Research Article

Anti-platelet aggregation effects of extracts from Arbutus unedo leaves

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Abstract
It is well known that platelet hyperactivity is a risk factor for cardiovascular diseases such as atherosclerosis, stroke and myocardial infarction. This study aimed to examine the effects of extracts enriched in flavonoids obtained from Arbutus unedo leaves on platelet aggregation. Rat platelets were prepared and incubated in vitro with different doses of the tested extracts, and aggregation was trigged by physiological agonists. Platelet treatment with increasing concentrations (0.1 - 1 mg/ml) of diethyl ether extract (genins = free flavonoids) or ethyl acetate extract (heterosidic flavonoids) inhibited platelet aggregation evoked by thrombin in a concentration-dependant manner. The IC₅₀ values were 0.22 ± 0.03 and 0.36 ± 0.05 mg/ml for genins and heterosidic flavonoids respectively. Treatment with Arbutus unedo extracts also significantly reduced the initial rate of platelet aggregation. At 1 mg/ml, the rate inhibition was 97.8 ± 0.74 and 90.8 ± 1.55 % for genins and heterosidic flavonoids respectively. In addition, flavonoids significantly inhibited platelet aggregation induced by ADP, collagen or epinephrine. We conclude that Arbutus unedo extracts show antiaggregant effects due mainly to flavonoids. These results may partly explain the traditional use of Arbutus unedo leaves for the treatment of cardiovascular disorders such as hypertension.

Keywords
Platelets; Aggregation; Arbutus unedo; Cardiovascular disease; Flavonoids

Introduction
Platelet hyperactivity is thought to play a crucial role in the development of cardiovascular disorders such as strokes and myocardial infarction (Dogne et al. 2002; Wong et al., 2010). The high incidence of cardiovascular disorders and the limited tolerability of the main antiplatelet agents currently used (Van De Graaff and Steinhubl, 2001) has stimulated research into the prevention of platelet hyperactivity by several means including medicinal plants (El Haouari and Rosado, 2016). The interest in the use of medicinal plants has been attributed to their good accessibility and to the believe that most of them are better tolerated than conventional drugs (Izzo et al., 2016).

Arbutus unedo L. (strawberry tree, Ericaceae family) is an evergreen shrub with a height usually smaller than 5 m and mainly typical
of Mediterranean climate (Delgado-Pelayo et al., 2016). Its fruits are spherical berries about 15-20 cm in diameter and ripening in autumn. The tree carries at the same time flowers and mature fruits, since the ripening of the fruits takes all the year (Males et al., 2006). In Oriental Morocco, A. unedo is commonly used in the traditional medicine for the treatment of hypertension and diabetes (Ziyyat et al. 1997). It is reported that the leaves of the plant are used as a diuretic, urinary antiseptic, anti diarrheal, anti-inflammatory, antioxidiant, astringent and depurative (Pabucçuoglu et al., 2003; Mariotto et al., 2008; Oliveira et al., 2009). Previous studies have shown that the aqueous extract of A. unedo exhibited vasorelaxant, antidiabetic and antiaggregant actions (Legssyer et al., 2004; Mekhfi et al., 2004; Bnouham et al., 2007). Studies regarding the chemical composition of leaves and fruits indicate the presence of phenolic acids, flavonoids, tannins, anthocyanins and vitamins which may be responsible for the pharmacological properties of the plant (Miguel et al., 2014). Thus, the current study was designed to investigate the effect of extracts obtained from A. unedo leaves on platelet aggregation.

Materials and methods

Plant material

Leaves of A. unedo L. (Ericaceae) were collected from Taza region (Morocco) and the collected plant was identified by Pr. B. Haloui, from the Department of Biology, Faculty of Sciences (Oujda, Morocco), where a voucher specimen (n° 14 ZL) is deposited.

Extraction of flavonoids

150 g of dried and powdered A. unedo leaves were first degreased with hexane (380 ml) using the Soxhlet refluxing apparatus for 10 h. After filtration, the degreased vegetal material has then undergone an extraction with a mixture of acetone (180 ml) and water (260 ml) under reflux during 10 h. After filtration, the filtrate was evaporated in vacuo to remove acetone. The aqueous solution has been recovered and washed with petroleum ether (2 x 100 ml) by decantation to eliminate lipids and chlorophylls. For the extraction of free flavonoids (or genins), the recovered aqueous solution was extracted with diethyl ether (3 x 100 ml). The remaining aqueous solution was then extracted with ethyl acetate (3 x 100 ml) to isolate the heterosidic flavonoids (El Haouari et al., 2006). The yield of extractions of the two types of flavonoids was 2.1 % and 0.003 % for heterosidic flavonoids and genins and respectively.

Animals

The animals were supplied by the Faculty of Sciences, University Mohamed 1st, Oujda, Morocco. They were kept in a polycarbonate cages in environmental conditions with free access to food and water and under 12h light/12h dark (light period 7:00 AM–7:00 PM). All manipulations concerning the animals were carried out in an ethical manner according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health.

Preparation of washed platelets suspension

Blood was collected by catherization of the abdominal aorta in rats (mean weight: 287.2 ± 13.6 g) anesthetized with ether. The anticoagulant used is Acid-Citrate-Dextrose (ACD) (9:1, v/v). Washed platelets were prepared as described by Tomita et al. (1983). Blood was centrifuged at 230 g for 15 min to obtain platelet rich plasma (PRP). The latter has been recentrifuged at 400 g for 15 min. Platelet-poor plasma (PPP) was eliminated and the platelet pellet was delicately resuspended in a washing buffer (NaCl 137 mM, KCl 2.6 mM, NaHCO₃ 12 mM, MgCl₂ 0.9 mM, Glucose 5.5 mM, Gelatine 0.25 %) at pH 6.5. Washed platelets were finally suspended in a suspension buffer (NaCl 137 mM, KCl 2.6 mM, MgCl₂ 0.9 mM, Glucose 5.5 mM, Gelatine 0.25 %, Hepes 5 mM, pH 7.4) at a final concentration of 5 x 10⁵ platelets/ml. The platelet count was performed under an optical microscope and aggregation tests were carried out within 3 hours to avoid loss of aggregability.

Platelet aggregation study

Platelet aggregation was studied using an aggregometer apparatus (Chronolog, Havertown, PA). The platelet suspension (400 µl) was preincubated in the absence (control) or presence of the tested extract for 1 min at 37 °C, and then the platelet aggregation was initiated by the addition of the agonist. The mixture is stirred at 1000 rpm and aggregation was monitored during 5 minutes. Dose-response curves of the platelet aggregation with the different extracts are realized. The percentage of inhibition (%) was calculated using the following equation:

\[ \text{Inhibition} \% = \frac{A - B}{A} \times 100 \]

A= Maximum aggregation of washed platelets in the absence of the plant extract (control).
B= Maximum aggregation of washed platelets in the presence of the plant extract.

The initial rate of platelet aggregation (mm/min) was determined by the slope of the aggregation signal.

The IC₅₀ values (half maximal inhibition) was determined using linear regression method for each dose response study.

1http://www.nap.edu/readingroom/books/labrats/index.html
Acute toxicity
Five groups of six mice each (male and female, 21-36g) were constituted. Each group received orally the aqueous extract of *A. unedo* leaves in a single dose of 0, 3, 6, 8 and 12 g/Kg. Animals were observed for gross effects and mortality during 15 days. To determine the toxicity of the extract, we have adopted the method described by Lorke (1983).

Reagents
Thrombin (from bovine plasma), ADP and Verapamil were purchased from Sigma Chemical Co. Acetylsalicylic acid was obtained from Sigma-Aldrich, Inc. (Germany). Epinephrine was purchased from Acros Organics and Collagen was obtained from ICN Biomedicals, Inc. (USA).

Statistical analysis
All provided data were expressed as mean ± S.E.M. *IC*$_{50}$ was calculated using linear regression method. Student’s *t*-test was used to analyze the differences between values. Only a level of significance set at *P*<0.05 was accepted.

Results

**Effect of extracts from Arbutus unedo on thrombin-induced platelet aggregation**
Treatment of platelets with increasing concentrations (0.1 - 1 mg/ml) of the extract enriched in flavonoids, significantly inhibited thrombin-induced aggregation in a dose-dependent manner. Fig 1 shows typical tracings representing the effect of heterosidic flavonoids on thrombin (0.5 U/ml)-induced platelet aggregation. Fig 2 showed the percentage inhibition of aggregation by flavonoids at different doses. Significant responses of antiplatelet aggregation were observed with all the doses of flavonoids. At 1 mg/ml, there were 93.76 ± 2.11 % (n=4) and 86.04 ± 2.15 % (n = 5) inhibition with genins and heterosidic flavonoids respectively.

**Effect of extracts from Arbutus unedo on rate platelet aggregation**
Genins (0.1 – 1 mg/ml) and heterosidic flavonoids (0.1 – 1 mg/ml) also significantly (*P*<0.05) reduced the initial rate of thrombin-induced platelet aggregation (Fig 3). At 1 mg/ml, the initial rate of platelet aggregation was 1.24 ± 0.41 mm/min (n = 4) and 5.2 ± 0.65 mm/min (n = 5) corresponding to an inhibition of 97.8 ± 0.7 and 90.8 ± 1.5 % respectively for genins and heterosidic flavonoids. Genins appeared to be more effective than heterosidic flavonoids in inhibiting both the initial rate (p<0.01) and the extent of aggregation.

**Effect of extracts from Arbutus unedo on platelet aggregation induced by different agonists**
In order to have an idea on the action mechanism of the tested extracts, the antiplatelet activity of
the extracts enriched in flavonoids was verified with different platelet agonists including ADP, collagen and epinephrine. The obtained results showed that flavonoids from *A. unedo* reduced platelet aggregation in a concentration-dependent manner (Table 1). The analysis of the results (IC₅₀) indicated that genins are more effective than heterosidic flavonoids in reducing platelet aggregation evoked separately by different aggregating agents (Table 1). The positive controls: Acetylsalicylic acid (ASA) and verapamil showed a potent antiplatelet effect towards collagen-evoked aggregation (IC₅₀ = 0.39 ± 0.02 and 0.35 ± 0.02 mg/ml respectively) with a slight difference from flavonoids (Table 1).

**Discussion**

In this investigation, the *in vitro* effects of extracts enriched in flavonoids from *Arbutus unedo* on platelet aggregation evoked by physiological agonists were determined. Our results indicate that free flavonoids (genins) and heterosidic flavonoids significantly inhibited platelet aggregation induced by thrombin (0.5 U/ml) in a concentration-dependent manner. The IC₅₀ values for genins (0.22 ± 0.03 mg/ml) and heterosidic flavonoids (0.36 ± 0.05 mg/ml) were lower than that found previously with the crude aqueous extract (IC₅₀ = 1.8 ± 0.09 mg/ml) (Mekhfi *et al.*, 2006), indicating that the antiaggregant effect of *A. unedo* is mainly due to flavonoids. Moreover, the flavonoids showed more potent anti-platelet effect than the tannins isolated from the methanolic extract of *Arbutus unedo* leaves (Mekhfi *et al.*, 2006). In addition to the inhibition of the extent of aggregation, flavonoids significantly reduced the initial rate of platelet aggregation. These results are consistent with previous reports in which...
flavonoids showed antiaggregant activities in vitro and in vivo (Freedman et al., 2001; Son et al., 2004; El Haouari et al., 2006; Ghayur et al., 2011; El Haouari and Rosado, 2011; Ro et al., 2015; Liang et al., 2015; Lu et al., 2016). There are marked differences in the antiplatelet effect between genins and heterosidic flavonoids. This difference in efficacy could be explained by the chemical structures of the two types of flavonoids. Indeed, flavonoids exist in nature as aglycones, glycosides and methylated derivatives (Middleton, 1984). In the glycosylated form, at least one OH group of the aglycone is bound to one or more saccharides.

The antiaggregant effect of extracts enriched in flavonoids was verified with the use of various agonists: ADP, epinephrine and collagen. The obtained results showed that these compounds markedly reduced platelet aggregation evoked by the different agonists in a dose-dependent manner. This indicates that flavonoids act through non-specific mechanism. The common pathway relative to the cellular action of the different agonists (ADP, epinephrine, thrombin, and collagen) used in the present study is the increase in the concentration of the intracellular Ca²⁺, which can be explained either by its release from intraplatelet stores or through Ca²⁺ influx from the extracellular medium (Heemskerk et al., 1994; Rosado et al., 2004). Since flavonoids inhibit the aggregation evoked by different agonists, this indicates that these compounds may interfere with the Ca²⁺ signaling in activated platelets or blocked the binding of fibrinogen to its receptor in the platelet plasma membrane (glycoprotein GPⅡb-IIIa), the final and common steep of platelet aggregation. Accordingly, it has been shown that the antiplatelet effect of flavonoids may be attributed to the inhibition of Ca²⁺ influx and internal Ca²⁺ release (Formica and Regelson, 1995; Kelly et al., 1996; Kang et al., 1999). Moreover, it has been reported that flavonoids inhibited collagen, ADP and thrombin-induced platelet aggregation, and GPⅡb-Ⅲa expression in ADP and epinephrine-stimulated platelets (Kang et al. 2001; Rein et al., 2000a; 2000b; Pearson et al., 2002). Several other studies have shown that flavonoids modulated different cellular signaling pathways in platelets, including, calcium mobilization, ROS, phosphorylation /dephosphorylation of tyrosine kinase and and nitric oxide pathway (El Haouari and Rosado, 2011)

The acute toxicity study performed in mice showed that oral administration of a dose of the crude aqueous extract from A. unedo up to 1200 mg / kg cause no mortality and no signs of side effects, which demonstrate that the LD₅₀ for the oral administration of the A. unedo aqueous extract was higher than 1200 mg/kg body weight of mice. According to Loomis and Hayes (Loomis and Hayes, 1996), a chemical with an LD₅₀ of between 5,000 and 15,000 mg / kg is considered practically non-toxic. Thus, with an LD₅₀ more than 1200 mg / kg, A. unedo should be considered practically non-toxic in case of acute intake.

### Conclusion

In conclusion, our results indicate that Arbutus unedo leaves have antiaggregant effects in which flavonoids are mainly implicated. We also showed that the vegetal material administered orally has no toxic effect in mice. Further studies are necessary to elucidated antiplatelet mechanism of the tested plant and to isolate the active principle responsible for the antiplatelet activity. These results confirmed partially the traditional use of A. unedo against cardiovascular diseases.

### Acknowledgements

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### Conflict of Interest

The authors have declared that there is no conflict of interest.

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**Table 1.** IC₅₀ values (mg/ml) of extracts (genins and heterosidic flavonoids) from *Arbutus unedo* on platelet aggregation evoked by various agonists. Acetylsalicylic acid (ASA) and verapamil were used as positive controls. Values are expressed as means ± SEM (n = 6-11). (—), Not determined.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Genins</th>
<th>Heterosidic flavonoids</th>
<th>ASA</th>
<th>Verapamil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin (0.5 U/ml)</td>
<td>0.22 ± 0.03</td>
<td>0.36 ± 0.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>ADP (10 µM)</td>
<td>0.33 ± 0.03</td>
<td>0.39 ± 0.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Collagen (5 µg/ml)</td>
<td>0.29 ± 0.02</td>
<td>0.36 ± 0.05</td>
<td>0.39 ± 0.02</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Epinephrine (100 µM)</td>
<td>0.35 ± 0.02</td>
<td>0.4 ± 0.04</td>
<td>—</td>
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</tr>
</tbody>
</table>
References


