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ORIGINAL PAPER

Antithrombotic Effects of *Amaranthus hypochondriacus* Proteins in Rats

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Abstract Cardiovascular disease (CVD) is a major cause of disability and premature death throughout the world. Diets with antithrombotic components offer a convenient and effective way of preventing and reducing CVD incidence. The aim of the present work was to assess *in vivo* and *ex vivo* effects of *Amaranthus hypochondriacus* proteins on platelet plug formation and coagulation cascade. Amaranth proteins were orally administrated to rats (AG, 8 animals) and bleeding time was determined showing no significant difference compared with control rats (CG, 7 animals). However, results show a strong tendency, suggesting that amaranth proteins are involved in the inhibition of thrombus formation. Non-anticoagulated blood extracted from animals was analyzed with the hemostatometer, where AG parameters obtained were twice the values showed by CG. The clotting tests, thrombin time (TT) and activated partial thromboplastin time (APTT), presented a 17 and 14 % clotting formation increase respectively when comparing AG with CG. The *ex-vivo* assays confirm the hypothesis inferring that amaranth proteins are a potential antithrombotic agent.

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Abbreviations

AG	Rats fed with amaranth proteins
APTT	Activated partial thromboplastin time
BT	Bleeding time
CG	Rats that were fed with control diet
CVD	Cardiovascular disease
HG	Rats injected with heparin
PT	Prothrombin time
TT	Thrombin time

Introduction

Cardiovascular diseases produce an important number of deaths in the occidental world [1]. Consumption of food that helps the organism decreasing arterial pressure, thrombus formation tendency and oxidative stress could be an interesting strategy that leads to a lowering morbidity and a decrease of health costs [2].

Functional foods can contain components with proven physiological activity called bioactive compounds. Within this group of substances are food proteins and peptides encrypted in their sequences. Over the last years it is observed a growing tendency and interest in the utilization of peptides derived from food proteins due to its variety and remarkable multifunctionality [3–5]. Peptides could perform various bioactivities related to the cardiovascular system; antihypertensive, antithrombotic and antioxidant activities among them [5]. Recently it has been shown that proteins from *Amaranthus* seeds, a Mesoamerican pseudocereal, are able to perform some of these activities [6–8].

Blood coagulation is a complex process that involves the confluence of numerous factors and phenomena and consists of three overlapping phases that maintain the integrity of the circulatory system: vascular phase, platelet phase, and coagulation phase. The platelet phase, also called primary hemostasis, implies the adhesion, activation and aggregation of the platelets. The coagulation phase (secondary hemostasis) involves the participation of the coagulation factors, many of which are proteases synthesized in the liver as zymogens that in sequential order lead to the clot formation. This coagulation cascade consists of two triggering pathways, the extrinsic and the intrinsic one, both converging in the common pathway.

Antithrombotic activity of *Amaranthus mantegazzianus* seed proteins and other food protein peptides e.g. pork meat [9], rapeseed [10], and egg white [11] have been informed. A previous work [9] confirmed the presence of potential antithrombotic peptides contained in amaranth proteins or released after an alcalase and trypsin hydrolysis. The glutelin fraction exhibited the highest antithrombotic activity (IC_{50} = 80 ± 3 μg/mL) whereas the amaranth isolate presented lower activity only after proteolysis (IC_{50} = 10.87 ± 1.00 mg/mL). The aforementioned work and most of the above referred publications correspond to *in vitro* assay results, constituting a first approximation to the bioactivity study. *In vivo* and *ex vivo* assays provide a closer approach to the evaluation of this biological activity. In this context, the aim of this work was to study the potential antithrombotic activity of amaranth seeds proteins, assessing their effect on blood coagulation in rats fed with amaranth proteins. This study performed for the first time will provide a progress in the use of amaranth proteins as functional ingredients.

Materials and Methods

Plant Material and Samples Preparation

Amaranth Seeds *Amaranthus hypochondriacus* (cv Mercado) seeds harvested at Río Cuarto, Córdoba, Argentina.

Amaranth Flour Preparation Seeds were ground using a cyclone mill with a 1 mm mesh. The resulting flour was defatted with n-hexane (10 g flour/100 mL n-hexane) during 24 h, the five first hours with constant stirring and then overnight contact.

Preparation of Protein Isolates Amaranth isolates were obtained by methodology previously used in our laboratory [12]. Briefly, amaranth defatted flour was suspended in distilled water (10 g defatted flour/100 mL), pH 9, 1 h stirring, room temperature). Then, a centrifugation step was made (9,000×*g*, 10 °C, 20 min) and the supernatant was adjusted to pH 5 with 2 M HCl in order to precipitate the proteins at their isoeletric point. After another centrifugation step (9,000×*g*, 4 °C, 20 min) the precipitate was suspended in water (1:3 relation), neutralized and lyophilized. The isolate composition was determined. The protein content was determined by Kjeldahl (method 954.01 AOAC, 1990), using as the conversion factor 5.85 [13]. The methods used for measuring carbohydrates, ashes, water, and total dietary fiber were, respectively, anthrone after complete acid hydrolysis, heating in a muffle at 550 °C, drying in a stove at 105 °C until constant weight, and sequential enzymatic digestion by heat stable α-amylase, protease and amyloglycosidase according to AOAC (methods 923.03, 925.09, and 991.43; 1990). Every determination was performed in duplicate.

Animals Assay

Materials Male Wistar rats (strain WKAH/Hok) aged 11–12 weeks (270–340 g, LAE Facultad de Ciencias Veterinarias, UNLP, La Plata, Argentina). Diets were formulated according to the American Institute of Nutrition using AIN-93G as the control diet [14] in the laboratory of Nutrición y Bromatología, Facultad de Farmacia y Bioquímica, UBA, Buenos Aires, Argentina). Amaranth diet consisted in a partial replacement of the protein source, casein, for the amaranth protein isolate (Table 1). Lactic casein (Dilsa, Lanus, Argentina), L-cystine (Anedra, San Fernando, Argentina), soy oil (Molinos Río de La Plata, Victoria, Argentina), choline chloride (Sigma, St. Louis, MO, USA), microcrystalline cellulose (Sigma), dextrin (Cofem, Lima, Perú), vitamins and minerals were used for diets preparation.

Twenty two male Wistar rats were distributed in three groups: Control group (CG), Amaranth group (AG), and Heparin group (HG). CG ingested the control diet, while AG was fed with the amaranth diet (Table 1). HG consumed the control diet and after the two weeks experiment the animals were intraperitoneally injected with 250 UI of sodium heparin (Fada Pharma, CABA, Argentina). Food and water were given *ad libitum.*

Blood Extraction and Plasma Preparation Once the feeding period was completed animals were anesthetized with intraperitoneal injections of 25 mg/kg sodium pentobarbital (Sigma) and 2.5 mg/kg diazepam (Klonal, Quilmes, Argentina) and immobilized. Then, the blood was extracted by cannulation of the abdominal aorta artery. The blood was right away destined to hemostatometer assays. An aliquot of the blood from the animals was placed in conical tubes with 2.5 g/100 mL sodium citrate solution (1 part of sodium citrate and 9 parts of blood). The plasma was separated from blood cells by centrifuging at 2,500×g during 15 min at 20 °C and stored at −20 °C until further testing.

Table 1 Formulation of diets as recommended by the American Institute of Nutrition, AIN-93G [14]

	Ingredients (g/1000 g food)							
	Casein	Amaranth isolate	Minerals	L-cystine	Soy oil	Choline	Fiber	Dextrine
Control diet (C diet)	140.0	-	35.0	1.8	40.0	7.1	50.0	716.1
Amaranth diet (A diet)	64.7	89.8	35.0	1.8	39.1	7.1	50.0	702.5

Determination of Antithrombotic Activity

Bleeding Time The bleeding time (BT) is a measure that studies specifically primary hemostasis. Measures were performed using the protocol described by Stenberg et al. [15]. A small cut on the tip of the tail of the animals was undertaken while it was under anesthesia. After making the wound, bleeding was recorded by touching the area of the incision with a filter paper strip every 30 s. BT was defined as the time required to completely stopping the bleeding, without leaving bloodstains on the filter paper.

Hemostatometer In order to study different clotting parameters while blood is flowing, in a dynamic way, a hemostatometer was custom-built by Dr. Gustavo Rinaldi in the Facultad de Ciencias Médicas de la Universidad Nacional de La Plata following the descriptive details given by various authors [9, 16–18].

The principle of hemostatometry is shown in Fig. 1. The hemostatometer consists of a Harvard pump which infuses liquid paraffin, at a constant flow (0.2 mL/min), into a 37 °C thermostatized chamber where the non-anticoagulated blood sample extracted from the animals was previously placed. The blood is displaced by the liquid paraffin, resulting in blood flow through the polyethylene tubing (i.d. 1.6 mm). A pressure transducer records pressure changes in the system over time generating the hemostatograms. In order to maintain the basal pressure of the system at about 60 mmHg, the outflow tubing is submerged the required depth in a tube filled with mercury. While blood is flowing the tube is pierced with a fine needle at 90 s after blood withdrawal, resulting in 'bleeding' from the tube into surrounding warm saline, leading to an abrupt decrease of the initial pressure (Fig. 1b). Eventually, phenomena tending to occlude the injury of the tube stop the hemorrhage due to the formation platelet haemostatic plug in the holes, causing a recovery of the initial pressure in the system (first pressure increase, Fig. 1b). Subsequently, the flow in the tube stopped and the pressure increased, indicating total coagulation (second pressure increase, Fig. 1b). The recorded pressure changes reflected both the formation of the platelet plug and coagulation processes. The pressure recovery area was used to assess platelet activation and the time required to reach 100 mmHg of pressure was defined as the clotting time.

Clotting Tests The clotting tests were performed with clinically used commercial kits (Wiener Lab., Rosario, Argentina). The citrated plasma used (relation blood:citrate 9:1) was obtained from a cannula placed in the abdominal aorta of the rats. The extracted blood was centrifuged (2,500×*g*, 15 min, 20 °C) and frozen at −20 °C until use.

Activated Partial Thromboplastin Time (APTT) Test Citrated rat plasma (100 μL) was incubated for 1 min at 37 °C. The APTT reagent (100 μL) was added into the mixture and incubated for 3 min at 37 °C. The clotting time was determined after adding 100 μ L of 25 mM $CaCl_2$.

Prothrombin Time (PT) Test Rat plasma (100 μL) and the PT reagent were preincubated during 2–3 min at 37 °C. Then the plasma was added to the tube containing 200 μL of reagent PT and the clotting time was recorded.

Thrombin Time Test (TT) In order to determine TT, 200 μL of rat plasma were incubated 2 min at 37 °C and immediately the clotting time was determined after inducing the clotting by adding 200 μL of the TT reagent 2.3 NIH/mL.

Every determination was performed in triplicate.

Statistical Analysis

Results were evaluated statistically by using variance analysis (ANOVA) with the *post-hoc* Tuckey's test ($\alpha = 0.05$).

Results and Discussion

The percent composition of the protein isolate, used for amaranth diet preparation (Table 1) was determined. The content of proteins, water, ashes, carbohydrates and fiber expressed as % (*w*/*w*) was 71.3 ± 1.5 % (f = 5.85), 6.4 ± 0.6 %, 3.6 ± 0.1 %, 9.0 ± 0.8 %, 13.1 ± 0.9 %, respectively.

During the animal assay the average food consumption per rat *per* day was calculated for the three groups control, amaranth and heparin (Fig. 2a). CG consumed 18.7 ± 2.2 g diet/rat/day, whereas AG ingested 18.3 ± 3.1 g diet/rat/day, and HG consumed 17.2 ± 1.2 g diet/rat/day. Results show that

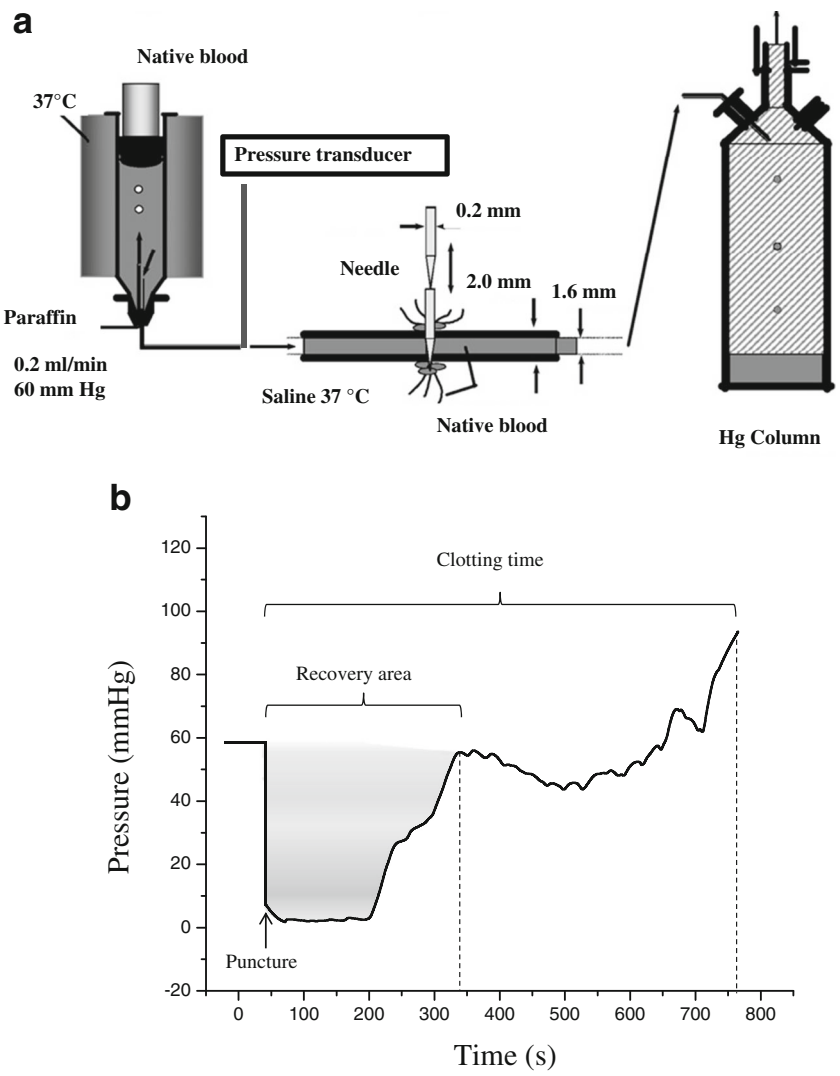

Fig. 1 **a.** Scheme of the hemostatometer used for *ex vivo* assays with blood of animals and **b.** Hemostatogram obtained with one of the rats. Recovery area and clotting time are shown in this figure

the average food consumption did not present significant difference between the groups, and the values were similar to those reported by Kalyani et al. [19]. Average weight of animals/week for the three groups was calculated (Fig. 2b). A slight increase of the average weight was observed for all the groups during the assay, varying between 300 and 400 g. This weight range is coincident with data from Cossio-Bolaños et al. [20] work, where Wistar rats growing curves were evaluated. Authors found average weights between 350 and 380 g for animals aged 11–13 weeks. Figure 2b shows significant average weight increases at the end of the assay in all the groups (Tukey, $\alpha = 0.05$), denoting a slight growth of the animals, which should continue until week 15 [20].

Bleeding Time

Bleeding time, a widely use clinical assay that studies platelet activity [21], is defined as the time required to stopping the bleeding generated by an intentional injury. This parameter increases when there is a deficiency or inhibition of the platelets, alterations in their adhesion to the blood vessel or alterations in their aggregation [22], all primary hemostasis phenomena. Figure 3a shows, as an example, three filter paper strips that belong to bleeding times recorded for the three groups. HG exhibited the longest bleeding time (420 s), followed by AG (330 s) and CG (210 s). This tendency was observed over all the determinations performed in the groups (Fig. 3b). The average bleeding time was 230 ± 10 s for CG, 270 ± 21 s for AG and 360 ± 24.5 s for HG. Even though HG exhibited a bleeding time significantly different compared to amaranth and control groups, there was no significant difference between CG and AG (Tukey, $\alpha = 0.05$).

Lavelle and MacIomhair [22] obtained bleeding times of 340.9 ± 25.7 s in a group of control rats, and 551.0 ± 31.8 s for an heparin group (200 UI/kg rat). Both times were higher than those detected in this work. The difference could be due to the

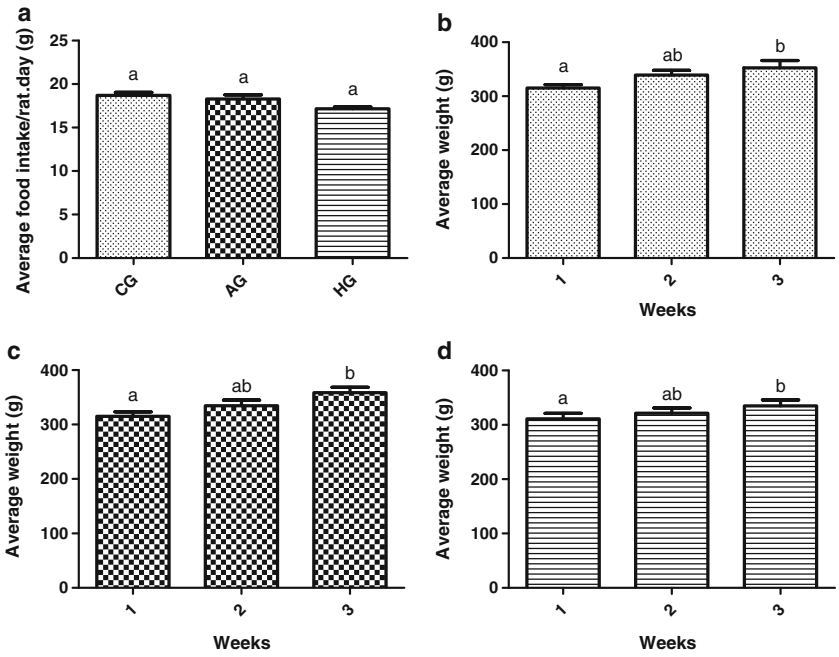

Fig. 2 Animal feeding. **a.** Average food intake per day *per* rat of control group (CG), amaranth group (AG), and heparin group (HG). Average weight *per* week of: **b.** control group, **c.** amaranth group, and **d.** heparin group. Error bars correspond to standard error of the mean. $n = 5–7$ *per* group of rats. Different letters over the bars indicate significant difference (Tukey, $\alpha = 0.05$) in each graphic

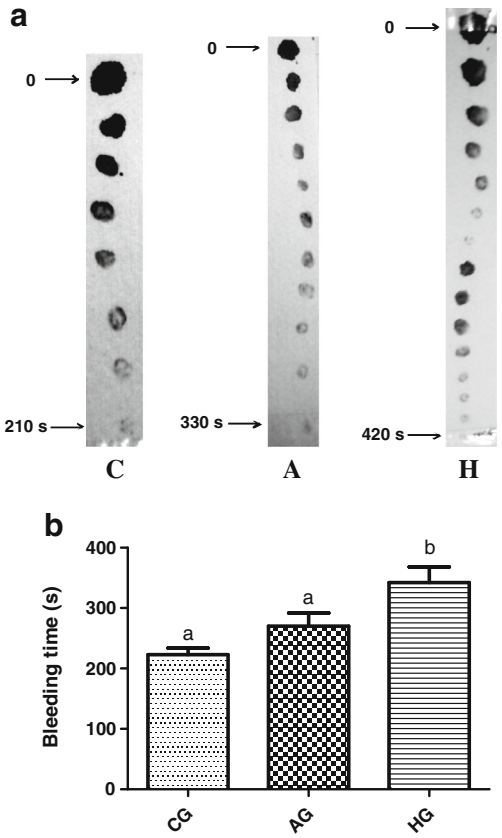

Fig. 3 Bleeding time in rats. **a.** Actual records pertaining to an animal of each group: control (C), amaranth (A), and heparin (H). **b.** Average bleeding times *per* group: control group (CG), amaranth group (AG), and heparin group (HG). Error bars correspond to standard error of the mean. $n = 5–7$ *per* group of rats. Different letters over the bars indicate significant difference (Tukey, $\alpha = 0.05$)

fact that Lavelle and MacIomhair used male and female Sprague Dawley rats. However, both works show that heparin produces longer bleeding times. Heparin is an anticoagulant that inhibits thrombin enzyme, whereas bleeding time is related with platelet function. Liu et al. [23] found bleeding times between 120 and 180 s for control group, and from 400 to 1200 s for rats administrated with different concentrations of an antiplatelet drug called Prasugrel, using an esophageal probe. Since Prasugrel inhibits platelet function, the results informed by Liu et al. [23] are not comparable to those obtained in this work.

The bleeding times shown, as well as those informed by Lavelle and MacIomhair [22], indicate a greater inhibition of the platelet aggregation when animals are heparinized. Other authors confirmed the fact that heparin, tough recognized for inhibiting reactions involved in the coagulation cascade, generate bleeding time extensions [22, 24]. Heparin inhibits thrombin, and it has been demonstrated that there are PAR family receptors in the platelets surface cleaved and activated by thrombin, suggesting that this enzyme activates human platelets [25]. Other explanation for this behavior could be that an arteriole from the tail is cut in the tail transection method. The arteriole contains a smooth muscle layer that causes vasoconstriction after injury [26]. The vasoconstriction could cause a slight blood accumulation previous to its outflow; hence, secondary hemostasis and thrombin inhibition would be also involved in the assay. According to Fritz et al. [27], amaranth has a vasodilator effect that could partially counteract the injury vasoconstriction.

Even though bleeding times of AG did not presented significant difference compared to CG, results show a strong tendency, suggesting that amaranth proteins are involved in the inhibition of the platelet plug formation.

Hemostatometer

Each animal provided a hemostatogram (Fig. 1b) and parameters related with the coagulation process were obtained. The recovery area (Fig. 4A) corresponds to the area enclosed between the register and a base line draw from the zero time, when the piercing of the tube was made (Fig. 1), to the time where the initial pressure of the system (60 mmHg) was reached. This parameter is associated with platelet plug formation, which generates the cessation of the 'bleeding' from the tubing by closing the orifice and causes an increase in the system pressure. The clotting time (Fig. 4B) is defined as the time required reaching 100 mmHg of pressure in the hemostatometer, moment in which total blockage of the blood flow is observed.

The average recovery area of AG animals resulted significantly higher compared to CG (Fig. 4A), whereas significant differences were not observed between AG and HG. Results indicate that the inclusion of amaranth isolate in the diet caused an increase in this parameter, similar to that generated by the use of an anticoagulant (heparin).

Recovery areas from hemostatograms have been informed by different authors. Shimizu et al. [9] obtained recovery area values from 5000 to 7000 mmHg/s for control rat blood, and values between 10,000 and 12,000 mmHg/s when blood blended with a protein hydrolysate from pig muscle was analyzed. Other authors [28] reported recovery area values in a range of 7500–10,000 mmHg/s with control rat blood, and 15000–45000 mmHg.s with blood blended with tomatoes extracts in a 9:1 (blood:extract relation). Satake et al. [18] obtained areas of approximately 5000 mmHg/s when blood of control rats was studied, and areas of 7500 mmHg/s when they analyzed blood of rats administrated with purified *Centella asiatica* extracts trough an oral intragastric tube. The recovery area values obtained in this work (CG: 6422 ± 1208 mmHg/s) resulted comparable to those informed in bibliography for control rat blood and higher values when blood of rats feed with amaranth diet was studied (AG: 13,618 ± 1, 454 mmHg.s). Results suggest that the amaranth protein isolate incorporated in the diet (9 %, *w/w*), ingested, digested and absorbed in the intestine, contains bioactive compounds that affect the behavior of the extracted blood. However, a synergic effect between casein and amaranth proteins cannot be discarded as there have been described peptides released from κ-casein able to inhibit platelet aggregation, due to the sequence homologies that exist in the fibrinogen γ-chain and κ-casein [29]. Hence, the control diet may exert an antithrombotic activity over CG animals, effect already demonstrated in humans [30].

The clotting time parameter is associated with secondary hemostasis, where fibrin monomers are generated from fibrinogen due to the action of thrombin enzyme in order to reach total coagulation of the blood. HG presented the highest clotting time (828.5 ± 46.8 s), which was an expectable behavior due to the fact that heparin is a widely used anticoagulant, as it inhibits thrombin (Fig. 4B). AG clotting time was 612.1 ± 32.4 s, while CG presented the lowest value, 330.9 ± 49.5 s (Fig. 4B). The three average clotting times were significantly different (Tukey, $\alpha = 0.05$).

Satake et al. [18] found differences between the clotting times of the animals control group and the group administrated with the most active *Centella asiatica* extracts (500 and 600–750 s, respectively). When potentially antithrombotic samples were blended with the blood, no difference was detected compared to controls [9, 28].

Clotting Tests

The thrombin time (TT) studies fibrinoformation phase as it measures the clotting time of a citrated plasma in presence of thrombin; prothrombin time (PT) detects alterations of the extrinsic pathway factors as it records the clotting time of a citrated plasma in presence of thromboplastin and Ca^{+2}; activated partial thromboplastin time (APTT) detects changes in the intrinsic pathway components and consists in the measurement of the clotting time of a citrated plasma in presence of thromboplastin phospholipid components (partial thromboplastin), an activator and Ca^{+2}.

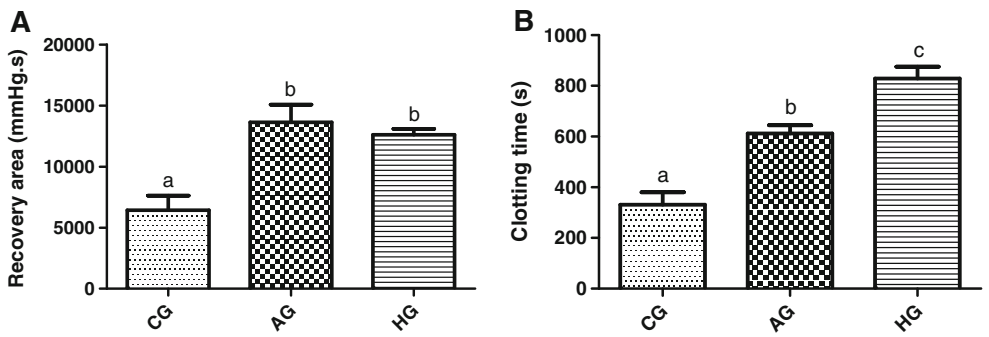

Fig. 4 Parameters obtained from hemostatograms. **A** Average recovery areas per group: control group (CG), amaranth group (AG), and heparin group (HG). **B** Average clotting times *per* group: control group (CG), amaranth group (AG), and heparin group (HG). Error bars correspond to standard error of the mean. $n = 5–7$ *per* group of rats. Different letters over the bars indicate significant difference (Tukey, $\alpha = 0.05$)

Rats have been a widely used animal model over the last years to study different clotting parameters [31–36], showing great variability in the results depending specially on the kits and rats used. PT, APTT and TT values obtained with CG animals plasma in this work were shown in Table 2.

The TT presented a significant increase in HG (Tukey, $\alpha = 0.05$), this was an expectable result as heparin inhibits specifically the thrombin enzyme. This TT is similar to that informed in the work of Prezoto et al. [32] where plasma of heparinized rats (300 UI/Kg) was higher compared to the TT recorded when analyzing plasma from control rats. Plasma from AG also presented an increment in the TT (Tukey, $\alpha = 0.05$), however this increase was not comparable to that detected in the HG. TT of AG showed a 17 % increment compared to animals from CG; higher extension than that informed by You et al. [34], who studied the effect of the batroxobin enzyme, from the venom of *Bothrops atrox moojeni*, blending it directly with the animals plasma, whereas Cho and Choi [33] reported a duplication of the TT in rats that received an intravenous injection of a protease from *Spirodela polyrhiza*. It must be taken into consideration that the bioactive substance studied by these authors was administrated in a more direct way, whereas in the present study amaranth proteins were ingested by the animals.

The APTTs detailed in Table 2 from HG and AG animals presented significant differences between them, and also compared with those obtained when CG plasma was studied (Tukey, $\alpha = 0.05$); the HG values correlate favorably with Prezoto et al. [32]. In our experiments APTT showed a 14 % increment over CG, and even though it was a significant increase, it was slightly inferior compared with the values informed by Cho and Choi [33], where APTT results doubled when plasma from animals injected with the protease *Spirodela polyrhiza* was analyzed.

The PT from AG rats presented in Table 2 showed no significant difference (Tukey, $\alpha = 0.05$) compared with CG, while PT from HG resulted significantly different. These data is consistent with that informed by Prezoto et al. [32].

The clotting test data presented would indicate that peptides from amaranth, released during the protein digestion, alter in some way the common pathway of coagulation (fibrin formation phase), and could also generate changes over the intrinsic pathway. The results would confirm inhibitory clotting effects from amaranth peptides due to an increase in the clot formation time of AG animals compared with CG, however, platelet aggregation effects cannot be discarded according to the recovery area and bleeding time findings. Recent studies in our laboratory confirmed antithrombotic effects of amaranth protein hydrolysates obtained performing a simulated gastrointestinal digestion method (not shown results) and with commercial proteases using the *in vitro* microplates method [8–11].

In conclusion, relying on *in vitro*, *in vivo* and *ex vivo* studies, our work suggests the presence of bioactive peptides encrypted in amaranth proteins. The peptides, released from their proteins during the gastrointestinal digestion, could perform an antiplatelet or anticoagulant effect, confirming the fact that amaranth proteins have a possible application as an antithrombotic sample. It should be highlighted that the protein isolate was administrated by incorporating it in the diet of the animals, implying this that the released active peptides during digestion were absorbed in the intestine and passed through the circulatory system in order to exercise its antithrombotic effect.

Other authors informed that amaranth hydrolysates generated the vasodilatation of isolated aortic smooth muscle [28]. The vasodilatation is a phenomenom that affects collaterally the obstructive circulatory events. When vasoconstriction decreases, blood flows, and platelets as well as serum factors will move more freely than when vessels are constricted. The potential vasodilatory effect of amaranth proteins could have an impact on this work only on the bleeding time, due to the fact that the transection tail method implies the cut of an irrigating arteriole; in the other assays, *ex vivo* and *in vitro*, the smooth muscle activity did not show effect. Although our findings indicated that the bleeding time of AG did not present significant difference compared with CG, a tendency is observed and the vasodilatation effect informed by Fritz et al. [27] cannot be discarded.

Conclusions

Our findings suggest that amaranth proteins contain encrypted peptides that, once released by gastrointestinal digestion, are able to perform an antiplatelet or/and anticoagulant effect. In

Table 2 Clotting tests performed with the plasma of rats that consumed control diet (Group C and Group H) and amaranth diet (Group A)

	Prothrombin time (PT, s)	Thrombin time (TT, s)	Activated partial thromboplastin time (APTT, s)
Group C	20.6 ± 2.3^{a}	96.4 ± 4.3^{a}	43.3 ± 2.9^{a}
Group A	22.6 ± 2.0^{a}	113.1 ± 5.6^{b}	49.4 ± 6.0^{b}
Group H	36.3 ± 11.0^{b}	$> 600^{c}$	89.8 ± 8.0^{c}

Different superscript letter in a same column corresponded at different values (Tukey, $\alpha = 0.05$)

the *ex vivo* assays, the antiplatelet effect was observed when the area of recovery, associated with the platelet plug formation phase, was analyzed, showing significant differences between AG and CG (13,618 ± 1,454 and 6422 ± 1,208 mmHg/s, respectively). The anticoagulant effect was detected in the clotting time values, where AG clotting time resulted greater than CG (612.1 ± 32.4 and 330.9 ± 49.5 s respectively). This parameter, associated with the coagulation cascade, suggests that amaranth proteins have inhibitory effects on the coagulation phase of hemostasis. This result correlates with those obtained with *in vitro* assay results informed by Sabbione et al. [8] and with the clotting tests performed, where significant increases of the clot formation were observed for the TT assay with the plasma of the animals fed with the amaranth diet. Even though the bleeding time in the *in vivo* assay did not exhibit significant differences between AG and CG, results support the idea that amaranth proteins exert antithrombotic activity. In this regard, it seems likely to confirm that amaranth proteins are a potential antithrombotic agent.

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Compliance with Ethical Standards

Conflict of Interest Authors, A.C. Sabbione, G. Rinaldi, M.C. Añón, and A. Scilingo, declare that they have no conflict of interest.

Ethical Approval All experimental procedures were in accordance with the ethical standards approved by CICUAL, Facultad de Ciencias Médicas de la Universidad Nacional de La Plata Ethics Committee. All efforts were made to minimize the number of animals used and their suffering. This article does not contain studies with human participants performed by the authors.

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