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Argania spinosa Leaves and Branches: Antiaggregant, Anticoagulant, Antioxidant Activities and Bioactive Compounds Quantification

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Argania spinosa

Aggregation

Coagulation

Antioxidant activity

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ABSTRACT

Thrombocytes, also known as platelets, are crucial in maintaining the balance between blood clotting. Platelet hyperactivity and oxidative stress are the primary factors contributing to cardiovascular complications. Antithrombotic therapy remains one of the most effective treatments, but various potential side effects hinder its effectiveness, including the risk of haemorrhage. Intense research has been conducted on medicinal plants to discover the natural antithrombotic compounds. *Argania spinosa*, commonly known as the argan tree or argan oil tree, is a native species of southwestern Morocco. This study evaluated the primary and secondary hemostasis and antioxidant activity of leaf and branch aqueous extracts of *A. spinosa* and also assessed the phytochemical composition of these extracts. Platelet aggregation assay was performed using washed platelets stimulated with thrombin. For plasmatic coagulation, activated partial thromboplastin time and prothrombin time were measured using the poor plasma method. Bleeding time was evaluated by inducing bleeding at the tip of a mouse tail. The antioxidant activity of the extracts was determined through the DPPH, β-carotene, and FRAP methods. The presence or absence of the secondary metabolites was carried out with the help of specific reagents, and the quantitative analysis was carried out using spectrophotometric and colorimetric methods. The study results revealed the presence of phenols, total flavonoids, cardiac glycosides, tannins, and coumarins type of secondary metabolites in both types of aqueous extracts and a higher concentration of

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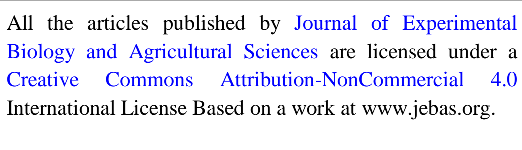
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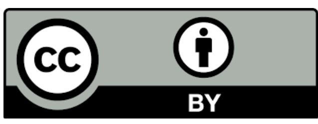

these was recorded in the leaves extracts. Both aqueous extracts significantly reduced *in vitro* thrombin-induced platelet aggregation, extended tail bleeding time, prolonged activated partial thromboplastin and prothrombin time and exhibited remarkable antioxidant activity. The leaf extract of *A. spinosa* exerts significant effects against thrombotic manifestations and could be a promising source of new antithrombotic compounds.

1 Introduction

Thrombotic complications related to cardiovascular diseases consist of the abnormal formation of a blood clot (thrombosis) at the level of the vessel, and its constitute is the same, which involves mechanisms as in normal hemostasis. This phenomenon causes ten million deaths yearly despite several effective antithrombotic remedies (Rosendaal and Raskob 2014). This led the researchers to put more effort into developing new natural remedies rich in bioactive compounds with fewer side effects. Ethnobotanical studies conducted in Morocco have demonstrated the antithrombotic, antiplatelet and anticoagulant potentials of various herbal extracts such as *Ageratum conyzoides*, *Brownea grandiceps Jacq* and *Lamiophlomis rotata* (Ebrahimi et al. 2020). In addition, various previous laboratory studies have shown the antiaggregating activity of *Juglans regia* (Amirou et al. 2018), *Arbutus unedo, Cistus ladaniferus* and *Urtica dioïca* (El Haouari and Mekhfi 2017; Mekhfi et al. 2004), and *Petroselinum crispum* (Gadi et al. 2009).

"Argane" is the vernacular name of *Argania spinosa,* belonging to the family Sapotaceae, also known as the "tree of life". The 10th May of each year is declared International Day of *Argania spinosa* by the United Nations. *A. spinosa* is a tree native to the Souss-Massa Region of Morocco and is found across approximately 800,000 hectares in this area. Other trees of the same species have also been discovered in the Orientale Region of Morocco. It is a drought and heat resistance tree characterized by a height of 8 to 10 meters and a life period of 150 to 200 years (Rammal et al. 2009). The local population widely uses this tree for therapeutic, food and cosmetic purposes (Moukal 2004). Several studies have shown that Argan oil may have remedies for diseases such as diabetes (Bnouham et al. 2008) and cardiovascular diseases (Cherki et al. 2006). Another experimental study also showed that argane oil could benefit by exercising an antiplatelet and antithrombotic effects (Mekhfi et al. 2012).

Besides the oil's intense phytotherapeutic and nutritional values, the other parts of *A. spinosa* remained unexploited. Hence, this study was focused on the leaves and branches of *A. spinosa* in the Oriental Region of Morocco. According to available research, no studies have been conducted on these parts to test their effects on platelet aggregation and plasmatic coagulation. Therefore, this study aimed to assess the *in vitro* effects of aqueous extracts of leaves and branches of *A. spinosa* on platelet aggregation, bleeding time and plasmatic coagulation in rats. Studies of *in vitro* antioxidant activity of both aqueous extracts were also evaluated to elucidate their mechanism of action, and the qualitative and quantitative phytochemical compositions were also determined.

2 Materials and Methods

2.1 Preparation of crude aqueous extracts of *Argania spinosa*

The preparation of *A. spinosa* crude aqueous extracts (CAE) was carried out from the leaves and branches of the tree. After washing and drying in the oven at 40°C, the leaves of *A. spinosa* are crushed in a blender to make leaves powder. After this, 300 mL of boiled distilled water was mixed with the 30 g of leaves powder. The mixture was left for 30 minutes and then filtered with filter paper. The obtained filtrate was concentrated under vacuum with a rotary evaporator (Instruments Heidolph, Germany) at 45°C and then dried overnight in the oven at 40°C.

For the branches, the CAE was prepared by decoction. After washing and drying, 40 g of small branches were heated in 400 mL of distilled water until boiling. Then, the solution was filtered, concentrated with the rotary evaporator at 45°C and dried in the oven overnight at 40°C. The yield Y of each extract was calculated using the following formula:

$$Y\ (\%) = X/Z \times 100$$

Y: Yield expressed as a percentage.

X: Final weight of dry extract in grams.

Z: Initial weight of dry plant part (leaves or branches) in grams.

All extracts were aliquoted and stored at -4°C until use.

2.2 Qualitative determination of phytochemical compounds

The qualitative screening study for various extracts was conducted to determine the presence or absence of some secondary metabolites such as alkaloids, flavonoids, tannins, cardiac glycosides and coumarins.

2.2.1 Tests for alkaloids

For evaluating the presence of alkaloids in CAE, the Dragendorff test was used, for this, addition of Dragendorff reagent to particular extracts results in the formation of a dark precipitate, which shows the presence of alkaloids (Raal et al. 2020).

2.2.2 Tests for flavonoids

Aluminium chloride ($AlCl_3$) reagent test was used for recording the presence of flavonoids in all extracts of *A. spinosa.* These extracts were treated with a few drops of the $AlCl_3$ reagent solution, and the formation of green colour indicates the presence of flavonoids and colour detection was performed using fluorescence at 370 nm (Jesus et al. 2018).

2.2.3 Tests for tannins

A Ferric chloride ($FeCl_3$) test was used to evaluate the presence of tannins. For this, 1 % $FeCl_3$ is added to the sample. The development of a blue-green colour indicates the presence of tannins (Jesus et al. 2018).

2.2.4 Tests for cardiotonic heterosides

Cardiotonic heterosides were identified using the Liebermann-Buchard reaction, which involves mixing acetic anhydride and sulfuric acid with a portion of aqueous extract. The formation of a green coloring indicates the presence of the steroidal nucleus characteristic of cardiotonic compounds (Jesus et al. 2018).

2.2.5 Tests for coumarins

The extracts were treated with a few drops of 10% NaOH solution (Alkaline reagent test). The formation of yellow color indicates the presence of coumarins (Buvaneswari et al. 2011).

2.3 Determination of total phenolic content

Total phenolic (TP) was determined according to the method of Folin-Ciocalteu (Hagerman 1988), with minor modifications. 0.25 mL of Folin-Ciocalteu reagent and 0.5 mL of (2%) Na_2CO_3 solution were mixed with 0.5 mL of each CAE. After adjusting the volume with 3.5 mL of distilled water, the mixture was stirred carefully and incubated at room temperature in the dark for 90 minutes. The absorbance of the mixture was then measured by a spectrophotometer (Beijing Rayleigh Analytical Instrument, China) at 750 nm. The standard curve was prepared by using gallic acid as a standard at 0, 25, 50, 75, 125, 250, 500 µg/mL. The content of TP was expressed as "g gallic acid equivalent per 100 g dry extract". All experiments were repeated three times.

2.4 Determination of total flavonoid content

Total flavonoid contents (TF) were determined using a colorimetric method (Mu et al. 2010). 1 mL of $AlCl_3$, dissolved in 2% methanol, was added to 1 mL of each extract. After 30 min of incubation at room temperature, the absorbance of the mixture was determined by a spectrophotometer at 415 nm. A standard curve was performed using rutin as a standard at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µg/mL. The concentration of the TF amount was expressed in "g rutin equivalent per 100 g dry extract. Each treatment has been repeated three times.

2.5 Antioxidant assays of *A. spinosa* extracts

2.5.1 DPPH Free Radical Scavenging Activity

This test aimed to evaluate the antiradical potential of *A. spinosa* leaves and branches CAE against a stable free radical of 1, 1-diphenyl-2- picrylhydrazyl (DPPH). This assay followed the method given by de la Rosa et al. (2011) with few modifications. 1 mL of the DPPH solution was added to a final concentration range for each CAE. The samples were incubated for 30 minutes in darkness at room temperature. The absorbance was measured with a spectrophotometer at 517 nm. Ascorbic acid (AA) was used as a standard antioxidant at 25, 50, 100, 200, 400, 800, and 1000 µg/mL. All measurements were performed in triplicate. The scavenging effect is calculated according to the following equation:

$$\text{Radical Scavenging Activity (\%)} = [(A_o - A_t) / A_o] \times 100$$

A_o represents the absorbance of DPPH solution without extract; A_t represents the absorbance of the test extract mixed with DPPH solution.

2.5.2 β-Carotene Bleaching Test

This study aimed to evaluate the protective potential of leaves and branches CAE against the bleaching of β-carotene by linoleic acid degradation products. A solution of β-carotene was prepared by dissolving 2 mg in chloroform and then mixed with 20 mg of linoleic acid and 200 mg of Tween-80. The chloroform was removed by rotary evaporator at 40°C. The dry β-carotene/linoleic acid emulsion was reconstituted by adding 100 mL of distilled water with vigorous stirring. From this emulsion, 0.2 mL was transferred into different test tubes containing the sample solution. The first absorbance of the samples (t_0) was immediately read at 470 nm. Then, all samples were incubated in a water bath at 50°C with continuous stirring. Two hours later, a second absorbance was recorded. Butylated hydroxyanisole(BHA) was used as standard at 25, 50, 100, 200, 400, 800, 1000 µg/mL, and all the measurements were performed three times. The percentage of residual color is calculated according to the following formula:

$$\text{Residual color (\%)} = 100 - [((\text{initial OD} - \text{sample OD}) / \text{initial OD}) \times 100]$$

Where initial OD represents the absorbance before incubation, sample OD represents the absorbance after 2 hours of incubation.

2.5.3 Ferric Reducing Antioxidant Power (FRAP) of extracts

The iron-reducing activity reduces Fe^{3+} present in the potassium ferricyanide ($K_3Fe(CN)_6$) complex in Fe^{2+}. Ferric ion-reducing

power from extracts was determined by following the protocol of Bekkouch et al. (2019). Various concentrations of extracts have been prepared (25, 50, 100, 200, 400, 800, 1000 µg/mL). 1.25 mL phosphate buffer (pH=6.6) and 1.25 mL of 1 % potassium ferricyanide were added to 0.5 mL of each extract. The mixture was incubated at 50°C for 20 minutes. Then, 1.25 mL of 10% trichloroacetic acid was added to the test sample, and subsequently, the mixture was centrifuged at 3000 rpm for 10 minutes. After raising the supernatant, it is mixed with 1.25 mL of distilled water and 0.25 mL of ferric chloride solution (0.1%); the absorbance of the solution was read at 700 nm. AA was used as a standard control (25, 50, 100, 200, 400, 800, 1000 µg/mL), all measurements were carried out in triplicate.

2.6 Experimental Animals

Male and female Wistar rats and Albinos mice used in the studies were raised in the Department of Biology at the Faculty of Sciences animal house (Mohammed the First University, Oujda, Morocco) at a temperature of 22±2°C, 12 hours light/dark cycle and with free access to water and food. All animals in these experiments comply with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (2012, Guide for the Care and Use of Laboratory Animals), where a minimal statistically valid number of rats and mice was respected.

2.7 *In vitro* platelet study

2.7.1 Washed Platelet Preparation

Washed platelets (WP) were prepared as described by Mekhfi et al. (2008). After light ether anaesthesia, rat blood was collected by catheterization of the abdominal aorta in a plastic tube containing (9:1, v/v) an anticoagulant solution (170 mM of trisodium citric acid, 130 mM of citric acid, and 4% dextrose). Centrifugations separated the platelet-rich plasma (PRP) from other blood cells. After noting its volume, the PRP was centrifuged again to obtain a pellet of platelets. The supernatant, known as platelet-poor plasma (PPP), was discarded, and the platelet pellet was resuspended in a washing buffer equal to the initial PRP volume. A last centrifugation was conducted, and the platelet pellet was then suspended in a calculated volume of a final buffer (NaCl 137 mM, KCl 2.6 mM, $MgCl_2$ 0.9 mM, Glucose 5.5 mM, $CaCl_2$ 1.3 mM, Gelatin 0.25%, Hepes 5 mM, pH of 7.4) to achieve a constant concentration of 5×10^5 washed platelets/mm^3.

2.7.2 *In vitro* Platelet Aggregation Study

In vitro measurement of platelet aggregation was performed using a semi-automatic aggregometer (Helena, USA) under a constant temperature (37°C) and at a stirring speed (1000 rpm), and AggroLink Software piloted the results. To study the effect of *A. spinosa*, 250 µL of the washed platelets were incubated for one minute in an aggrometer tube with CAE (1 g/L). The control tube contained only WP suspension without plant extract. Then, platelet aggregation was triggered by adding an aggregant agonist, the thrombin at 0.5 U/mL, and the aggregation signal was recorded for at least 5 minutes. Two parameters were deducted from the original traces of platelet aggregation. The computer software automatically calculated the amount of aggregation (%) in the absence and presence of plant extracts. The following formula calculates the amount of inhibition of aggregation (%):

$$\text{Inhibition (\%)} = (A\text{-}B/A) \times 100$$

A: Maximum aggregation in the absence of the extract (control)

B: Maximum aggregation in the presence of the extract

2.8 Bleeding time determination

Bleeding time (BT) overall reflects the primary hemostasis process. Male and female Albino mice (18-22 g) were divided into six groups, with 5 mice per group. A single oral administration was performed as follows: the control group received distilled water (1 mL/100 g), the four test groups received branches and leaves CAE from the two regions (1 g/Kg), and the positive control group received acetylsalicylic acid (ASA) (30 mg/Kg). Intraperitoneal anaesthesia with sodium pentobarbital (50 mg/kg) was applied one hour after the treatment. The animals were then placed on a heating plate at 37°C. Afterwards, the mouse's tail was disinfected with alcohol and sectioned 1.5 cm from its tip with a scalpel. The stopwatch was started as soon as the first drop of blood appeared. When no drops came out, the stopwatch was stopped, and the BT (seconds) was noted.

2.9 *In vitro* anticoagulant activity

2.9.1 Platelet Poor Plasma Preparation

The blood sample was taken using the abdominal aorta catheterization method in anaesthetized rats (250-300 g). The blood was placed in a plastic tube containing the anticoagulant (3.8% trisodium citrate, 1/9, v/v), and centrifuged immediately at 3000 rpm for 20 minutes to recover platelet-poor plasma (PPP) (Amirou et al. 2022).

2.9.2 Determination of coagulation times

Coagulation tests are performed on the PPP using an automatic coagulometer "CS-2100i-sysmex". 100 µL of PPP and 50 µL of different *A. spinosa* extracts (1 g/L) or 50 µL of distilled water were incubated at 37°C for 5 minutes. A 50 µL aliquot of the mixture is taken and re-incubated for 2 min at 37°C. The coagulation process is triggered by adding 100 µL of the reagent

Table 1 Yields of the extraction of *A. spinosa* leaves and branches

Plant site	Part used	Yield (%)
Chwihia	Leaves	26.06
	Branches	4.32
Oujda city	Leaves	14.52
	Branches	1.07

Table 2 Qualitative phytochemical screening of the different *A. spinosa* crude extracts

Collected sample	Alkaloids	Flavonoids	Tannins	Coumarins	Cardiac glycosides
ASLC	-	+++	++	++	+++
ASBC	-	+	-	-	-
ASLO	-	+++	++	++	++
ASBO	-	++	+	-	+

ASLC - *A. spinosa* leaves from Chwihia site; ASBC - *A. spinosa* branches from Chwihia site; ASLO - *A. spinosa* leaves from Oujda site; ASBO - *A. spinosa* branches from Oujda site; "+++" Heavily present; "++" Moderately present; "+" Slightly present; "-" Not present.

Thromborel S to determine the prothrombin time (PT). To determine the activated partial thromboplastin time (APTT), 50 μL of the reagent CK PREST is added, and the mixture is incubated for 3 minutes. Then, the reaction is triggered by adding 50 μL of calcium. Heparin, an anticoagulant molecule, was used as a reference positive control for clotting tests.

2.10 Statistical analysis

The obtained data was analyzed using GraphPad Software (version 5.01, GraphPad Software, Inc.). For all studies, the results were expressed as mean ± SEM. 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).

3 Results

3.1 Yields of aqueous extractions

To prepare the *A. spinosa* leaves and branches, CAE samples were collected from the Chwihia and Oujda sites. The yields of leaves and branches CAE were determined with the weight of dry plant material. As per the results presented in Table 1, the highest yields were obtained from the leaves rather than the branches. In addition, *A. spinosa* samples collected from the Chwihia's site showed higher yields than the Oujda site.

3.2 Qualitative phytochemical screening of the crude extracts

The presence of the secondary phytochemical compounds was evaluated by using specific reagents. From the qualitative phytochemical screening, it was reported that the flavonoids are the most represented secondary metabolites, followed by cardiac glycosides, tannins and coumarins, while the presence of the alkaloids wasn't detected in any CAE (Table 2). In addition, leaves of *A. spinosa* seem richer than branches, especially in *A. spinosa* branches of the Chwihia, where nearly all compounds don't exist.

3.3 Quantitative phytochemical evaluation of polyphenolic and flavonoid compounds

Total polyphenols and flavonoids compounds were quantified in all CAE of *A. spinosa* and expressed in equivalent milligrams of gallic acid and rutin per 1 g of dry extract weight, respectively. As presented in Table 3, total polyphenols were more concentrated in our extracts than flavonoids, and in general, *A.spinosa* branches CAE were poorer than the leaves, regardless of the collection site.

Table 3 Total polyphenols and flavonoids of *A. spinosa* crude aqueous extracts.

Extracts	Polyphenols compounds (mg eq Gallic acid/g dry ext)	Flavonoids compounds (mg eq Rutin/g dry ext)
ASLC	162.5±1.8	49.6±0.8
ASBC	59.3±1.3	33.8±0.9
ASLO	160.3±5.4	34.2±0.8
ASBO	150.4±1.1	22.0±2.1

ASLC - *A. spinosa* leaves from Chwihia; ASBC - *A. spinosa* branches from Chwihia; ASLO - *A. spinosa* leaves from Oujda, and ASBO - *A. spinosa* branches from Oujda; All data are represented by mean ± SEM (n = 3).

3.4 Antioxidant activities of the crude extracts

Three methods used for the antioxidant measurement were DPPH, β-carotene and FRAP. Table 4 presents the IC_{50} values for the four types of CAE. They ranged between 294 and 2500 µg/mL, obtained with the FRAP method. Depending on the plant part, leaves seem to have the most robust antioxidant activity, with IC_{50} values smaller than in branches, except for ASLO and ASBO extracts in the β-carotene test. On the other hand, all extracts' values are higher than those of the antioxidant references, AA and BHA.

Table 4 Half inhibition concentration ($IC_{50;}$ µg/mL) values for leaves and branches of *A. spinosa* obtained with three antioxidant methods

	IC_{50} (µg/mL)		
Extracts	DPPH	β-carotene	FRAP
ASLC	434.4 ± 2.8	380.5 ± 1.5	357.1 ± 0.01
ASBC	735.2 ± 15.2	433.6 ± 6.7	2500 ± 0.3
ASLO	419.8 ± 5.6	425.8 ± 16.2	294.1 ± 0.1
ASBO	454.5 ± 10.5	406.5 ± 6.1	555.5 ± 0.1
AA	137.6 ± 0.1	-	33.6 ± 0.1
BHA	-	144.7 ± 1.1	-

ASLC - *A. spinosa* leaves from Chwihia; ASBC - *A. spinosa* branches from Chwihia; ASLO - *A. spinosa* leaves from Oujda, and ASBO - *A. spinosa* branches from Oujda; AA - ascorbic acid; BHA - Butylated Hydroxyl Anisole; Data are presented as mean ± SEM; Number of independent experiments = 3.

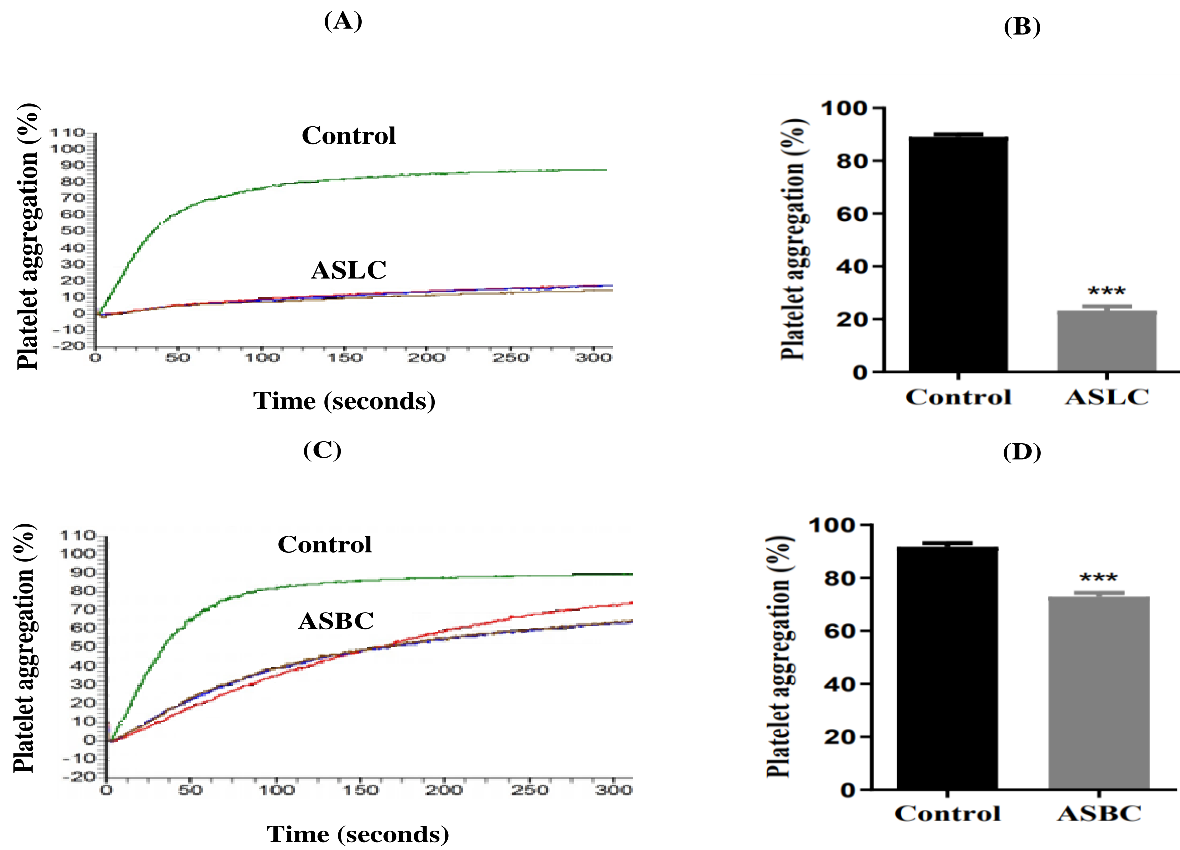

Figure 1 Original plots (A, C) and histograms (B, D) representing the effect of the crude aqueous extracts of *A. spinosa* leaves and branches (1 g/L) on *in vitro* thrombin-induced platelet aggregation; the values given are means ± SEM; Number of independent experiments 6-8; ASLC - *A. spinosa* leaves from Chwihia; ASBC - *A. spinosa* branches from Chwihia; *** ($p<0.001$) vs control

3.5 *In vitro* effect of aqueous extracts of *A. spinosa* on platelet aggregation

This study evaluates the *in vitro* effect of the *A. spinosa* leaves and branches CAE from the two regions on rat platelet aggregation. One minute after preincubation with extract (1 g/l), the WP aggregation was evoked by thrombin (0.5 U/mL). As shown in Figure 1, the aggregation control was around 90%. While *A. spinosa* leaves (ASLC) and branches (ASBC) extracts collected from the Chwihia region caused a significant reduction in aggregation amount as compared to the control ($p<0.001$). The calculated amounts of inhibition were 76.1 ± 1.6 and 17.97 ± 1.4 for ASLC and ASBC, respectively. The comparison of these values showed that ASLC was statistically more effective ($p<0.001$) on aggregation inhibition than ASBC.

Figure 2 shows the *in vitro* effect of *A. spinosa* leaves (ASLO) and branches (ASBO) CAE collected from the Oujda site on thrombin-induced platelet aggregation compared to the control (without extract) (≈ 95%). The two extracts reduced the platelet aggregation significantly ($p<0.001$). The amount of inhibition was similar in both extracts, 66.1±8.3 % and 68.7±4.0 % for ASLO and ASBO, respectively.

3.6 Effect of aqueous extracts of *A. spinosa* on tail bleeding time

In control, the BT is about 150 seconds (n=5) and after treatment with the *A. spinosa* leaves CAE from Chwihia and Oujda sites (1 g/kg), significantly extended ($p<0.001$, and $p<0.01$ respectively) the BT (Figure 3). Among the tested extracts, the ASLC effect is

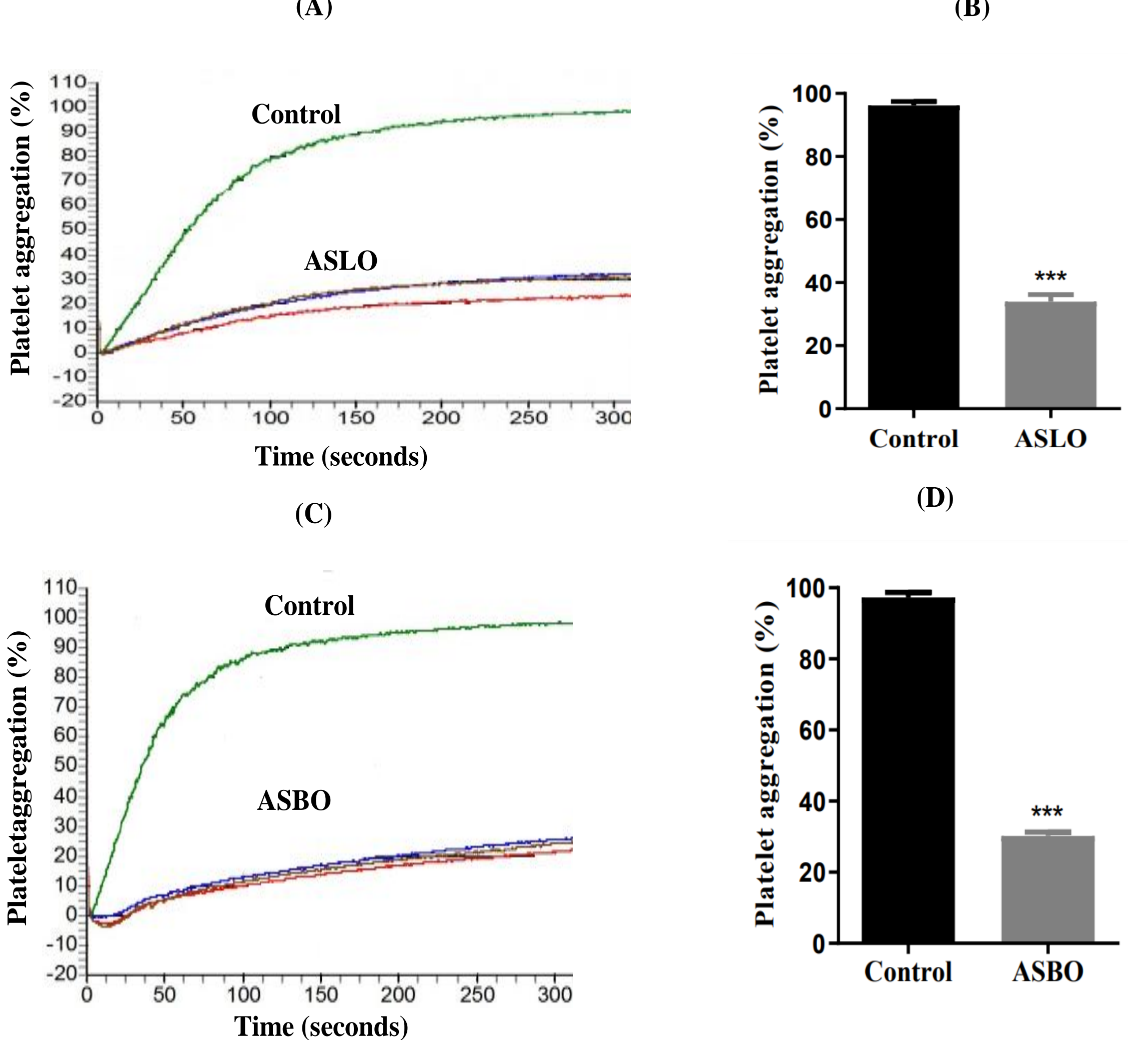

Figure 2 Original plots (A, C) and histograms (B, D) representing the effect of the crude aqueous extract of *A. spinosa* leaves and branches (1 g/L) on *in vitro* thrombin-induced platelet aggregation in rats; the number of independent experiments 6-8, ASLO: *A. spinosa* leaves from Oujda and ASBO: *A. spinosa* branches from Oujda, significance level at *** $p<0.001$ vs control.

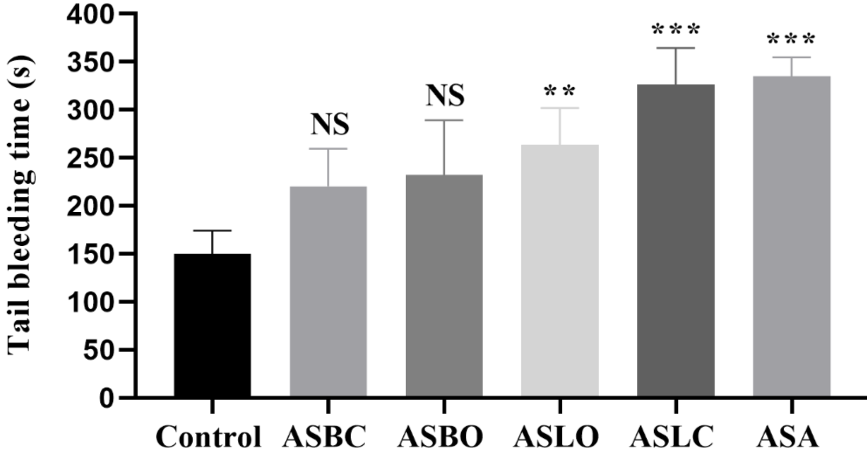

Figure 3 Effect of crude aqueous extracts of leaves and branches of *A. spinosa* (1 g/kg) on tail bleeding time in mice; Number of independent experiments 5; ASLC - *A. spinosa* leaves from Chwihia; ASBC - *A. spinosa* branches from Chwihia; ASLO - *A. spinosa* leaves from Oujda and ASBO - *A. spinosa* branches from Oujda; ASA - acetylsalicylic acid; ***p*<0.01, ****p*<0.001 compared to control.

Table 5 *In vitro* effect of aqueous extracts of leaves and branches of *A. spinosa* on coagulation times

Sample	PT (s)	APTT (s)
Control	10.3±0.3	37.0±1.4
ASLC	17.9±0.8***	51.4±3.5***
ASBC	9.6±0.2^{NS}	39.6±3.5^{NS}
ASLO	26.7±2.6***	60.4±2.7***
ASBO	9.9±0.2^{NS}	48.4±0.1*
Heparin (0.4U/ml)	10.7±0.2^{NS}	77.6±1.4***

ASLC - *A. spinosa* leaves from Chwihia; ASBC - *A. spinosa* branches from Chwihia; ASLO - *A. spinosa* leaves from Oujda and ASBO - *A. spinosa* branches from Oujda; PT - Prothrombin time; APTT - Activated partial thromboplastin time; **p*<0.05, ****p*<0.001 compared to control; Number of independent experiments = 5-6.

comparable to the ASA. While, in the case of branches, the results show an increase in BT, these differences did not reach significance.

3.7 *In vitro* effect of aqueous extracts of *A. spinosa* on plasmatic coagulation

As presented in Table 5, the two leaf extracts (1 g/l) significantly (p<0.001) prolonged the PT and APTT compared to the control. However, the branch extracts didn't change these coagulation times, except for the ASBO action on the APTT (p<0.05).

4 Discussion

Blood platelets or thrombocytes play a key role in the hemostasis process, specifically in primary hemostasis. In case of a vascular lesion, their mission is to prevent bleeding and plug the vascular injury by forming a platelet thrombus. Thus, several sequential mechanisms involving these cells are triggered: sub-endothelium adhesion, activation, shape change, granular secretion, and finally, platelet aggregation. On the other hand, abnormal platelet hyperactivity linked to many diseases (arterial hypertension, diabetes) is widely described in literature (El Haouari and Rosado 2016), which contribute to the development of thrombotic complication, ischemic stroke, coronary heart disease, myocardial infarction, and cerebral vascular accident (Morel et al. 2010). Several antithrombotic drugs have been developed to treat or prevent these adverse effects. Unfortunately, they cause severe side effects, including hemorrhagic disorder, ulcers, allergies, and hepatic and renal toxicities (Li et al. 2020). So, intense research and efforts are deployed to develop new antiplatelet or antithrombotic molecules with less or no side effects.

In this context, this study focused on *A. spinosa*, an endemic tree of Morocco. The choice of this plant is mainly based on ethnobotanical studies that have shown its wider use in various traditional medicines for treating several diseases by the Moroccan population. The tree is widely known for its oil and therapeutic virtues. Many experimental investigations have already proven that *A. spinosa* oil exert several beneficial actions against

cardiovascular (Cherki et al. 2006) and metabolic disorders, such as hypocholesterolemic (Berrougui et al. 2003), antioxidant and antidiabetic (Ben Mansour et al. 2018), antiplatelet aggregation and antithrombotic (Mekhfi et al. 2012).

In the present study, to explore additional information, leaves and branches of *A. spinosa* were collected from two different localities of the Oriental Region of Morocco (Chwihia and Oujda sites). After exploring their phytochemical composition, the CAE of leaves and branches of *A. spinosa* were tested on platelet aggregation, bleeding time, and plasmatic coagulation and evaluated their antioxidant power. Results of the phytochemical analytical study revealed that alkaloids are absent in both types of aqueous extract. However, flavonoids are present in all extracts with different concentrations. Tannins, phenols, and cardiac glycosides have been identified only in three extracts, i.e. ASLC, ASLO and ASBO. While the coumarins are present in the same amount in *A. spinosa* leaves collected from both sites. These phytochemicals are known for their therapeutic importance because they have many biological roles. Indeed, flavonoids exert antibacterial activity, phenols are antioxidants, and tannins have antiviral and healing effects (Rai et al. 2013).

The quantification of polyphenols and flavonoids is commonly done through spectrophotometric methods (Folin-Ciocalteu test and aluminium trichloride test). The study results revealed a high content of polyphenols in the leaves aqueous extracts of both regions, although a remarkable amount of flavonoids was observed in the CAE of the leaves of Chwihia. These results are consistent with the previous scientific works on the same plant (Charrouf and Guillaume 1999; Mercolini et al. 2016). Indeed, some flavonoids, quercetin and myricetin, have been isolated from the leaves of *A. spinosa* (Charrouf and Guillaume 1999). Other phytochemical studies have shown that *A. spinosa* leaves are rich in various phenolic compounds such as epicatechina, catechin, myricetin, quercetin, rutin, myricetin 3-O-galactoside and melatonin (Mercolini et al. 2016).

The literature has reported that flavonoids have exceptional antioxidant potential and act by their anti-inflammatory and anti-thrombogenic effects that would be potentially effective against chronic diseases (Nijveldt et al. 2001). For this reason, this study tested the effect of polyphenolic and flavonoid compounds rich *A. spinosa* extracts against platelet aggregation, bleeding time, plasmatic coagulation and antioxidant activity.

Further, antioxidant activity should not be decided based on a single antioxidant test model. Several *in vitro* test procedures are performed to evaluate antioxidant activities with samples of interest (Alam et al. 2013). Therefore, we used three tests with different antioxidant principles in this study. The ability of the extracts to give a hydrogen atom or an electron has been assessed using DPPH (Tepe et al. 2005). The potential of *A. spinosa* extracts to delay lipid peroxidation was evaluated using a β-carotene/linoleic acid bleaching test and FRAP reducing power test, which assesses the ability of the extracts to reduce the Fe^{3+}ion to Fe^{2+} ion. Results of the study showed that except for Chwihia branches extract, all extracts exhibit a strong ability to scavenge oxygen free radicals. Results of the study suggested that leaves extracts are more effective than branches extract. There is a well-established link between antioxidant activity and the abundance of these bioactive chemicals. However, certain limits must be considered when comparing the content and composition of polyphenols in Argan leaves and branches aqueous extract and their antioxidant activity. The synthesis of secondary metabolites is an ingenious strategy plants use to control their environment and adapt to biotic and abiotic conditions. Therefore, the geographical origin, the period of collection of plant material and the phenotype of the Argan tree are the major parameters that have a significant impact on the chemical composition of the plant (Bourhim et al. 2021), which could explain the difference in activity between the same plant from two different regions.

This study investigated the effect of different extracts of *A. spinosa* (1 mg/ml) on thrombin-induced platelet aggregation and bleeding time under *in vitro* conditions. Contrary to the Chwihia branches extract, which exhibits a shallow antiplatelet effect, all other extracts demonstrate a significant reduction in platelet aggregation and a prolonged tail bleeding time. Similar studies on some medicinal plants have been conducted and reported that inhibition of platelet aggregation is not always associated with a change in bleeding time. Mekhfi et al. (2008) showed that *A. spinosa* oil inhibits platelet aggregation without affecting bleeding time. However, Ferreira et al. (1999) reported that in addition to its antiplatelet effect, isolated quinones of *Auxemma oncocalyx Taub* cause a significant decrease in bleeding time. This difference in the results suggested that different compounds have different modes of action. Based on all these data, the tested aqueous extracts of *A. spinosa* (leaves and branches) have a more potent antiaggregating effect than its oil. This can be attributed to their difference in solubility. Indeed, *A. spinosa* oil could act just at the level of the cell membrane, while the constituents of aqueous extracts can penetrate inside the cell. According to Rand et al. (1988), fatty acids can regulate platelet function by modulating the number of their precursors and changing cell membrane fluidity.

In the cascade of coagulation, activated partial thromboplastin time and prothrombin time are the basic blood measurements to assess the risk of bleeding and thrombosis. APTT is linked to the intrinsic and/or common pathways of plasma coagulation and is used to detect deficiencies in factors II, V, VIII, IX, X, XI and XII. PT mainly explores clothing factors of the extrinsic pathway (Kim et al. 2013; Sayari et al. 2016). The current study showed that leaves

of *A. spinosa* significantly prolong the PT and APTT, suggesting that these extracts exert an intense action on the extrinsic and intrinsic pathways of plasmatic coagulation.

Functional abnormalities of platelets or thrombopathies produce hemorrhagic clinical manifestations and an extension of bleeding time similar to thrombocytopenia. The hemorrhagic syndrome may affect one or more steps, including adhesion, secretion, activation, aggregation and coagulant hemostasis activity (Nurden et al. 2007). In the current study, we suggest that the extension of bleeding time induced by *A. spinosa* leaves would be caused in part, probably, by the inhibition of platelet aggregation. Consequently, these extracts may be able to prevent cardiovascular and thrombotic complications. This shows this plant's special interest, which is widely consumed by the Moroccan population.

Epidemiological studies suggested that high consumption of polyphenols is associated with reduced risk of cardiovascular disease (Nardini et al. 2007). Furthermore, sterols can increase the release of prostacyclin "PGI2" by vascular smooth muscle cells. This molecule acts as a platelet antiaggregator by stimulating adenylate cyclase and cAMP production (Awad et al. 2001). Another study shows that sterols present in margarine significantly inhibits platelet aggregation and adhesion time after collagen activation (Kozłowska-Wojciechowska et al. 2003). Margarine-containing sterols can play a vital role in the prevention of cardiovascular diseases.

Flavonoids, whose richness is demonstrated by both types of extracts prepared in this study, and their beneficial properties against cardiovascular risk are well documented (Kumar and Pandey 2013). According to Pignatelli et al. (2000), flavonoids quercetin and myricetin inhibited platelet aggregation induced by different agonists such as ADP, arachidonic acid and collagen.

Many routes, such as arachidonic acid way, phospholipase C signalling pathway, mobilization of intraplatelet Ca^{++} and other enzymatic activities, are proposed to explain the antiaggregant action of flavonoids. These compounds may act by stopping the thromboxane A_2 production by blocking the cyclooxygenase "COX", phospholipase A_2 or the thromboxane A_2 synthetase (Faggio et al. 2017) or inhibiting the interaction of thromboxane A_2 with its receptor (Guerrero et al. 2005). In addition, Nardini et al. (2007) reported that reduced phospholipase C activity may be leading to the breakdown of the cytoplasmic inositol trisphosphate (Sheu et al. 2004). Finally, the cytoplasmic Ca^{++}, which plays a key role in platelet activity, is indeed reduced, and it was reported that quercetin has been shown to inhibit calcium mobilization (Pignatelli et al. 2000). On the other hand, flavonoids are also able to activate adenylate cyclase and guanylate cyclase or inhibit phosphodiesterases. These enzymatic activities inhibition can promote the accumulation of cAMP and cGMP. This accumulation causes a decrease in granular secretion and, thus, an inhibition of platelet aggregation (Landolfi et al. 1984).

Moreover, Faggio et al. (2017) demonstrate that polyphenols enhance the synthesis of nitrogen monoxide. By stimulating guanylate cyclase, this molecule causes an increase in the level of intraplatelet cGMP leading to an inhibition of platelet aggregation. Tyrosine kinase proteins contribute massively to regulating platelet function from the initial phase of activation to the final step of aggregation, and an *in vitro* study has shown that quercetin reduced phosphorylation (Hubbard et al. 2003).

Finally, the activation of blood platelets produces some reactive oxygen derivatives via NADPH oxidase activation. These reactive oxygen derivatives can stimulate platelet aggregation by activating a kinase protein or inhibiting tyrosine phosphatase (Nardini et al. 2007). Inhibition of platelet aggregation by phenolic compounds is achieved by reducing endogenous peroxides or storing endogenous antioxidants. Flavonoids can trap (or recover) free radicals such as superoxide anion hydroxyl radicals by transferring hydrogen (Procházková et al. 2011). A previous study showed that flavonoids such as quercetin and catechin decrease collagen-induced human platelet aggregation by inhibiting the platelet production of hydrogen peroxide (Pignatelli et al. 2000). In the same way, Freedman et al. (2001) reported that the consumption of grape juice significantly decreases the production of superoxide anion by platelets.

Other mechanisms are also proposed, such as the inhibition of the binding of Von Willbrand factor to its specific receptor and also the change in the conformation of the IIb-IIIa glycoprotein fibrinogen receptor, responsible for the aggregation of the platelets (Mruk et al. 2000).

Conclusion

In conclusion, the results of this study showed an *in vitro* antioxidant, antiaggregant and anticoagulant effect of two aqueous extracts of *A. spinosa* (leaves and branches) harvested from the Oriental Region of Morocco. These activities may be related to many active phytochemical Compounds present in the extract. These original results suggest that *A. spinosa* may be considered a vibrant source of antiplatelet, anticoagulant and antioxidant compounds, capable of preventing thrombotic complications of cardiovascular diseases.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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