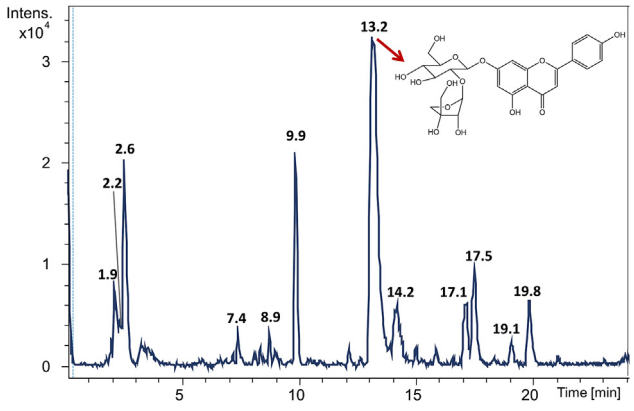

Fig. 2. Base peak chromatogram (LC-MS) of the aqueous aerial parts extract from *Petroselinum crispum* var. *neapolitanum* Danert (PC; 2 mg/mL) in the negative ion mode.

Peak corresponding to apiin, whose structure is shown, is indicated with a red arrow. Proposed structures for major peaks: Rt 1.9 min: sucrose, Rt 2.2 min: citric acid, Rt 2.6 min: unknown, Rt 7.4 min: coumaroyl hexoside I, Rt 8.9 min: methylquercetin dihexoside, Rt 9.9 min: coumaroyl hexoside II, Rt 13.2 min: apiin, Rt 14.2 min: methylluteolin hexoside-pentoside, Rt 17.1 min: coumaroyl derivative I, Rt 17.5 min: coumaroyl derivative II, Rt 19.1 min: coumaroyl derivative III, Rt 19.8 min: coumaroyl derivative IV.

shown *in vitro* antiplatelet activity at the concentration of 500 μM. The antiplatelet effect was also demonstrated *ex vivo* (rabbits, for 2 weeks at 5 mg/kg) without effect on blood coagulation.[23] However, in another study, *p*-coumaric acid produced a decrease of blood clotting time in rats after three days of treatment at 3 mmol/kg (492 mg/kg). It also reduced partial thromboplastin time (aPPT) of rabbits treated with 0.75 mmol/kg (123 mg/kg) for three days.[24] Nevertheless, these effects were observed at higher doses than the one at which antiplatelet activity was shown and may not be relevant for the activity of PC extract.

Apiin, the most abundant flavonoid in PC extract, corresponded to 1.35 ± 0.15% of its dry weight according to the quantification by HPLC-DAD. Previous reports showed that apiin and malonyl-apiin are the major phenolic compounds in different parsley extracts. Other apigenin and luteolin glycosides were also previously identified in PC.[17,19,25]

Despite the fact that the aglycone apigenin is not a major substance in PC extracts,[19,25] there are reports on the presence of apigenin in human plasma and urine after consumption of parsley. In this study, neither HPLC-DAD-MS/MS (Fig. 2) nor ^{1}H NMR (Fig. S2) analyses showed evidence of the presence of apigenin. Thus, we can suppose that apiin and other apigenin glycosides are metabolized to apigenin, its respective aglycone.[26]

Apigenin has been previously recognized as an antiplatelet and antithrombotic agent.[3,27] Additionally, data from aggregation studies evidenced that apigenin exhibited a dose-dependent inhibition of ADP-induced aggregation,[3] collagen and arachidonic acid induced aggregation.[27]

Moreover, the use of formulations based on flavonoid glycosides for the treatment of thrombosis and other circulatory diseases has been proposed.[28]

3. Conclusions

We demonstrated for the first time that an aqueous extract of parsley aerial parts extract has a significant antithrombotic activity in rats, either by intravenous or oral administration, in a deep venous and arterial thrombosis model. Orally, its effect in preventing arterial thrombosis was 5 times more important. Our findings proved that parsley extract is an effective antithrombotic agent, potentially useful for thrombosis prevention by oral route. In resume, the parsley entire phenolic composition rather than an isolated single compound, at least partially, may explain its antithrombotic profile.

4. Material and methods

The experimental section is available in supplementary data.

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CRediT authorship contribution statement

Flávia Serra Frattani: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Mariane Assafim:** Conceptualization, Investigation, Methodology, Writing - original draft. **Livia Marques Casanova:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Jacqueline Elis de Souza:** Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. **Douglas Siqueira de Almeida Chaves:** Data curation, Methodology, Writing - review & editing. **Sônia Soares Costa:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Russolina Benedeta Zingali:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Visualization, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2020.04.003.

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