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Research Article

ANTI-COAGULANT ACTIVITY OF *RUBUS ULMIFOLIUS* EXTRACTS FROM JIJEL, ALGERIA

Saliha Hireche [1,2], Rachid Belhattab[1,3*], Asma Cherbal [2,4], Mohammed Kebieche[5],

[1] *Laboratory of Applied Microbiology, Ferhat Abbes Setif1 University, 19000 Setif, Algeria*
[2] *Department of Molecular and Cell Biology, Faculty of Nature and Life Sciences, Mohammed Seddik Benyahia University, 18000 Jijel, Algeria*
[3] *Department of Biochemistry, Faculty of Nature and Life Sciences, Ferhat Abbes Setif1University, 19000 Setif, Algeria*
[4] *Laboratory of Biomathematics, Biophysics, Biochemistry and Scientometry, Faculty of Nature and Life Sciences, University Abderrahman Mira, 06000 Bejaia, Algeria.*
[5] *Faculty of Nature and Life Sciences, University of Batna2, 05000 Batna, Algeria*.

**Corresponding Author:*
E-mail: rbelhat@yahoo.fr

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ABSTRACT. The anticoagulants used such as heparins and anti-vitamin K derivatives have life-threatening side effects. Hence, there is an increasing need to identify new anticoagulants from plants, which constitute the major source of drugs for the treatment of many diseases. Such natural substances may act with no side effects for clinical use. *Rubus ulmifolius* is an Algerian medicinal plant that is used traditionally to cure some diseases. Yet, there is no report regarding its anticoagulant activities. So this work was conducted to study the anticoagulant potential of hydro-methanolic and hydro-ethanolic *Rubus ulmifolius* extracts through activated partial thromboplastin time (APTT) and prothrombin time (PT) assays. In this study, hydro-methanolic and hydro-ethanolic extracts could significantly prolong the time for the blood to clot estimated by by the APTT assay indicating its ability to inhibit the intrinsic pathway of the coagulation cascade with all concentrations used and the anticoagulant effect persist at an approximate concentration of 195 µ g/mL of plasma. A significant difference was observed between results of the two extracts ($p < 0.01$) where the hydro- methanolic extract is the best. Perhaps this result is due to the richness of the hydro-methanolic extract with polyphenols compared to the hydro-ethanolic one. The two extracts also affect the extrinsic pathway of coagulation with some concentrations. The combined effect on the two pathways was also observed with some concentrations, which is suggested to cause a qualitative or quantitative fibrinogen defect.

Keywords: *Rubus ulmifolius, hydro-methanolic, hydro-ethanolic, anticoagulant activity, phenolic compounds.*

INTRODUCTION

The hemostatic system consists of platelet aggregation, coagulation and fibrinolysis. As a host defense mechanism, it preserves the integrity of the high pressure closed circulatory system in mammals in case of vascular damage [1, 2]. the coagulation process is a result of sequential reactions in which thrombin as a final enzyme converts soluble fibrinogen into insoluble fibrin [3, 4]. Thrombosis occurs when blood constituents form an abnormal mass named clot which blocks the vascular system of a living animal. When it happens in the deep veins, it is referred to as deep vein thrombosis (DVT) [5]. it is well

documented that several diseases such as atherosclerosis, ischemic heart disease and stroke, as well as rheumatoid arthritis, hyperuricemia, and various inflammatory conditions are probably due to thrombosis [2]. In developed countries, morbidity and mortality are often caused by thromboembolic disorders such as pulmonary emboli, deep vein thrombosis, strokes and heart attacks [3, 6]. All coagulation cascade aspects as well as primary hemostasis and fibrinolysis can be affected [7]. The coagulation cascade dysregulation and the subsequent formation of intra-alveolar or systemic fibrin clots have been demonstrated in both humans and animal models and constitute the main findings in coronavirus infections associated with severe respiratory disease [8]. Viral infections are associated with coagulation disorders. Recently, numerous studies have shown that coagulation dysfunction is developed in patients with severe novel corona virus pneumonia (NCP) whereas the incidence of venous thromboembolism (VTE) in those with severe NCP is 25% (20/81), which may be related to poor prognosis [9]. Drugs such as heparin and anti-vitamin K derivatives are anticoagulants with deleterious life-threatening side effects [6]**.** Usually, the treatment of thrombotic diseases requires thrombolytic therapy and mechanical interventions. The inconvenients of this with current thrombolytic therapy include slow and incomplete thrombolysis and frequent bleeding complications [4]. It's worthy to note that acute gastrointestinal bleeding is associated with the use of oral anticoagulant therapy [10]**.** Heparin is widely used in patients with arterial thrombosis especially when coumarin derivatives cannot be given. Even though VTE remains the main indication, heparin's ability to potentiate fibrinolysis and stimulate TFPI release can be of benefit. [11, 12]**.** Blood coagulation inhibitors were isolated from some medicinal plants, originating from Asian countries: Korea, China, Indonesia and Malaysia like Piper betle [13]. Medicinal plants are used to treat several disorders in different systems such as thromboembolic diseases; they have shown anticoagulant/ antithrombotic activity and such plants claimed in the traditional system still remain to be scientifically investigated [6]**.** The target of this study is to evaluate the anticoagulant activity of hydro- methanolic and hydro-ethanolic *Rubus ulmifolius* (Rosaceae family) extracts due to its traditional uses at local level.

MATERIALS AND METHODS

Chemicals

Sodium carbonate, gallic acid, quercetin, aluminum chloride, and Folin–Ciocalteau phenol reagent were purchased from Sigma–Aldrich GmbH (Sternheim, Germany) and used for the determination of phenolic compounds. The solvents (methanol and ethanol) used for the extraction were from Sigma-Aldrich and were of analytical reagent grade. PT reagent (Hemostat thromboplastin-SI. Human, Germany), aPTT reagent (Human, Germany), calcium chloride solution (Human, Germany) were used for the determination of anticoagulation activity.

Plant extracts preparation

Plant material was identified by Dr. Hanane Khennouf from Department of Environment and Agronomy Sciences of the University of Jijel.80 g of the grounded dried plant's aerial part was weighed using an electronic balance and flooded with 800 ml of methanol-water solution at 8: 2 (V / V). Stir and mix the mixture manually several times and leave to rest at room temperature for 24 hours. The mixture is filtered using Whatman

paper N°1 and filtrate is concentrated using a rotavapour Heidolphvu 2000 at temperature below 60^0C. This process is repeated three times using a new solvent. Ethanolic extract is prepared in the same way by replacing methanol with ethanol.

Determination of Plant Extract Yield

The yield of evaporated dried extracts based on dry weight basis was calculated from the following equation:

$$\text{Yield (g/100 g of dry plant material)} = (W1 \times 100) / W2$$

Where W1 was the weight of the extract after the solvent evaporation and W2 was the weight of the dry plant material [14].

Determination of phenolic compounds

A. Determination of total phenolic compounds: The dosage of total polyphenols was determined by spectrophotometry, according to the colorimetric method using the Folin-Ciocalteu reagent. The protocol used is based on that described by [15] with some modifications. Briefly, in test tubes, a volume of 0.2 ml of the 125 µg / ml extract was mixed with 1.5 ml of Folin-Ciocalteu reagent diluted 10 times (1/10). After vortexing and incubating for 5 min at room temperature and in the dark, 1.5 ml of 7.5% (W/V) sodium carbonate (Na_2CO_3) solution were added to the mixture. The latter was stirred and stored at room temperature for 90 min. The absorbance is read at 750 nm against a blank containing no extract but is subjected to all the previous steps. To determine the concentrations of total phenols in the extract, a standard calibration curve was carried out in parallel under the same operating conditions with different concentrations of gallic acid (200 µg / ml, 100 µg / ml, 50 µg / ml, 25 µg / ml, 12.5 µg / ml, 6.25 µg / ml and 3.125 µg / ml) obtained from a stock solution prepared at 200 µg / ml. The total phenol content was expressed in mg of gallic acid equivalent (GAE) per g of crude extract. The analysis was performed in triplicate and the mean value was calculated.

B. Determination of total flavonoids: The flavonoid content was determined according to the method of [16, 17], with some modifications. 1.5 ml of extract with a concentration of 125 µg / ml was added to 1.5 ml of 2% $AlCl_3$ (w/ v), the mixture is stirred and then incubated in the dark and at room temperature for 30 min, the absorbance was measured at 430 nm against a blank which contains all the constituents except the extract using a spectrophotometer. The results are expressed in mg quercetin equivalent / g of crude extract with reference to the standard curve for quercetin carried out in parallel under the same operating conditions withdifferent concentrations of quercetin (200 µg / ml, 100 µg / ml, 50 µg / ml, 25 µg / ml, 12.5 µg / ml, 6.25 µg / ml and 3.125 µg / ml) obtained from a stock solution prepared at 200 µg / ml.

C. Determination of flavones and flavonols: The determination of flavones and flavonols was carried out according to the colorimetric method described by [18] with some modifications. A volume of 1 ml of the plant extract solution (125 µg / ml) was mixed with 1 ml of a 2 % (w/v) aluminum chloride solution ($AlCl_3$) and 3 ml of 5% CH_3CO_2Na were added. The mixture was vigorously stirred and then incubated in the dark for 30 min at room temperature. The absorbance is then read at 440 nm against a control prepared under

the same conditions and not containing extract of the plant. The amount of flavones and flavonols was expressed in mg of quercetin equivalent (EQ) per gram of crude extract (mg QE / g) with reference to the quercetin calibration curve carried out in parallel under the same operating conditions with different concentrations of quercetin (200 µg / ml, 100 µg / ml, 50 µg / ml, 25 µg / ml, 12.5 µg / ml, 6.25 µg / ml and 3.125 µg / ml) obtained from a stock solution prepared at 200 µg / ml. The analysis was performed triplicate and the mean value was calculated.

Anticoagulant activity of methanolic and ethanolic extract of plant

The anticoagulant activity was determined by combining [19] and [20] methods with slight modifications. To evaluate the extrinsic and the intrinsic pathways of coagulation, PT and APTT were measured, respectively. The tests were conducted in a fully automated coagulometer (Biomerieux)).

Plasma preparation: Blood samples were collected from healthy volunteers their age is 22 years old, anti-coagulated using 3.2% sodium citrate in a polypropylene container (9 parts of blood to 1 part of sodium citrate solution) and immediately centrifuged (Jouan centrifuge) at 3000 RPM for 10 min, and plasma was separated and pooled.

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PT: The Thromboplastin reagent (Bialobo S-F2160 France), which is reconstituted by distilled water, is then incubated in a water bath at 37 ° C at least 10 minutes before the start of the test. The different concentrations of hydro- methanolic or hydro- ethanolic extracts were diluted in PBS to give different concentrations (12.5, 6. 25, 3. 125, 1. 562, 0. 781, 0.3 90 and 0. 195 mg/ mL of plasma, final concentration). In a test tube, 50 µl of plasma were incubated for 3 minutes at 37 °C, then 50 µl of the previously obtained methanolic or ethanolic extract were added just before completing the volume with 100 µl of the total tromboplastin-calcium reagent. For the control, the methanolic or ethanolic extract is replaced with 50 µl prewarmed PBS. All experiments were carried out in triplicates

APTT: To determine this time, we use the partial thromboplastin with activator reagent (Bialabo SA FO2160 France), which is reconstituted by distilled water, and incubated in a water bath at 37^0 C at least 10 minutes before the start of the test. In the same conditions, 0.02 M of calcium chloride ($CaCl_2$) were used and incubated in a water bath at 37^0C at least 10 minutes. 50 µl of plasma were placed in the test tube. After incubating for 3 minutes at 37 °C, 50 µl of the partial thromboplastin solution were added with activator and the content was mixed rapidly. The mixture was then incubated for another 3 minutes. For the samples, 50 µl of the methanolic or ethanolic extract were added to the contents of the test tube just prior to the addition of calcium chloride solution. For the control the extract is replaced with 50 µl prewarmed PBS. All experiments were carried out in triplicates.

Statistical analysis

Results are given as Mean± SD (Standard Deviation) of three independent replicates. Statistical comparisons of the results were determined by ANOVA followed by Tukey's or Sidak's multiple comparisons tests using Graphpad Prism version 7.00 for windows; GraphPad Software, La Jolla California USA. Results of anticoagulant activity were

considered significant at $p<0.05$ whereas those of the extraction yield and phenolic compounds content were considered significant at $p< 0.001$.

RESULTS

The percentage yields of dry plant material extraction and phenolic content are summarized in Table 1. The total phenolic and flavonoid compounds contents were estimated in methanolic and ethanolic extracts of *Rubus ulmifolius* according to the gallic acid (Fig.1) and quercetin (Fig.2) calibration curves respectively, methanolic extracts showed higher content compared to ethanolic extract. Whereas flavones and flavonols contents (Fig.3) were expressed in quercitin equivalent and showed ethanolic extracts with higher contents compared to methanolic extract.

Table1. *Yield of extraction and phenolic compounds contents of the hydro-ethanolic and the hydro-methanolic extracts*

Plant extracts	Yield (g/100g dry plant)	Poly phenols (mg GAE/g of extract)	Flavonoids (mg QE/g of extract)	flavones and flavonols (mg QE/ g of extract)
Hydro-ethanolic	28.675 ±0.035	306.42±2.7427	605.6±00	136.24±6.5589
Hydro-methanolic	28.75±0.053ns	441.48±4.0712***	649.6±5.6568***	135.03±0.4199ns

Results were compared using two-way ANOVA followed by Sidak's multiple comparisons test. ns $p>0.999$, ***<0.001.

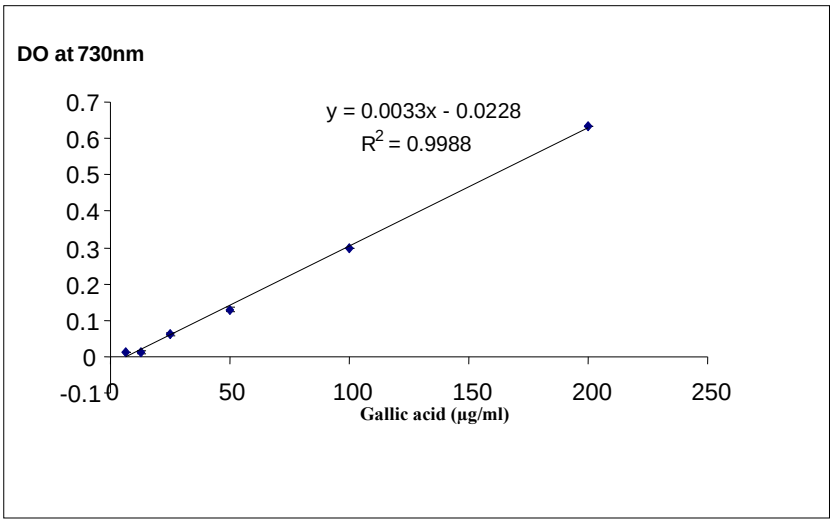

Fig. 1. *Gallic acid calibration curve used for polyphenols determination*

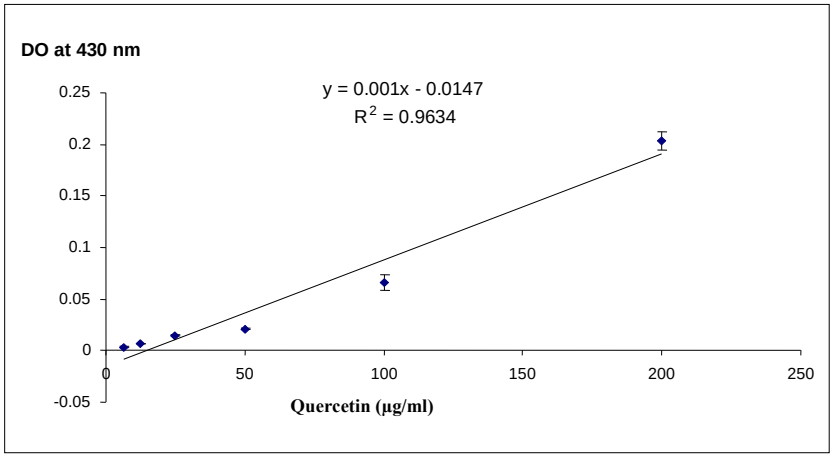

Fig.2. *Quercetin calibration curve for flavonoids determination*

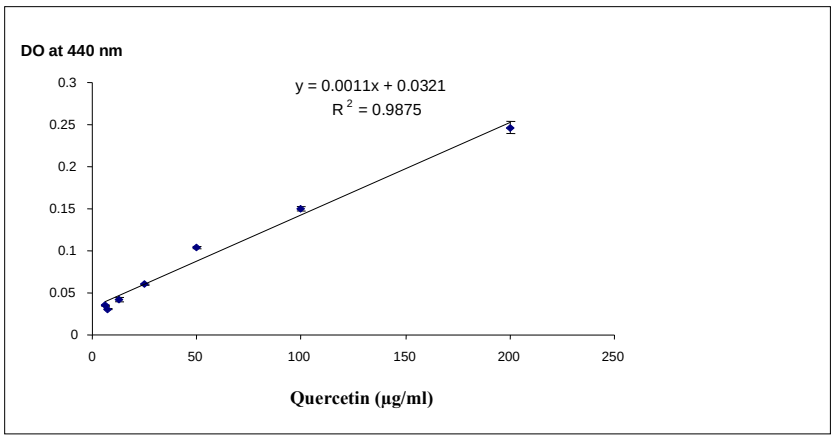

Fig.3. *Quercetin calibration curve for flavones and flavonols determination*

The anticoagulant activity of *Rubus ulmifolius* hydro-methanolic and hydro-ethanolic extracts was measured by a coagulometer in AP and APTT test. Results showed that the two examined extracts prolonged PT (Fig.4) and aPTT (Fig.5) differently.

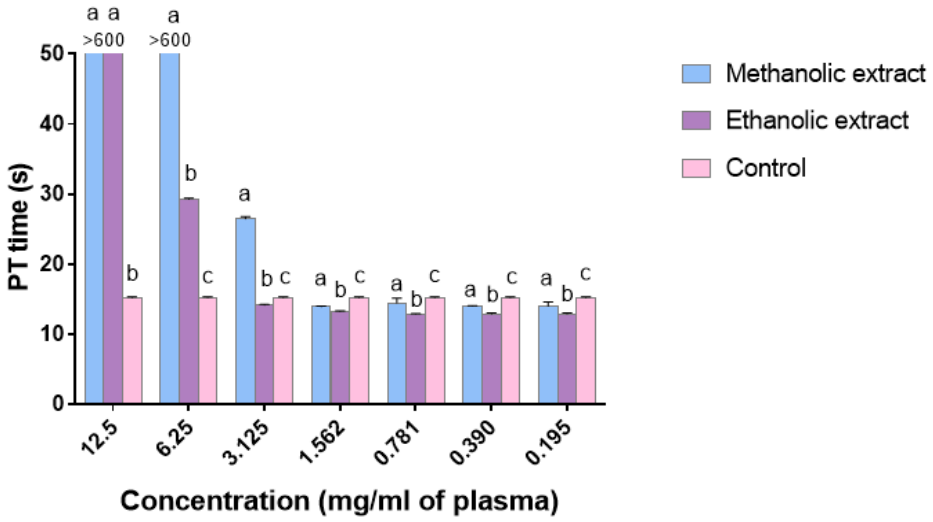

Fig.4. *The anticoagulant activity of Rubusulmifoliushydro-methanolic and hydro ethanolic extracts measured by coagulometer in PT test. Each bar indicates the mean ± S.D. of three separate assays. Comparison was carried out between groups using two-way ANOVA followed by Tukey's multiple comparisons test. Bars labeled with the same letter present no significant difference at p<0.05.*

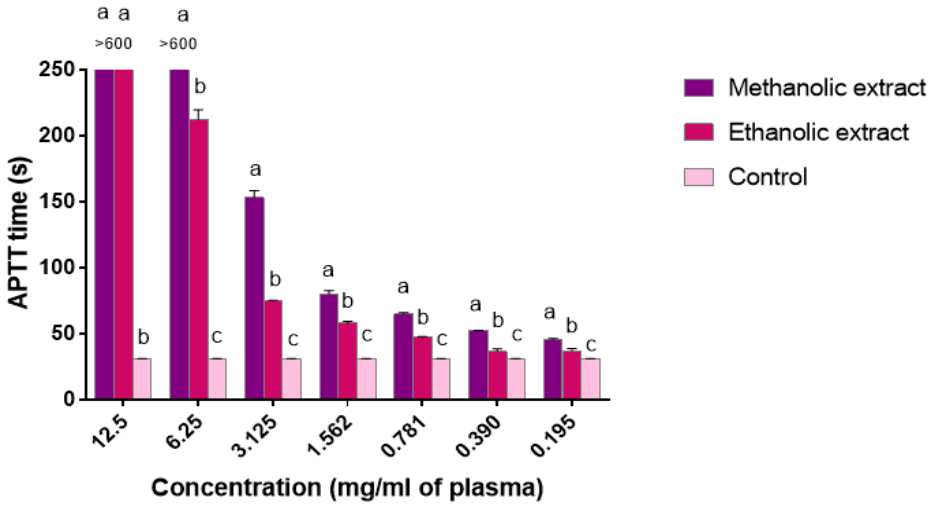

Fig.5. *The anticoagulant activity of Rubus ulmifolius hydro-methanolic and hydro-ethanolic extracts measured by coagulometer in APTT test. Each bar indicates the mean ± S.D. of three separate assays. Comparison was carried out between groups using two-way ANOVA followed by Tukey's multiple comparisons test. Bars labeled with the same letter present no significant difference at p<0.05.*

DISCUSSION

In this study, the main objective was to determine the anticoagulant effect of a selected candidate plant. The blood coagulation system is a necessary process to stop excessive bleeding and to prevent homeostasis imbalance. Coagulation is an important causative factor in diseases such as stroke, ischemia and COVID-19 pandemic [21; 22]. Moreover, determining the active constituents of the plant in order to produce plant-based anticoagulant compounds and medicines would be helpful.

Since the link between cancer and hypercoagulation is established and several changes in prothrobotic direction caused by severe COVID-19 pneumonia [23. 24], drugs able to treat cancer and having potential antithrombotic/anticoagulant activity would be ideal especially when they are of plant origin. In view of the fact that plants are a safe drug source against clotting, our plant extracts were studied for the first time in order to find alternatives to harmful anticoagulants, as the need for these anticoagulants increased by increasing the deadly viral diseases in the world as a whole.

Various models exist to study *in vitro* anticoagulant activity include effects on prothrombin time (PT), activated partial thromboplastin time (aPTT), and thrombin time (TT) among others are used as models to study *in vitro* anticoagulant activity [25]. The standard clotting times for PT and aPTT are between 12.5 and 13.7 seconds and between 31 and 39 seconds respectively [26]. Inhibition of intrinsic blood coagulation pathway factors such as F V, VIII, IX, XI, XII is measured by APPT [27]. It has been shown that aPTT prolongation is due to a deficiency or inhibition of one or more of the intrinsic factors of blood coagulation pathways (prekallikrein, high molecular weight kininogen, factors XII, XI, IX, and VIII) [28].The two examined extracts of *Rubus ulmifolius* prolonged aPTT for all concentrations relative to the normal control at (Almost with all concentrations at $P<0.01$). This indicates that this two plant extracts may cause a deficiency or inhibitory effect on the intrinsic pathway clotting factors [28]. The two extracts exhibited significant prolonged time of intrinsic clotting of blood at all concentrations and this effect persist at a concentration as low as 195 µ g/mL but the methanolic one was shown to be the most active extract (Figure 3 and 4). It has been shown that an isolated PT prolongation is a possible cause of extrinsic pathway (FVII) deficiency or inhibition, but mild factor X, V, and II deficiencies are also possible causes [28]. The two examined extracts of *Rubus ulmifolius* prolonged PT for 12.5 and 6.25mg/ml concentrations relative to the normal control ($P<0.01$). Except methanolic extract of *Rubus ulmifolius* prolonged PT for 3.125 mg/ml concentration relative to the normal control ($P<0.01$). aPTT and PT prolongation time is due to a deficiency or inhibition of the common pathway coagulation factors (factor X, V, and II), or fibrinogen defect [28]. At 12.5 and 6.25mg/ml concentrations it has got a PT and APTT prolongation times due to a deficiency or inhibition of the common pathway coagulation factors (factor X, V, and II), or fibrinogen defect [28].We note a decrease in PT time at the concentrations ranged between 1.562 and 0.195 mg/ml relative to the normal control ($P<0.01$). These results suggest that the extracts have accelerated the coagulation cascade through the activation of several clotting factors of the extrinsic pathway at these concentrations. Also, this coagulation activity could be due to the proteolytic action on fibrinogen. Secondary metabolites of plant origin constitute a potential source of anticoagulants [29]. Some studies have indicated that phenolic acid compounds show better anticoagulant activity [30]. As shown in table1, extraction with methanol was found to provide the highest phenolic contents value (441.4848±4.0712 mg GAE/g dry weight) as compared with extraction by ethanol (306.4242±2.7427 mg GAE/g dry weight)

(P<0.001) this is in agreement with the study of [31] which reported the efficiency of methanol in polyphenols recovery compared to ethanol [31]. The findings were likely in agreement with [32] who found methanol more efficient solvent for extracting phenolic compounds from rosemary leaves as compared to ethanol and water [32]. Due to the rich methanolic extract of the plant with polyphenols, it gives better anticoagulant activity than ethanolic one. Likewise, flavonoids contents in samples extracts, expressed as mg QE/g of dry extract, differ in significant way among solvents and the highest flavonoids contents were found in methanolic flowers extracts [31].In our study the highest flavoinoids (649.6±5.6568mg QE/g of dry weight) content were found in hydro-methanolic extract compared to the hydro-ethanolic one (605.6±00mgQE/g of dry weight) (P<0.001) (Table 1).

CONCLUSION

To our knowledge, it is the first work that deals with in vitro anticoagulation activity of *Rubus ulmifolius* extracts have been established. The extracts have a potential anti-coagulant activity which could be used to extract anticoagulants used as treatment. This research is part of the research that is useful in searching for active substances in the case of viral infectionthathavebeenshowntocausethrombosis, especially the current epidemic of Corona virus (COVID-19).

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