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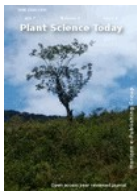

Research Article

Anti-platelet aggregation effects of extracts from *Arbutus unedo* leaves

Mohammed El Haouari[1,2] and Hassane Mekhfi[3]

[1]Centre Régional des Métiers de l'Education et de la Formation de Taza (CRMEF – Taza), BP 1178 - Taza Gare, Morocco.

[2]Laboratoire des Matériaux, Substances Naturelles, Environnement & Modélisation (LMSNEM), Faculté Polydisciplinaire de Taza, Université Sidi Mohamed Ben Abdellah, Fès, Morocco.

[3]Laboratoire de Physiologie et Ethnopharmacologie, Département de biologie, Faculté des Sciences, Université Mohamed Premier, BP 717, 60000 Oujda, Morocco.

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Correspondence
Mohammed El Haouari
✉ elhouarim@yahoo.fr

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 (hetrosidic flavonoids) inhibited platelet aggregation evoked by thrombin in a concentration-dependant manner. The IC_{50} 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

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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, antidiarrheal, anti-inflammatory, antioxidant, astringent and depurative (Pabuçcuoglu *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 Soxlhet 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 repectively.

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[1].

Preparation of washed platelets suspension

Blood was collected by catheterization 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, $NaHCO_3$ 12 mM, $MgCl_2$ 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, $MgCl_2$ 0.9 mM, Glucose 5.5 mM, Gelatine 0.25 %, Hepes 5 mM, pH 7.4) at a final concentration of 5 x 10^8 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 IC_{50} values (half maximal inhibition) was determined using linear regression method for each dose response study.

[1]http://www.nap.edu/readingroom/books/labrats/index.html

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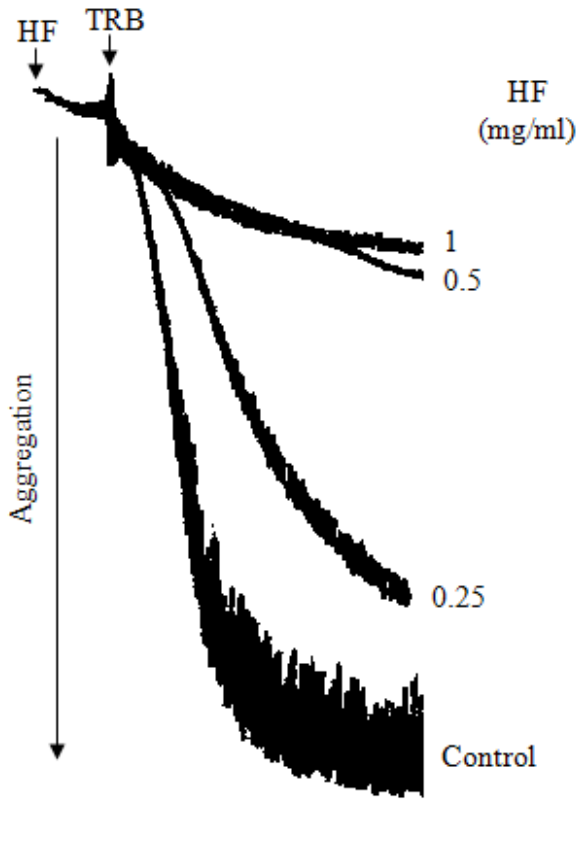

Fig 1. Effect of flavonoids on platelet aggregation. Example of original traces showing inhibition of thrombin-induced platelet aggregation by various concentrations of heterosidic flavonoids (HT) *in vitro*. Washed platelets were incubated with different concentrations of flavonoids for 1 min at 37°C and then stimulated by thrombin (TRB) 0.5 U/ml. Additions were indicated by arrow. Control is platelet aggregation without extract.

Acute toxicity

Five groups of six mices 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 then hetrosidic 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

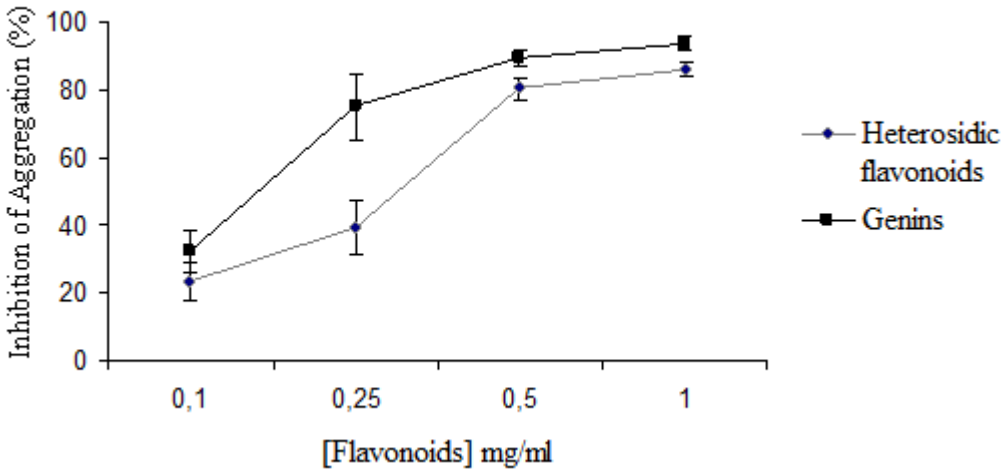

Fig 2. Effects of flavonoids (genins and heterosidic flavonoids) from *A. unedo* leaves on thrombin-evoked platelet aggregation. Washed rat platelets were preincubated for 1 min with increasing doses of flavonoids at 37°C before stimulation with thrombin (0.5 U/ml). Values represent the percent inhibition and are presented as mean ± S.E.M. (n = 4-8).

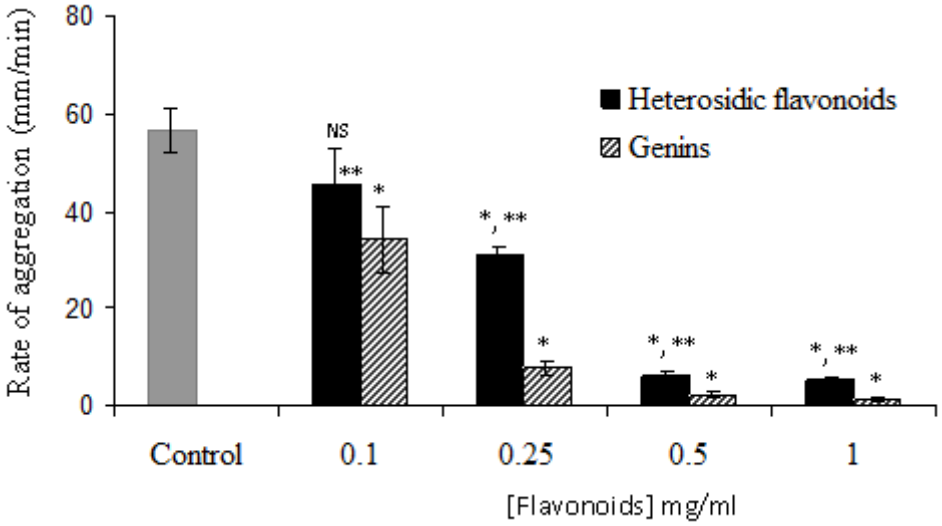

Fig 3. Effect of flavonoids from *A. unedo* leaves on the initial rate of platelet aggregation. Washed platelets were preincubated with increasing doses (0.1 - 1 mg/ml) of flavonoids for 1 min at 37°C before stimulation with with thrombin (0.5 U/ml). Values are expressed as mean ± S.E.M. (n = 4-13). *p<0.05 *vs*. control; **p<0.05 vs. genins. NS: Not significant

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 (IC_{50}) 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 (IC_{50} = 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-dependant manner. The IC_{50} 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 (IC_{50} = 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

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Table 1. IC_{50} 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.

	IC_{50} (mg/ml)			
	Genins	Heterosidic flavonoids	ASA	Verapamil
Thrombin (0.5 U/ml)	0.22 ± 0.03	0.36 ± 0.05	—	—
ADP (10 µM)	0.33 ± 0.03	0.39 ± 0.05	—	—
Collagen (5 µg/ml)	0.29 ± 0.02	0.36 ± 0.05	0.39 ± 0.02	0.35 ± 0.02
Epinephrine (100 µM)	0.35 ± 0.02	0.4 ± 0.04	—	—

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 sacharides.

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 Ca^{2+}, which can be explained either by its release from intraplatelet stores or through Ca^{2+} influx from the extracellular medium (Heemskerk *et al*., 1994; Rosado *et al*., 2004). Since flavonoids inhibit the aggregation evoked by different agonits, this indicates that that these compounds may interfere with the Ca^{2+} signaling in activated platelets or blocked the binding of fibrinogen to its receptor in the platelet plasma membrane (glycoprotein (GP)IIb-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 Ca^{2+} influx and internal Ca^{2+} 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 LD_{50} 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 LD_{50} of between 5,000 and 15,000 mg / kg is considered practically non-toxic. Thus, with an LD_{50} 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.

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Conflict of Interest

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

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