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ANTI-AGGREGATORY AND ANTI-COAGULANT ACTIVITIES OF *POLYGONUM CUSPIDATUM* EXTRACTS

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Summary

Anti-Aggregatory and Anti-Coagulant Activities of *Polygonum cuspidatum* Extracts

Polygonum cuspidatum, also called Cốt khí củ in Vietnam, is a well-known traditional plant with many approved pharmacological effects. In the current study, various extracts from *P. cuspidatum* rhizomes were tested for their anti-thrombotic activity on human blood for the first time. The platelet aggregation triggered by collagen was measured in human platelet-rich plasma. The blood clotting time was determined using three factors of the coagulation system: activated partial thromboplastin time (APTT), prothrombin time (PT), and thrombin time (TT). The results showed that the ethyl acetate extract (PC-EA) displayed the most potent inhibitory effect against collagen-induced platelet aggregation, with the percentage of inhibition increasing from 28.03% at 0.2 mg/mL to 82.38% at 0.8 mg/mL. Moreover, this extract also expressed a more potent anti-coagulant activity than the *n*-hexane and ethanol extracts ($p < 0.05$). In addition, PC-EA was the only sample that prolonged the blood clotting time in a dose-dependent manner for both APTT and TT assays. However, this extract could slightly extend the PT at 0.8 mg/mL with 13.57 ± 0.48 s compared to 12.13 ± 0.17 s of DMSO 0.1% used as the negative control ($p < 0.05$). The ethanol fraction from 0.4 mg/mL possessed considerably higher APTT and TT values than the negative control, meanwhile, the *n*-hexane display no effects on the blood coagulation by any pathways. In conclusion, the ethyl acetate was the most active fraction from *P. cuspidatum*. Therefore, this extract could provide a source of promising phytoconstituents used in further prevention and treatment of thrombosis-associated diseases.

Keywords: *Polygonum cuspidatum, Anti-aggregatory activity, Anti-coagulant activity, Thrombosis.*

1. Introduction

The World Health Organization (WHO) stated that cardiovascular diseases (CVD) are the leading cause of death globally [1]. Thrombosis, or the formation of blood clots in blood arteries, is the main source of CVD [2]. The main antithrombotics include antiplatelet drugs aiming to reduce platelet aggregation and anticoagulant agents focusing on the suppression of blood clot development. Conventional antithrombotic medications are powerful, however, also linked to several negative consequences [3]. Therefore, seeking new effective antithrombotic agents from natural resources with fewer side effects is required for further treatment of CDV.

Polygonum cuspidatum Sieb. *et* Zucc (*P. cuspidatum*), belonging to the family Polygonaceae, is a well-known medicinal plant largely growing in many Asia countries. In the literature, many pharmacological effects *in vitro* and *in vivo* of this plant have been approved including the anti-inflammatory effect [4], anticancer activity [5], neuroprotective effect [6], and hepatoprotective effect [7]. Moreover, previous studies documented that several polyphenolic compounds such as resveratrol, polydatin, quercetin, emodin, and their derivatives were the key active phytoconstituents in *P. cuspidatum* [8]. A few reports on the cardioprotective action of polydatin have been published, justifying its ability to prevent heart failure and cardiac hypertrophy caused by

pressure overload [9] or to lower the expression of angiotensin and avoid damage from cardiac ischemia [10]. Another study by Gavriil et al. demonstrated that the consumption of a supplement containing *P. cuspidatum* extract decreased significantly the platelet-activating factor-triggered platelet aggregation [11]. However, the inhibitory effect related to the thrombosis-associated diseases of *P. cuspidatum* and its components was still limited.

Therefore, the present study aimed to evaluate the anti-thrombotic effect of *Polygonum cuspidatum* extracts for the first time. This activity is composed of two primary assays investigated on human plasma: the anti-aggregatory and anticoagulant effects.

2. Material and Methods

2.1. Chemicals

Aspirin, collagen, heparin, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Blood clotting reagents including APTT, PT, and TT were obtained from Dade Behring Marburg GmbH (Marburg, Germany).

2.2. Plant material

Dried roots of *Polygonum cuspidatum* were harvested in Thai Nguyen province in February 2023. The plant was identified by Dr. Nguyen Quoc Binh, Vietnam National Museum of Nature, Vietnam Academy of Science and Technology.

One gram of *P. cuspidatum* root powder was soaked twice using sonication for 30 minutes with 10 mL of each solvent with increasing polarity: *n*-hexane (HX), ethyl acetate (EA), and ethanol 80% (ET). The corresponding extracts (PC-HX, PC-EA, and PC-ET) were completely evaporated under reduced pressure and then stocked at 4°C before testing.

2.3. Blood samples preparation

The study received approval from the Ethics Committee of the School of Medicine and Pharmacy at Vietnam National University, Hanoi, Vietnam (document number 02/2020/CN-HĐĐĐ). Healthy participants in the study, aged 18 to 35 years, are nonsmokers, free of any medications for at least three weeks, and have fasted overnight at least 8 hours before the test. Whole blood (10 mL) after drawing from venous was put into test tubes containing 3.2% sodium citrate as the anticoagulant agent. After that, the blood samples were centrifuged for 10 minutes respectively at 500 rpm and then at 3000 rpm to collect platelet-rich plasma (PRP) and platelet-poor plasma (PPP), according to the protocol in the Department of Hemostasis, National Institute of Hematology and Blood Transfusion. All plasma samples were utilized up to three hours after collection.

2.4. Antiplatelet aggregation assay

This experiment was done following the technique of Le et al. [12]. 50 µL of PC-HX, PC-EA, and PC-ET (at a range of concentrations from 0.2 to 0.8 mg/mL in DMSO 0.1%) and 450 µL of PRP were slightly mixed and incubated for 3 min at 37°C. Then, the platelet aggregation was produced by adding 1 µL of collagen at 2 µg/mL. The percentage of aggregation of the negative control (X) and samples (Y) was measured by a Chrono-Log 530 VS aggregator (USA). The inhibition of platelet aggregation (%I) was calculated as follows:

$$\%I = (1 - Y/X) \times 100\%$$

The experiment used DMSO at 0.1% and aspirin at 0.1 mg/mL as the negative and positive controls, respectively.

2.5. Anticoagulant assay

Mixtures containing 50 µL of plant extracts at varying concentrations (final at 0.2, 0.4, and 0.8 mg/mL) and 450 µL of PPP were made, then incubated for five minutes at 37°C. Subsequently, clotting reagents (APTT, PT, and TT) were then added to measure the time that plasma has started to clot. APTT and PT values respectively represent the clotting time produced by the intrinsic and extrinsic pathways, while TT refers to the common pathway in which, fibrinogen was transformed into fibrin to form a blood clot. DMSO 0.1% was used as the negative control. Heparin at 0.2 IU/mL (for APTT and TT parameters) or 2 IU/mL (for PT parameter) was used as the positive control.

All experiments were carried out in triplicate with three different blood samples.

2.6. Data analysis

Results were expressed as the means ± standard deviation. Data were statistically compared using one-way ANOVA tests, and Tukey post-tests in the GraphPad 9.5.0 software. Pearson correlation was analyzed to determine the statistical relationship between concentrations and activities of samples.

3. Results and discussion

3.1. Anti-aggregatory activity of P. cuspidatum extracts

The inhibitory effect of different *P. cuspidatum* extracts on collagen-induced aggregation were demonstrated in Fig. 1. It is noted that all samples exhibited a dose-dependent manner activity from 0.2 to 0.8 mg/mL (Pearson correlation, $r > 0.95$, $p < 0.05$). Among the three fractions, PC-EA was shown to express the highest anti-aggregatory effect, followed by PC-ET and PC-HX. In more detail, at 0.8 mg/mL, the percentage of inhibitions obtained by PC-EA, PC-ET, and PC-HX was 82.38%, 48.61%, and 17.82% respectively. However, all these values were remarkably smaller than aspirin at 0.1 mg/mL used as the positive control ($p < 0.05$).

In 2003, a group of Korean scientists found similar results as indicated in the present study by proving that the ethyl acetate extract from *P. cuspidatum* was able to prevent collagen-induced platelet aggregation more effectively than other extracts [13]. In addition, polydatin, a main bioactive compound isolated from *P. cuspidatum*, was shown to possess a promising anti-platelet aggregation activity [14]. However, a very limited report was published in the literature on the anti-aggregatory effect of *P. cuspidatum* and its phytoconstituents.

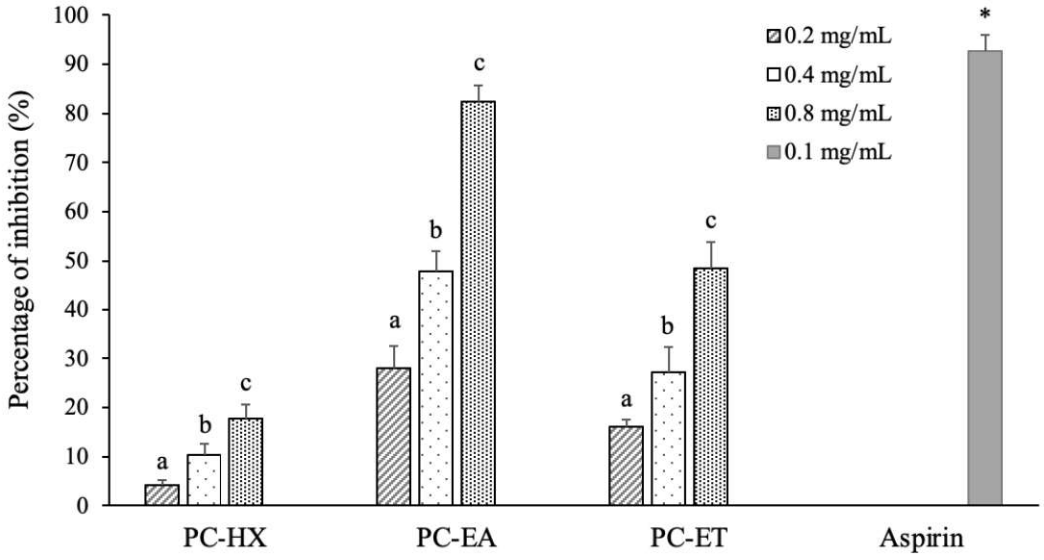

Fig. 1. Inhibitory effect of *P. cuspidatum* extracts on collagen-induced aggregation
PC-HX: the *n*-hexane extract, PC-EA: the ethyl acetate extract, PC-ET: the ethanol extract, aspirin: the positive control.
Different letters indicate significant differences among concentrations of the sample fraction,
*: $p < 0.05$: significant difference between aspirin and other samples.

3.2. Anti-coagulant effect of P. cuspidatum extracts

Like the anti-aggregatory effect, three fractions of *P. cuspidatum* were tested for their anti-coagulant activity at three concentrations of 0.2, 0.4, and 0.8 mg/mL. In this experiment, the blood clotting time was determined by three factors corresponding to three pathways: APTT is a functional measure of the intrinsic pathway of the coagulation system, PT is the time taken to form a blood clot in the extrinsic pathway with the presence of sufficient calcium, while TT measures the time when fibrinogen convert into fibrin in the solid state under the action of thrombin [12]. In the APTT and TT tests, heparin served as the positive control was used at the concentration of 0.2 IU/mL, which was able to prolong the clotting time by 1.5-2 times compared to the negative control. In addition, many previous studies have shown that heparin also influences the extrinsic pathway by increasing the activity of coagulation inhibitors on this pathway [15],[16]. Therefore, after examining several concentrations (including 0.2 IU/mL), the authors found that heparin at a concentration of 2 IU/mL showed the ability to significantly prolong the clotting time in the PT test. Similar results of heparin on the three coagulation pathways were also demonstrated in

several recent articles published by Le et al. [12],[17],[18].

Effect of P. cuspidatum extracts on activated partial thromboplastin time (APTT)

In Table 1, it is seen that PC-HX at all concentrations did not prolong the clotting time compared to the negative control ($p > 0.05$). In contrast, PC-EA expressed a strong anti-coagulant activity from 0.2 mg/mL to 0.8 mg/mL when APTT increased from 34.40 s to 40.81s, respectively. Meanwhile, PC-ET showed significantly higher APTT than DMSO 0.1% only from 0.4 mg/mL (34.15 s) to 0.8 mg/mL (38.71 s). In general, the ethyl acetate fraction possessed the most remarkable anti-aggregatory effect, followed by the ethanol fraction and the *n*-hexane fraction. This finding is also consistent with the result obtained by Nguyen et al. when they investigated similar experiments on *Canna generalis* extracts [17].

Effect of P. cuspidatum extracts on prothrombin time (PT)

In this assay, most of the samples tested were not able to extend the clotting time compared to the negative control (PT 12.13 s, $p > 0.05$). Only PC-EA at 0.8 mg/mL displayed a significant anti-coagulant effect through the extrinsic pathway (PT 13.57s, $p < 0.05$ compared to DMSO 0.1%). However, the clotting time of this extract was still considerably shorter than the one of the positive control, heparin with 31.21 s.

Effect of P. cuspidatum extracts on thrombin time (TT)

Similar to APTT and PT assays, PC-HX expressed no extending effect on blood coagulation's common pathway since it was not able to prolong TT compared to the negative control at all three concentrations tested ($p > 0.05$). Meanwhile, PC-ET only displayed an anti-coagulant activity at 0.8 mg/mL (TT, 18.49 ± 1.25 s vs. 14.31 ± 1.03 s of DMSO 0.1%, $p < 0.05$). Particularly, PC-EA extended the blood clotting time in a dose-dependent manner with TT rising from 13.63 s at 0.2 mg/mL to 21.60 s at 0.8 mg/mL (Pearson correlation, $r > 0.90$, $p < 0.05$). The positive control, heparin at 0.2 IU exhibited remarkably a longer TT (32.30 s ± 1.30) than all samples tested ($p < 0.05$).

Taking into consideration all the results mentioned above, it is worth noting that the ethyl acetate extract was shown to be the most active fraction of *P. cuspidatum* in inhibiting both platelet aggregation and blood coagulation. In our previous documents, the ethyl acetate fraction from different medicinal plants such as *Kaempferia parviflora* [12], *Canna generalis* [17], *Canna edulis* [18], or *Canna warszewiczii* [19], was also more potent than other fractions. In addition, several compounds isolated from the ethyl acetate extract of those plants were justified to be very potent in preventing platelet aggregation and prolonging the blood clotting time. For instance, the active compounds composed of 5,7,4′-trimethoxyflavone and 5-hydroxy-3,7,4′-trimethoxyflavone from *K. parviflora*; 4-ketopinoresinol and indole-3-carboxylic acid from *C. generalis*; epimedokoreanone A and nepetoidine B from *C. edulis*. In the current study, *P. cuspidatum* could serve as a source of phytoconstituents having potential in the treatment of thrombosis-associated diseases. In the upcoming studies, molecular docking analysis will be investigated to predict the potential interactions between the main components in *P. cuspidatum* and target receptors in the blood coagulation pathways. The results will help find the bioactive compounds from this plant and their mechanism of action.

Table 1. Anti-coagulant activity of *P. cuspidatum* extracts

Sample	Concentration (mg/mL)	APTT (s)	PT (s)	TT (s)
PC-HX	0.2	28.70 ± 1.08 (#)	11.76 ± 0.59 (#)	14.65 ± 0.52 (#)
	0.4	29.63 ± 0.24 (#)	12.08 ± 0.81 (#)	14.38 ± 0.64 (#)
	0.8	31.05 ± 0.69 (#)	12.16 ± 0.29 (#)	14.59 ± 0.50 (#)
PC-EA	0.2	34.40 ± 2.11 (*)(#)	12.20 ± 0.92 (#)	13.63 ± 0.87 (#)
	0.4	37.27 ± 1.35 (*)(#)	12.76 ± 0.59 (#)	17.72 ± 1.31 (*)(#)
	0.8	40.81 ± 1.04 (*)(#)	13.57 ± 0.48 (*)(#)	21.60 ± 1.47 (*)(#)

Sample	Concentration (mg/mL)	APTT (s)	PT (s)	TT (s)
PC-ET	0.2	31.05 ± 0.59 (#)	12.90 ± 1.16 (#)	14.75 ± 0.89 (#)
	0.4	34.15 ± 1.16 (*)(#)	11.60 ± 0.47 (#)	15.26 ± 0.38 (#)
	0.8	38.71 ± 1.73 (*)(#)	12.01 ± 0.85 (#)	18.49 ± 1.25 (*)(#)
DMSO	0.1%	30.95 ± 1.17 (#)	12.13 ± 0.17 (#)	14.31 ± 1.03 (#)
Heparin	0.2 IU	47.01 ± 1.90		32.30 ± 1.30
	2 IU		31.21 ± 0.82	

APTT: activated partial thromboplastin time, PT: prothrombin time, TT: thrombin time, PC-HX: the *n*-hexane extract, PC-EA: the ethyl acetate extract, PC-ET: the ethanol extract, DMSO: the negative control, heparin: the positive control, *: $p < 0.05$ compared to the negative control, #: $p < 0.05$ compared to the positive control.

4. Conclusion

Different extracts from *Polygonum cuspidatum* were evaluated for their anti-aggregatory and anti-coagulant activities for the first time. It was shown that the ethyl acetate fraction possessed the highest inhibitory effect against collagen-induced platelet aggregation. In addition, this extract was able to prevent blood coagulation through all tested pathways by elongating APTT, PT, and TT values in comparison with the negative control. The ethanol extract only extended two factors APTT and TT, while the *n*-hexane did not provide any changes in the blood clotting time. In conclusion, the ethyl acetate exhibited better anti-thrombotic activity than the ethanol and the *n*-hexane extracts. Therefore, further investigation on the ethyl acetate extract needs to be carried out to seek active phytochemicals and their mechanism of action in the treatment of cardiovascular diseases related to blood clot formation.

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POTENTIAL ACTIVITIES OF *ARTEMISIA VULGARIS* METHANOL EXTRACT AGAINST NTERA-2 CANCER STEM CELLS

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Summary

Potential Activities of *Artemisia vulgaris* Methanol Extract Against NTERA-2 Cancer Stem Cells

Cancer stem cells are the major cause of tumor initiation and metastasis, which can lead to drug resistance and recurrence in cancer patients. The search for natural chemicals that can inhibit the development of cancer stem cells is of interest to many scientists. *Artemisia vulgaris* L. (in Vietnamese named Ngai cuu) is known for traditional medicine to treat diseases such as diabetes, antiseptic, stomach aches, and cancer prevention. This study aims to evaluate the biological effect of methanol extract from *A. vulgaris* on the NTERA-2 cancer stem cell line. The activity of the extract was illustrated through cytotoxicity assay using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) method on monolayer and three-dimension cell culture models and *in vitro* migration assay. The extract of *A. vulgaris* exhibited inhibitory activity on NTERA-2 cells with an IC_{50} value of 18.31 µg/mL and reduced spheroid formation at the concentration of 500 µg/mL. Besides, this extract significantly reduced cell migration ability at the concentration of IC_{50} and $2xIC_{50}$. Consequently, *A. vulgaris* crude methanol extract presented obvious effects on NTERA-2 cancer stem cells, providing a new direction for investigating natural compound ingredients of this plant for more efficient cancer treatment.

Keywords: *Artemisia vulgaris, NTERA-2, Cytotoxicity, Tumorsphere formation assay, Scratch assay.*

1. Introduction

Cancer stem cells (CSCs) are a subpopulation of tumor cells with characteristics of both stem cells and cancer cells: proliferation, self-renewal, and differentiation. CSCs are thought to be associated with tumor recurrence and metastasis. Although chemotherapy and radiation can suppress large cancer populations and shrink tumors, those are not sufficient to eliminate thoroughly CSC populations. Therefore, residual CSCs would initiate and develop new tumors. The most effective strategy for cancer treatment was to combine drugs that target non-CSC and CSC tumor cells, respectively [1]. The discovery of new chemotherapy drugs to prevent tumor recurrence is necessary to overcome drug resistance caused by CSCs.

Artemisia vulgaris L. *(*AV*)*, a popular species belonging to the *Artemisia* genus from the Asteraceae family, distributes widely in natural habitats worldwide, particularly in tropical countries such as China, Indonesia, Vietnam… [2]. This plant contains various rich chemical compositions such as sterols, flavonoids, phenolic acids, and coumarins which are responsible for many biological activities. In ancient times, *Artemisia vulgaris* L. has been traditionally used to treat menstruation- and pregnancy-related ailments, heal wounds, gout, leg tiredness, and fever [3]. At present, a lot of scientific researchers have proved that this species possesses antioxidant [4], antihypotensive [5], anticancer [6], anti-inflammatory [7], antifungal [8], and antibacterial activities [9].

However, no reports exhibit the biological effect of the crude methanol extract from *Artemisia vulgaris* L. (VAM) on cancer stem cells. Therefore, in this project, we study the potential effects of VAM against proliferation, stemness, and migration properties of NTERA-2 pluripotent