Antithrombotic activity of *Juglans regia* root bark

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Abstract:

Thrombus formation in cardiovascular diseases is one of the important mechanisms responsible for death and disability. Plants have long been used as traditional remedies for the treatment of many diseases.

In this study, we investigated the antithrombotic, antiaggregant, and anticoagulant activities of flavonoids isolated from *Juglans regia* root bark.

The antithrombotic activity was evaluated on collagen plus epinephrine induced acute pulmonary thromboembolism model of mice and murine platelet aggregation was induced by different agonists. Clotting parameters including activated partial thromboplastin time, prothrombin time, and thrombin time in the platelet-poor plasma were determined *in vitro* by using a coagulometer in the presence and absence of flavonoid fractions.

HPLC analysis of two flavonoids-rich fractions revealed the presence of Galic acid and Rutin and the amount of phenolic and flavonoids compounds were higher for both fractions.

Flavonoids extracted from *Juglans regia* exhibited an antithrombotic activity (80% of protection) by preventing significantly ($p<0.01$) the paralysis or death induced by an intravenous injection of collagen and epinephrine. In addition, these compounds significantly inhibited platelet aggregation ($p<0.001$) and prolonged the three plasmatic coagulation times ($p<0.05$; $p<0.01$; $p<0.001$).

Flavonoids isolated from *Juglans regia* exert a potent antithrombotic effect *via* antiaggregant and anticoagulant actions. *Juglans regia* could be a precious source of antithrombotic compounds.

Keywords: Acute thromboembolism; Coagulation; Flavonoids; *Juglans regia*; Platelet aggregation.

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1. Introduction

Related to a vascular injury, an hemorrhage (bleeding) is generated, and in order to stop this flow a physiological response occurs by forming a blood clot, called hemostasis. In this process, the platelets play a crucial role (primary and secondary hemostasis). However, under abnormal physiological conditions, hemostasis may also cause thrombosis (Hou et al. 2015).

Thrombotic diseases represent a major cardiovascular complication that affects patients across the globe (Kim et al. 2011). A variety of antithrombotic drugs, including antiplatelet (aspirin, clopidogrel and ticlopidine), anticoagulant (warfarin and heparin), and fibrinolytic (streptokinase and tissue plasminogen activator) have been developed to prevent or treat thrombus formation (Gogoi et al. 2018 ; Zhou et al. 2019). Nevertheless, these remedies have side reactions such as gastrointestinal symptoms and hemorrhage (Zhou et al. 2019).

Medicinal plants are an abundant source of new bioactive molecules. Isolation and identification of pharmacologically active compounds from plants continue today. Development of antithrombotic agents from these plants has little side effects and has attracted much interest among researcher community (Wang et al. 2011).

Among the components synthesized by plants, there is a large family of polyphenolic elements called flavonoids. They provide several benefits for human health because of their high pharmacological activities (Yao et al. 2004). It was found that flavonoids such as quercetin, rutin, and hesperidin are most commonly studied for their cardiovascular effects. Furthermore, resveratrol has shown an anti-atherosclerotic and antithrombotic effect by inhibiting adhesion, activation, and aggregation of platelet (Du et al. 2016). In addition, flavonoids might be a promising source of novel inhibitors against thrombus formation (Choi et al. 2015a).

Juglans regia (*J. regia*) L. (Walnut) belonging to the family of Juglandaceae, has been applied in traditional medicine since ancient times (Nael Abu and Al-wadaan 2011). All parts of the plant, such as root, stem, bark, leaves, and seeds, are medicinally important and are used in folk medicine with depurative, anthelmintic, laxative, detergent, astringent, diuretic and antimicrobial activity (Deshpande and Salvekar 2011). It is cultivated throughout eastern Asia, southern Europe, northern Africa, the United States, and western South America. The root bark of *J. regia*, locally known as ‘Swak’ in Morocco, is used for cleaning and sparkling

teeth. Moreover, phytochemical studies showed the presence of bioactive compounds such as phenolic compounds, particularly flavonoids (Zhao et al. 2014 ; Santos et al. 2013).

There are a few studies on the effect of *J. regia* on hemostasis. An *in vitro* anti-platelet effect of *J. regia* hull extract has been reported (Meshkini and Tahmasbi 2017). Furthermore, we recently found an *in vitro* antiaggregant and anticoagulant effect of the crude aqueous extract of *J. regia* root bark (Amirou et al. 2018).

To continue our investigation, in this study, we tried to isolate flavonoids from *J. regia* root bark and explore their antithrombotic effect against an experimental thromboembolism model *in vivo* and then tried to elucidate their action mechanism by exploring primary (platelet aggregation) and secondary hemostasis (plasmatic coagulation).

2.Materials and methods

1.1. Chemicals

The standards (rutin, kaempferol, quercetin, catechol, and gallic acid) were obtained from Sigma (USA). Methanol and formic acid were obtained from Merck (Darmstadt, Germany). Arachidonic acid was purchased from CALBIOCHEM (USA), adenosine-5'-diphosphate (ADP) from Verum Diagnostica GmbH (Germany), thrombin from Sigma (Germany), collagen calf skin type III from Sigma (USA) and epinephrine from Acros Organics, NJ, USA.

1.2. Experimental animals

Mice were used for *in vivo* thrombosis model experiments. Wistar rats were used for platelet aggregation and plasmatic coagulation tests. Animals were provided from Faculty of Sciences, Oujda, Morocco) and were bred and housed under pathogen free conditions. They were kept under laboratory conditions: 12 h light/dark cycle, temperature (22 ± 2) °C and with a standard diet and water.

The study was conducted according to the NIH guidelines for the Care and Use of Laboratory Animals established by the US Department of Health and Human Services (NIH publication no 85–23, revised 1985).

1.3. Collection and identification of plant material

The plant material (root bark of *J. regia*) was collected from M'semrir (province of Tinghir in the Southern Morocco). Professor Mohammed Fennane from the Scientific National Institute

(Rabat, Morocco) performed authentication. A voucher specimen (HUMPO 149) was deposited in the herbarium at University Mohammed the First (Oujda, Morocco).

1.4. Flavonoids extraction

The extraction of flavonoids was done according to the method of Akroum et al. (2010). The roots bark (250 g) was dried and grounded into powder by using a blender. The obtained powder was macerated with ethanol-water (80:20, v/v) three times for 3 days at room temperature. The solvent was evaporated at 45 °C under reduced pressure (Rotavapor, Heidolph Instruments, Germany). The obtained fraction was mixed with distilled water (200 mL).

Afterward, the water-soluble fraction was extracted three times with solvents (1:1, v/v), and concentrated under vacuum. The first extraction was performed with petroleum ether to take out the non-phenolic compounds. The second was done with diethyl ether to extract the aglycones or free flavonoids (FAF) and the last extraction was carried out with the ethyl acetate, which extracted the heterosidic flavonoids (FHF). The extraction yield was 0.24% and 0.92% for aglycones and heterosidic flavonoids, respectively. The aglycones and heterosidic flavonoids were solubilized in distilled water and kept at -20 °C until use.

1.5. Determination of total phenolic content

The total phenolic content was determined by Folin-Ciocalteu assay following the method described by Noumi et al. (2011) with slight modifications. An aliquot (0.5 mL) of sample extract was added to 0.25 mL of the Folin-Ciocalteu reagent, and 0.5 mL of Na_2CO_3 solution (2 g/100 mL). The amount of 3.75 mL of distilled water was added to adjust the final volume at 5 mL and was mixed thoroughly. After incubation of all samples for 90 min at 23 °C in dark, the absorbance was measured at 750 nm against a blank containing 0.5 mL of water, 0.25 mL of the Folin-Ciocalteu reagent and 0.5 mL of Na_2CO_3 solution, by using a spectrophotometer (Rayleigh Vis-7220G, Beijing, China). Gallic acid was used as a standard and the total phenolic content was expressed as mg of gallic acid equivalents per 1 g of the dry extract sample. The content of total phenolic was taken in triplicate for all samples.

1.6. Determination of total flavonoids

The total flavonoid content was determined according to the aluminium chloride colorimetric method (Gloria A Ayoola et al. 2008) with slight modifications. Briefly, 1 mL of 2% $AlCl_3$ in methanol was added to 1 mL of the test sample. After 30 min incubation at room temperature, the absorbance of the reaction mixture was determined spectrophotometrically at 415 nm.

Rutin was used as a standard and the total flavonoid content was expressed as mg of rutin equivalents per 1 g of dry extract. The analyses were performed in triplicate.

1.7. HPLC analysis of phenolic and flavonoids compounds

The analysis of sample was carried out by using HPLC "Waters Alliance 2695 system (Waters Co., Milford, MA, USA)" with C18 (250 mm × 4.6 mm, particle size 5 µm) reversed phase column. The plant extract (20 µL) was separated at the room temperature and a flow rate of 1 mL/min using two phases: the mobile phase A composed of 1 % of formic acid and Milli-Q water [10:90 (v/v)] and the phase B composed of Milli-Q water, acetonitrile and formic acid [40:50:10 (v/v/v)]. Samples were eluted with the following gradient: 0 min: 88% A+12% B, 20 min: 70% A+30% B, 30 min: 0% A+100% B, and 45 min: 88% A+12% B. All the samples and mobile phase were filtered through 0.45 µm membrane filter (Millipore). Solutions of standard references were prepared at a concentration of 5 mg/mL for rutin, kaempferol, quercetin, catechol, and gallic acid. Quantification was carried out by integration of the peaks using the external standard method at 283 nm for gallic acid, catechol and at 365 nm for rutin, kaempferol, and quercetin.

1.8. Collagen and epinephrine induced acute thromboembolism model

Acute thromboembolism was induced on male mice according to the method of Mekhfi et al. (2012). For this study, heterosidic flavonoid-rich fraction was chosen because of its high yield rate and high solubility in distilled water.

Three experimental groups were studied (n=10). Group 1 was treated with distilled water (control, 1 mL/100 g body weight/day), group 2 was given 250 mg/kg/day of heterosidic flavonoid-rich fraction and group 3 was treated with acetylsalicylic acid (ASA, positive control, 30 mg/kg/day). All treatments were performed orally for one week. One hour after the last treatment, pulmonary thromboembolism was induced by injecting a mixture of collagen (12 mg/kg) and epinephrine (1 mg/kg) into the tail vein. The behavior of animals was observed for 15 min and the mortality and/or paralysis (loss of function of the hind limbs) of mice was noted. Surviving mice were sacrificed immediately after the experiment.

The results were reported as the percentage of protection against collagen and epinephrine induced thrombosis, and expressed as:

Protection (%) = [1 – (number of animals paralyzed or dead / number of total animals)] × 100

1.9. Histology of lungs

Samples of the lung from mice were collected and fixed in 10% formaldehyde. The tissue specimen was embedded in paraffin and sectioned in 5 µm thickness using a microtome. The histology slides were stained by hematoxylin and eosin and then analyzed for the presence of thrombo-emboli within the pulmonary vasculatures. Samples were then visualized using a light microscope coupled to a camera and a computer (Optika microscope, Italy).

1.10. Platelet aggregation

1.10.1. Preparation of murine washed platelets

The platelet aggregation study was carried out according to a method reported by Amirou et al. (2018). Rats weighing 150-250 g were lightly anesthetized with ether. Blood was collected from the abdominal aorta under an anticoagulant: acid-citrate-dextrose (9:1, v/v) (citric acid 130 mM, trisodium citrate 170 mM, dextrose 4%). Blood was centrifuged for 15 min at 250 g to obtain platelet-rich plasma. Platelet-rich plasma was centrifuged for 8 min at 72 g to eliminate residual blood cells, and for 15 min at 820 g to obtain the platelet pellet. Thereafter, platelets were washed with washing buffer (NaCl 137 mM, KCl 2.6 mM, $NaHCO_3$ 12 mM, $MgCl_2$ 0.9 mM, glucose 5.5 mM, gelatin 0.25%, pH 6.5). After the last centrifugation (15 min, 820 g), the platelets were resuspended in the final buffer (NaCl 137 mM, KCl 2.6 mM, $MgCl_2$ 0.9 mM, glucose 5.5 mM, gelatin 0.25%, HEPES 5 mM, pH 7.4). For all experiments, washed platelets were adjusted to a constant concentration of 5×10^5 cells/mm^3 using a hemocytometer for cell counts and allowed to rest at 37°C for 30 minutes before platelet aggregation tests.

1.10.2. Measurement of platelet aggregation

Platelet aggregation was monitored on a four-channel optical aggregometer (Helena Laboratories, Beaumont, Texas USA) at 37 °C and under continuous stirring (600 rpm). Before each experiment, the aggregometer was calibrated by adjusting the light transmission to 0% with the blank (washing buffer). For the control, an aliquot of washed platelets was preincubated in the aggregometer cuvette for 1 min. Then the platelet aggregation was triggered by various platelet agonists: collagen (5 µg/mL), thrombin (0.5 U/mL), ADP (5 µM), or arachidonic acid (20 µM). For the fraction, washed platelets were preincubated with different concentrations of FHF or FAF for 1 min in the aggregometer cuvette before being stimulated with the same aggregating agents. Aggregation signals were recorded for at least 5 min and the maximum

aggregation (%) was given systematically by the aggregometer software (HemoRAM). The anti-aggregating amount (inhibition, %) was calculated according to the following formula:

Inhibition (%) = [(A-B)/A)] ×100, where A is the maximum aggregation of the control (without extract), and B is the maximum aggregation of washed platelets treated with the fraction of *J. regia*.

1.11. Plasmatic coagulation assay

In vitro clotting assays were carried out for determination of the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) using a semi-automatic coagulometer (Thrombostat, Behnk Elektronik, Norderstedt, Germany) according to the manufacturer's recommended protocols. In this part, the blood of rats collected from the abdominal aorta directly into citrated tubes (trisodium citrate 3.8%, 1/9; v/v) was centrifuged at 3000 rpm for 20 min, and the platelet-poor plasma was removed. Plasma mixtures [100 µL of the platelet-poor plasma added with 50 µL of different concentrations of flavonoids-rich fractions of *J. regia* (0.25; 0.5 and 1 mg/mL) or 50 µL of distilled water (control)] were incubated at 37 °C for 5 min before determination of APTT, PT, and TT.

For APTT assay, 50 µL of reagent (C.K PREST ®, Stago, France) added to 50 µL of the plasma mixture were incubated at 37 °C for 180 s. The reaction was initiated by the addition of 50 µL of calcium chloride (25 mM) and the APTT (s) was determined.

For TT assay, plasma mixture (50 µL) was incubated at 37 °C for 120 s. The reaction was started by adding 100 µL of reagent (NEOPLASTINE®CI, Stago, France) and the clotting time (s) was recorded.

For PT assay, plasma mixture (50 µL) was incubated at 37 °C for 60 s. The reaction was triggered by 100 µL of thrombin (Test Thrombin Reagent, Dade Behring, Marburg, Germany) and the TT (s) was determined.

Heparin (0.4 U/ml) was used as positive control for all coagulation test (APTT, PT, and TT).

All clotting time assays were performed with five replicates to estimate the mean clotting time.

1.12. Statistical analysis

Statistical analysis data were performed using GraphPad Software (version 5.01, GraphPad Software, Inc.). For thromboembolism study, the *Chi*-square test was used to determine significant differences between the control group and the groups treated with flavonoids-rich or ASA. For the other studies, the results were expressed as mean ± SD. Differences between

values were analyzed using two-way and analysis of variance (ANOVA) followed by Bonferroni's post-test. *: $P < 0.05$, **: $P<0.01$ ***: $P<0.001$, NS: $P > 0.05$, vs Control group.

2. Results

2.1. Total Polyphenol and flavonoid content of *J. regia* fractions

Flavonoids-rich fractions were analyzed for its phenolic content by using Folin-Ciocalteu reagent and for flavonoids by using aluminum chloride. Total polyphenol contents of fractions were 434.0 ± 6.7 and 460.4 ± 39.9 mg gallic acid equivalent/g of extract for the FAF and FHF, respectively. The flavonoid contents were 60.0 ± 1.5 and 50.0 ± 2.0 mg rutin equivalent/g of extract for the FAF and FHF, respectively.

2.2.HPLC analysis of flavonoids-rich fractions

The identification of the compounds of *J. regia* fractions was performed by comparing their retention times with those of standards (Figure 1). The HPLC analysis showed that the chromatographic peaks of heterosidic flavonoid (Figure 2: A and B) and aglycones flavonoid-rich fractions (Figure 2: C and D) were similar. Gallic acid and rutin as the major compounds were present in both fractions. However, other peaks remained to be identified by using other standards.

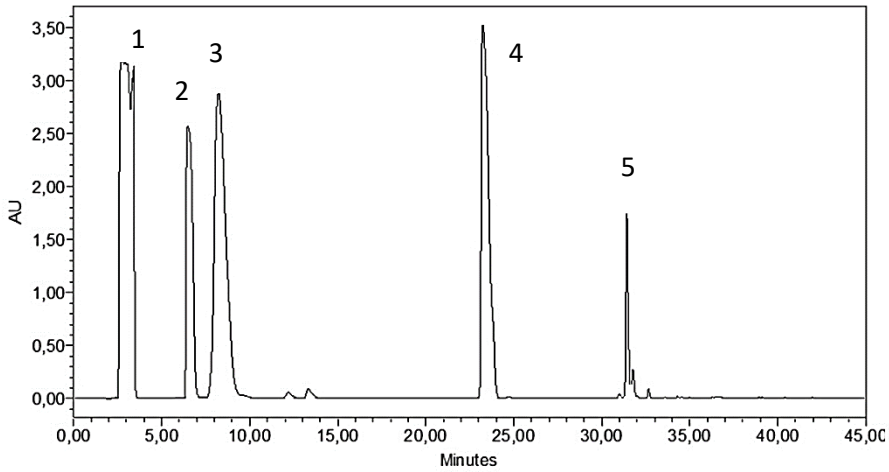

Figure 1. HPLC chromatographies of standards.

The peaks correspond to gallic acid (1), catechol (2), rutin (3), quercetin (4), and kaempferol (5). With retention time 2.78 min, 6.49 min, 8.31 min, 23.32 and 31.45 min, respectively.

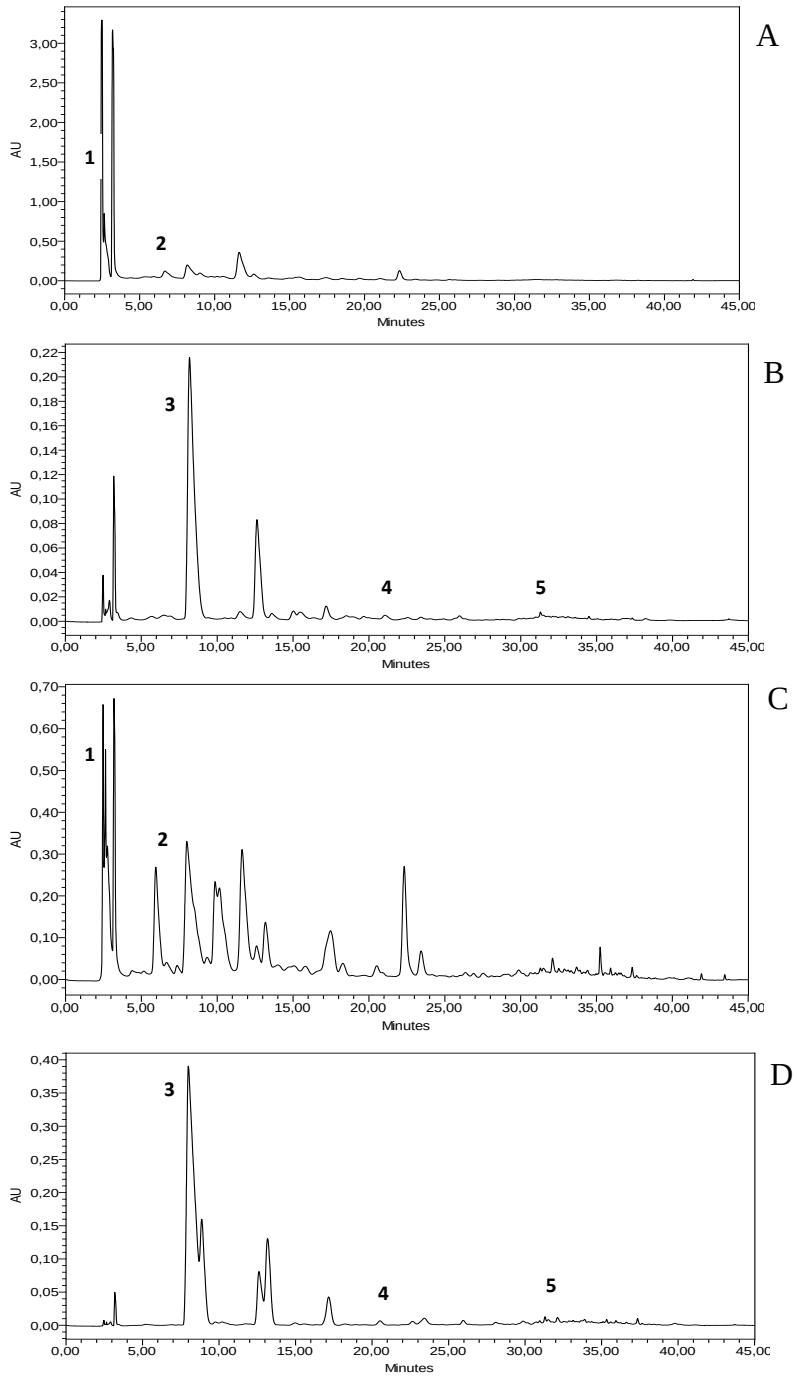

Figure 2. Chromatographic analysis of heterosidic flavonoids-rich fraction (A, B) and aglycone flavonoids-rich fraction (C, D) of *J. regia* obtained at 283 nm (A, C) and 365 nm (B, D).
The peaks correspond to Gallic acid (1), catechol (2), rutin (3), quercetin (4), and kaempferol (5).

2.1. Antithrombotic effect of FHF

In vivo antithrombotic activity was evaluated using the collagen and epinephrine induced acute thromboembolism model of mice. As shown in Table 1, for the negative control group, the intravenous injection of the thrombogenic mixture of collagen and epinephrine caused death and/or paralysis in 90% mice within 15 min (only 10% of protection), whereas FHF at 250 mg/kg/day was significantly effective ($P < 0.01$) in preventing collagen-epinephrine-induced thromboembolic death (80% of protection). As expected, ASA showed a significant protective effect of 80%.

Table 1. Effect of heterosidic flavonoid-rich fraction (FHF) on thromboembolism induced by collagen plus epinephrine in mice.

Experimental groups	Paralyzed or dead Animal number/total	Protection (%)
Negative control (distilled water, 1 ml/100 g/day)	9/10	10
Positive control (ASA, 30 mg/Kg/day)	2/10**	80
FHF (250 mg/Kg/day)	2/10**	80

** $p < 0.01$ as compared to negative control. ASA: Acetyl salicylic acid. FHF: heterosidic flavonoid-rich fraction. (n=10).

Histological study showed that the control animals injected with thrombogenic substances displayed some lung vessels totally or partly occluded by thrombi. In contrast, the mice treated with ASA (30 mg/kg/day) and with FHF (250 mg/kg/day) showed normal blood vessels (Figure 3).

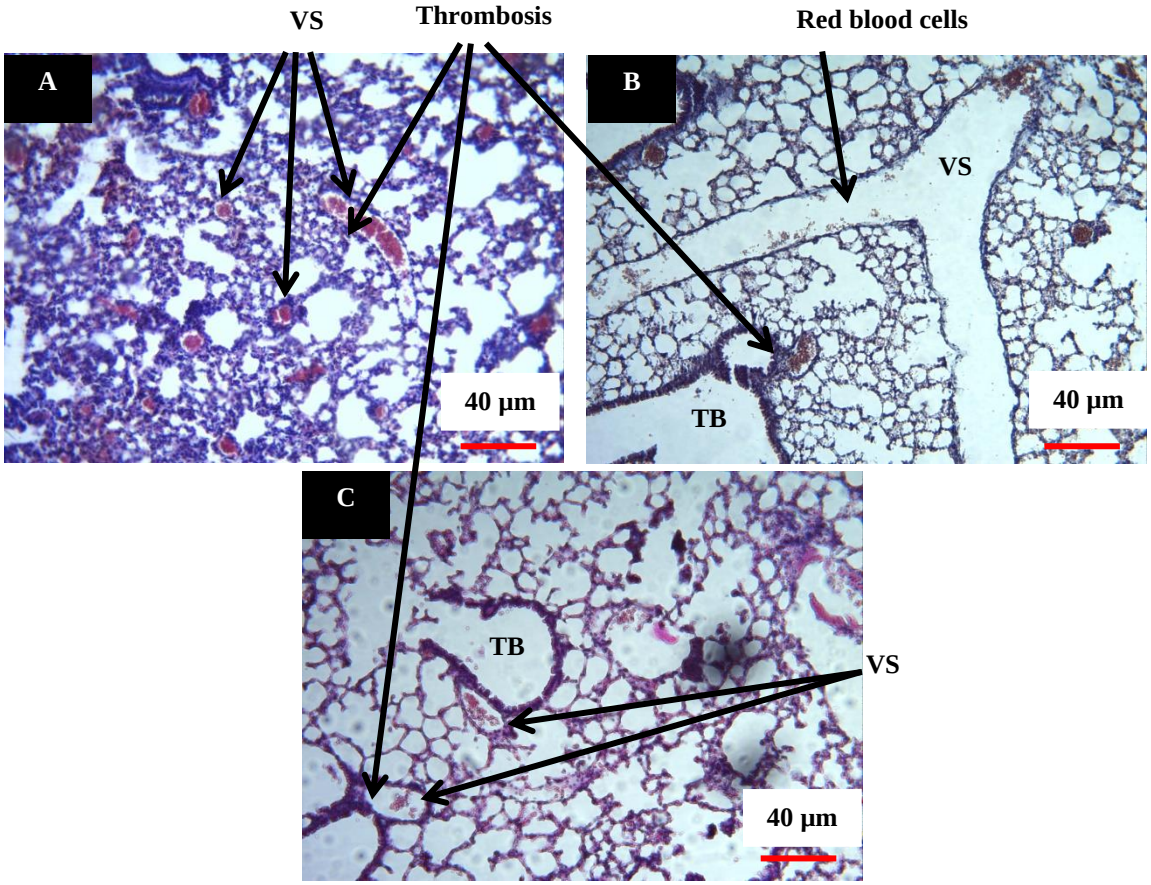

Figure 3. Histology of lungs. The intravenous injection of the thrombogenic mixture of collagen and epinephrine caused the formation of blood clot (thrombosis) in vessels for negative control group (A). The mice treated by ASA (Positive control group (B) and FHF group (C), showed the protective effect against thrombosis formation. VS: vessels, TB: terminal bronchiole. FHF: Heterosidic flavonoid-rich fraction.

2.2. Antiaggregant activity of flavonoid-rich fractions of *J. regia* root bark

The *in vitro* antiaggregant activities of the fractions are presented in Figure 4. The two flavonoid-rich fractions FHF and FAF at all doses significantly inhibited ADP, thrombin, collagen, and arachidonic acid-induced platelet aggregation in a concentration-dependent manner ($P < 0.001$). The percentages of inhibition varied between 64% and 96%.

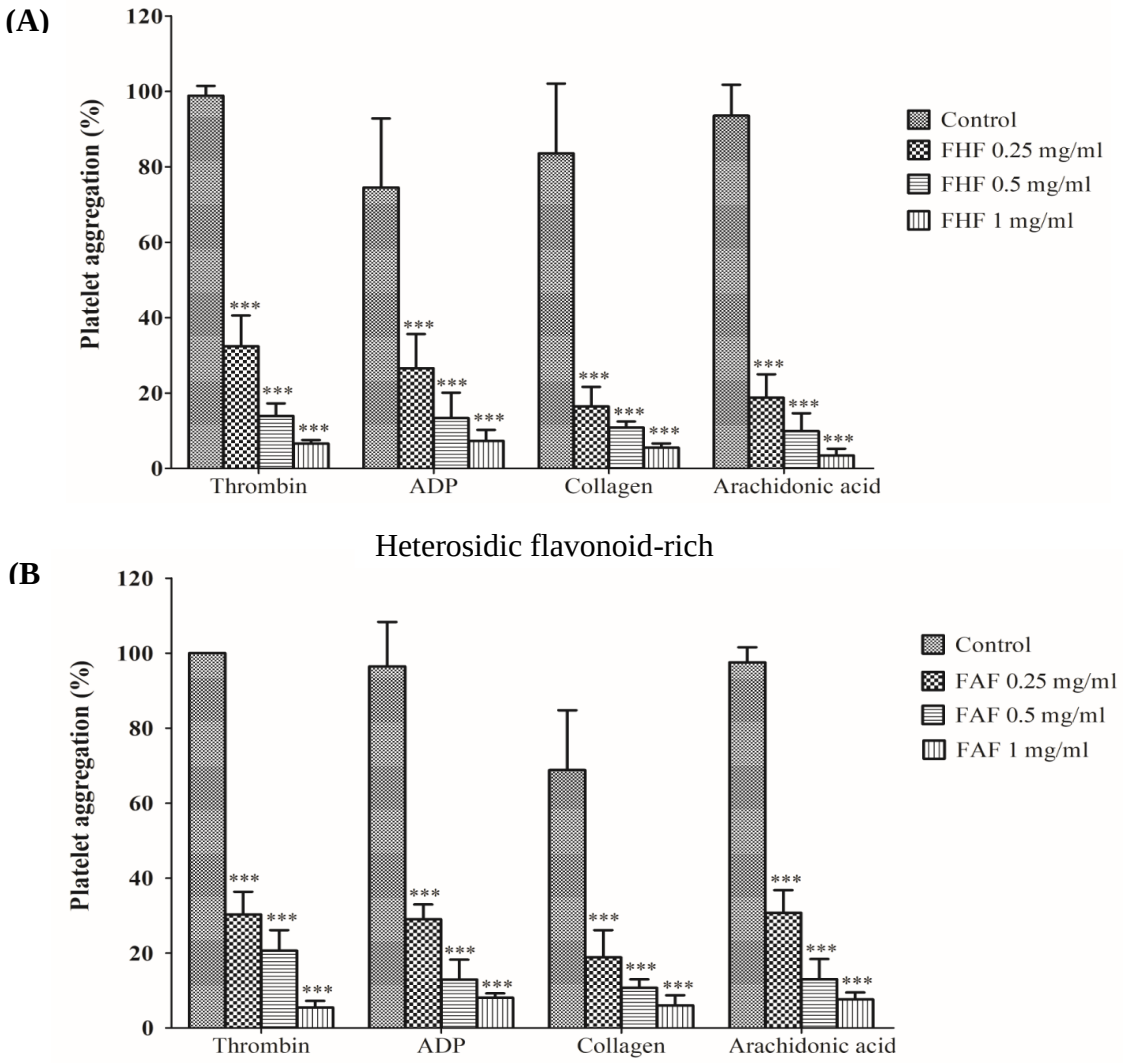

Figure 4. Effect of heterosidic flavonoids-rich fractions (A) and aglycones flavonoids-rich fractions (B) of *J.regia* on platelet aggregation induced *in vitro* by ADP (5 µM), thrombin (0.5 U/ml), collagen (5 µg/ml) and arachidonic acid (20 µM). Compared to control samples (washed platelets incubated without an extract) with *** $p < 0.001$, $n = 5$. FHF: Heterosidic flavonoid-rich fraction, FAF: Aglycone flavonoid-rich fraction.

2.3. Anticoagulant activity of flavonoid-rich fractions of *J. regia* root bark

As shown in Table 2, FHF was able to prolong significantly ($P < 0.001$) APTT in a concentration-dependent manner. However, this fraction showed a significant prolongation only for 0.5 and 1 mg/mL for PT and TT.

Similarly, FAF extended significantly ($P < 0.001$) the APTT at all tested concentrations, but its effect on PT and TT parameters was not significant, with only a weak prolongation at 0.25 and 0.5 mg/mL. As expected, heparin (0.4 U/mL), used as an anticoagulant drug, extended significantly all clotting times. In addition, no coagulation was observed for APTT and TT.

Table 2. *In vitro* anticoagulant activity of flavonoids-rich fractions.

	Dose	**PT**	**APTT**	**TT**
Distilled water	(1:1/v,v)	17.12 ± 0.94	13.96 ± 1.29	43.29 ± 4.91
FHF (mg/ml)	0.25	17.72 ± 0.52 NS	27.26 ± 2.51***	42.72 ± 2.49 NS
	0.5	28.74 ± 8.36 **	47.16 ± 6.36 ***	54.7 ± 7.05 **
	1	50.4 ± 4.1 ***	54.26 ± 7.56 ***	92.02 ± 4.83 ***
FAF (mg/ml)	0.25	15.9 ± 0.27 NS	25.7 ± 1.39 ***	51.65 ± 1.32*
	0.5	16.72 ± 0.72 NS	27.58 ± 0.9 ***	57.12 ± 11.42***
	1	18.2 ± 1.03 NS	35.26 ± 7.58 ***	46 ± 8.39 NS
Heparin (U/ml)	0.4	51.2 ± 9.74 ***	> 300	> 300

Mean ± standard deviation (SD), (n=5). > 300: No coagulation. * p< 0.05; ** p<0.01; ***p< 0.001 and NS: non-significant. PT: prothrombin time, APTT: activated partial thromboplastin time, TT: thrombin time, FAF: Aglycone flavonoids-rich fraction, FHF: Heterosidic flavonoids-rich fraction.

3. Discussion

Hemostatic and thrombotic disorders are the main causes of morbidity and mortality worldwide. Venous thromboembolism, which includes deep vein thrombosis and pulmonary embolism is a multifactor disease characterized by excessive formation of clots (Román et al. 2017).

Antithrombotic drugs could be classified into three categories including anticoagulant, antiaggregant, and fibrinolytic and are highly effective against arterial and venous thrombosis

(Hahn and Bae 2019). However, patients treated by antithrombotic drugs may suffer from undesirable side effects such as gastrointestinal bleeding and allergic reactions (Mirkov et al. 2016). Therefore, there is an increasingly urgent need for novel therapeutic approaches to reduce the current adverse effects of antithrombotic drugs without severely impairing their efficacy (Chen et al. 2015).

Medicinal plants served as a greater resource for new medication and attracted much interest from the scientific researchers all over the world. The World Health Organization has mentioned that medicinal plants could be used more effectively in health (WHO 2002). Currently, more endeavors have been devoted to finding some new natural molecules as antithrombotic drugs. Polyphenols are the largest group of secondary metabolites and they have been reported to have multiple biological effects. Flavonoids are a valuable class of polyphenols abundantly found in vegetables and more than 4 000 flavonoids have been isolated from plants and identified. In nature, flavonoids are mainly found as flavonoid glycosides (flavonoids connected to sugar residue) and flavonoid aglycones (Du et al. 2016 ; Koziara et al. 2019).

In the present study, we have investigated the phytochemical composition of flavonoids fractions of *J. regia* root bark and their antithrombotic effect by evaluating the *in vivo* protection against thrombosis formation, as well as *in vitro* platelet aggregation and plasmatic coagulation.

The flavonoids-rich fractions of *J. regia* root bark showed diversity in polyphenols and flavonoids. In addition to that, the HPLC analysis of flavonoids-rich fractions revealed that the major compounds were rutin and gallic acid. These results are similar to the studies performed by Stampar et al. (2006), who reported that gallic acid was the main phenolic compound, present at higher quantities than the other phenolic compounds from walnut husks. Moreover, rutin has been found with the greatest amount from green walnut (Cosmulescu et al. 2014).

Currently, a lot of researches are dedicated to the extraction, isolation, and identification of polyphenols and flavonoids from different parts of *J. regia* (Muzaffer and Paul 2018 ; Vieira et al. 2020). Yan et al. (2019) have identified 36 flavonoids compounds in flowers of *J. regia,* such as myricetin, quercetin, kaempferol, isorhamnetin, epicatechin, naringenin, avicularin, quercetin-3-L-rhamnoside, and juglanin.

Animal models of thrombosis have played a crucial role in the discovery of several compounds that are now successfully being used for the treatment and prevention of thrombotic diseases (Leadley Jr et al. 2000). Acute pulmonary thromboembolism *in vivo* model was used for this study to investigate the effect of heterosidic flavonoid-rich fraction on collagen-epinephrine stimulated platelet thrombus formation. This model was established by the intravenous injection of collagen-epinephrine (a thrombogenic mixture), and consequently triggered the massive activation of circulating platelets and the formation of thrombi in the pulmonary microcirculation leading to disseminated pulmonary microembolism and hind limb paralysis of the animal (Rajput et al. 2014).

Our results demonstrated that FHF of *J. regia* and ASA (a reference drug) were significantly effective in preventing collagen-epinephrine induced thromboembolic death (with 80% of protection). Lung histologic study validated these results. Indeed, we noticed that the blood vessels of the negative control group were totally or partly occluded by the formed thrombi, while they were still totally or partly opened in the FHF and ASA groups.

To try to understand the action mechanism of this antithrombotic activity, primary hemostasis (platelet aggregation) and secondary hemostasis (plasmatic coagulation) were explored *in vitro*.

In these experiments, the results showed that FHF and FAF of *J. regia* exhibited a strong inhibition of platelet aggregation triggered by different agonists (ADP, thrombin, collagen, and arachidonic acid) in a dose-dependent manner that activate platelets through different signaling pathways. These agonists interact with their specific receptors: P2Y1 for ADP, PARs (protease-activated receptor) for thrombin, and glycoprotein (GP VI) for collagen. Therefore, they initiate the platelet signalization by activating the phospholipase C [PLC (β/γ)] which hydrolyses phosphatidylinositol 4, 5-bisphosphate to diacylglycerol and inositol-1, 4, 5-triphosphate. Then, inositol-1, 4, 5-triphosphate increases intracellular calcium and results in shape change, secretion, and aggregation of platelets. Likewise, arachidonic acid plays an important role in platelet aggregation. Arachidonic acid is converted to thromboxane A2 (TXA2) by the action of enzymes COX1 and TXA2 synthase. After binding on its receptor (TP), TXA2 contributed to platelets shape change and activated fibrinogen receptors (GP IIb/IIIa, the most abundant glycoprotein in platelets), subsequently leading to platelet aggregation. The common final step of platelet aggregation is the activation of GP IIb/IIIa to which soluble fibrinogen binds and bridges platelets between them thus initiating the

formation of thrombus (Shin et al. 2017 ; Zine El Alaoui et al. 2020). The fact that flavonoids inhibit aggregation induced by the agonists suggests that these compounds act probably by preventing the binding of fibrinogen to its receptor, GP IIb/IIIa, and subsequently, suppress platelet aggregation. Moreover, the specific mechanism, which appears to be common for most of flavonoids compounds, seems to imply the inhibition platelet aggregation through the arachidonic acid-based pathway by the inhibition of COX-1 and thromboxane B2 production (Faggio et al. 2017).

For the secondary hemostasis, PT, APTT, and TT parameters were measured. They are basic blood tests for evaluating hemorrhage and thrombosis risks. APTT is related to the intrinsic and/or common pathways and is used to detect deficiencies of some coagulation factors: II, V, VIII, IX, X, XI, and XII. PT explores the extrinsic and/or common pathways and commonly used to evaluate the efficiency of other factors: I, II, V, VII, and X. Finally, TT explores the common and ultimate pathways which consists of the transformation of fibrinogen into fibrin under the action of thrombin (Sayari et al. 2016).

In these assays, we demonstrated that the two flavonoid-rich fractions of *J. regia* inhibited plasma coagulation. FHF exhibited a more intense effect than FAF. This inhibition was more pronounced and significant for APTT than PT or TT, suggesting that these compounds may negatively interfere with coagulation factors implicated specifically in the intrinsic and the common voices. However, FHF can be interfered also with the extrinsic pathway *via* the inhibition of the tissue factor (TF) or the complex TF-FVIIa. It was recently reported that flavonoids can inhibit excessive TF availability in the endothelium and then reduce the risk of thrombotic disease (Vazhappilly et al. 2019). All these results confirm our previous report obtained with the crude aqueous extract of *J. regia* root bark which also reduced platelet aggregation and extended clotting time (Amirou et al. 2018).

To explain the mechanism of action by which *J. regia* may exert its effect (antithrombotic, anti-aggregant, or anticoagulant), we must firstly consider the type of extract since any crude aqueous extract constitutes a complex mixture of several active compounds (Pal and Mitra 2006), not like isolated ones. These natural compounds could act individually or synergistically and therefore may affect one or several steps of primary and secondary hemostasis.

In our case, even isolated, flavonoids preserve their activities as in the crude aqueous extract of *J. regia* (Amirou et al. 2018). Several studies found that the flavonoids from plants could

have antithrombotic effect due to their anticoagulant and anti-platelet aggregation actions. For example, *Lindera obtusiloba* has been described to have antiplatelet and antithrombotic effects *in vitro* and *in vivo* (Kim et al. 2016). Zhou et al. (2019) has also demonstrated that the antithrombotic effect of *Celastrus orbiculatus* was related to the anticoagulant and antiplatelet effects. Other pathways of hemostasis such as platelet adhesion to the injured vascular endothelium and/or platelet secretion may be affected by the plant extract (Vaiyapuri et al. 2013 ; Gadi et al. 2012 ; Lee et al. 2013).

Furthermore, the major phenolic compound in the flavonoid-rich fraction of *J. regia* is rutin, which is commonly known for its property as antithrombotic. It is shown that rutin could inhibit thrombosis *in vitro* and *in vivo* through reduction of thrombin activity, inhibition of fibrin clotting, prolongation of APTT and PT, and decrease of platelet aggregation (Choi et al. 2015a). Similar data were observed with other flavonoids like quercetin and kaempferol, indicating that these components had antithrombotic effects (Lee et al. 2013 ; Choi et al. 2015b).

In the present study, we demonstrated that flavonoid-rich fractions of *J. regia* show a potent antithrombotic effect, which can be related, at least in part, to antiaggregant and anticoagulant activities. Other experiments are needed to further clarify the mechanism of action of these compounds like dosing the platelet thromboxane B2, studying the fibrinolytic activity. Moreover, unknown compounds present in *J. regia* fractions must be identified by further phytochemical analysis and tested for their hemostatic activities.

4. Conclusion

In conclusion, these results suggest that flavonoids-rich fractions of *J. regia* could be an important source of precious compounds that could be used as an alternative for new antithrombotic agents and helpful in preventing and treating thrombotic events such as myocardial or cerebral infarctions. Thus, modulation of this pathway may eventually constitute a target in the management of thrombotic events.

Abbreviations: APTT, activated partial thromboplastin time; ASA, acetylsalicylic acid; FAF, aglycones flavonoids-rich fraction;20) FHF, heterosidic Flavonoids-rich fraction; *J. regia*, *Juglans regia*; PT, prothrombin time; TT, thrombin time. COX-1, cyclooxygenase1; TXA2, thromboxane A2.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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