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

Flávia Serra Frattani a, Mariane Assafim b, Livia Marques Casanova c, Jacqueline Elis de Souza c, Douglas Siqueira de Almeida Chaves d, Sônia Soares Costa c, Russolina Benedeta Zingali b, **

a Laboratório de Hemostasia e Trombose (LHT), Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
b Laboratório de Química de Produtos Naturais Bioativos (LPN-Bio), Instituto de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
c Instituto de Bioquímica Médica Leopoldo de Meis, Programa de Biologia Estrutural, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
d Laboratório de Química de Produtos Naturais, Centro de Ciências Biológicas e da Saúde, Departamento de Ciências Farmacêuticas, Universidade Federal Rural do Rio de Janeiro, 23890-000, Seropédica, RJ, Brazil

** Corresponding author. Instituto de Bioquímica Médica Leopoldo de Meis, UFRJ, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ, 21941-902, Brazil.

* Corresponding author. Instituto de Pesquisas de Produtos Naturais CCS, UFRJ, Cidade Universitária, Ilha do Fundão, Rio de Janeiro, RJ, 21941-902, Brazil.

E-mail addresses: ssccostabh@gmail.com (S.S. Costa), zingali@bioqmed.ufrj.br (R.B. Zingali).

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
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. Atherosclerosis is predominantly associated with CVDs such as coronary artery disease, cerebrovascular disease, peripheral vascular disease and venous thromboembolism.

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.

Medical plants have been used empirically since antiquity and play an important role in primary health care in the less developed nations. Natural compounds are the first historical source of antithrombotic compounds (heparin, vitamin K antagonists); molecules obtained from plants provided some of the most original antithrombotic activity was not determined. Moreover, the chemical composition of their extract as well as the parsley variety used compared to heparin (Fig. S1C).

Previous study of Gadi et al. 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 compared to heparin (Fig. S1C).

### 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.

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).

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### 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 FeCl3-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.
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 C24H24O10. All of them showed fragments compatible with coumaric acid derivatives.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 (30-methoxyquercetin dihexoside, peak 8.9 min),17 luteolin 40-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 data20 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 chelate 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). Their properties are compatible with flavonoids previously described in parsley: isorhamnetin dihexoside (30-methoxyquercetin dihexoside, peak 8.9 min),27 luteolin 40-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 data20 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 reflect 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). Their properties are compatible with flavonoids previously described in parsley: isorhamnetin dihexoside (30-methoxyquercetin dihexoside, peak 8.9 min),27 luteolin 40-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 data20 and comparison with the spectrometric and chromatographic properties of this flavonoid previously isolated by our group.3

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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 (aPTT) of rabbits treated with 0.75 mmol/kg (492 mg/kg). It also reduced partial thromboplastin time (aPPT) of rabbits treated with 0.75 mmol/kg (492 mg/kg). 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.

Apin, 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 apin and malonyl-apin 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 1H NMR (Fig. S2) analyses showed evidence of the presence of apigenin. Thus, we can suppose that apin and other apigenin glycosides are metabolized to apigenin, its respective aglycone.26

Apigenin has been previously recognized as an antiplatelet and antithrombotic agent.27 Additionally, data from aggregation studies evidenced that apigenin exhibited a dose-dependent inhibition of ADP-induced aggregation,2 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

Flavia 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 Elise 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. Sonia Soares Costa: Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. Russolina Benedetta 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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