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In vitro assessment of the effect of *Undaria pinnatifida* extracts on erythrocytes membrane integrity and blood coagulation parameters of *Equus caballus*

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ABSTRACT

Objective: To study the activity of polysaccharides extracted from *Undaria pinnatifida* (*U. pinnatifida*) *in vitro* on red blood cell of *Equus caballus*, and compare it with heparin.
Methods: Algal extracts was tested at two different concentrations 10 mg/mL and 20 mg/mL. In all studies, using horse red blood cells, control experiments were carried out without extract. We evaluated the toxicity of algal extracts through trypan blue test and haemolysis test and anticoagulant action measured by activated partial thromboplastin time, prothrombin time and fibrinogen test.
Results: The polysaccharide extract of *U. pinnatifida* appeared to have no cytotoxic effect on the horse red blood cells. The values of prothrombin time, activated partial thromboplastin time and fibrinogen were significantly changed in the presence of the extract.
Conclusions: This study suggests a possible exploitation of *U. pinnatifida*, thriving in the lagoon of Venice, as a source of anticoagulant drug, with the aim of transforming waste into a valuable biomass.

1. Introduction

Heparin is the drug of choice in the prevention of thromboembolic disorders, due to its inhibition of thrombin and other enzymes in the coagulation system, but because of its side effects, research of alternative drugs is active. The development of antithrombotic algal polysaccharides would be beneficial because it would avoid the potential for contamination with prions or viruses in commercial heparins, which are obtained from the intestines of pigs and cattle. Then, the extracted algal sulfated polysaccharides could find complementary applications to heparin[1].

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The brown algae are known not only for their nutritional properties, but also as rich sources of polysaccharides with important biological activities, such as alginates and polysaccharides sulfonated (fucoidans). The polysaccharides play an important role in biomedical and pharmaceutical and are used as medicines due to their inherent biocompatibility and their potential cost-effectiveness. Most recognized and studied activities of sulfonated polysaccharides of brown algae are the anticoagulant properties, maybe similar to the action performed by heparin[2].

Over the past 15-20 years, many non-native species were found in the lagoon of Venice[3]. Among these, *Undaria pinnatifida* (*U. pinnatifida*) annually produces a high biomass. Fucans belong to the family of polysaccharides rich in sulphate L-fucose, typical of Phaeophyceae[4]. These contain fucose, sulfate and uronic acid, with a small percentage of galactose, xylose,arabinose and/or mannose and glucose. However, their composition changes depending on the algal species, the harvest season, and

also on the extraction procedures[5,6]. The fucans are present in cell walls in the form of homopolymers, or homofucans, and heteropolymers, or heterofucans. The fucans have activities similar to those of heparin, a drug that acts by accelerating the natural action of the inhibitor of thrombin, antithrombin[7]. Many studies have shown that this property is related to the content and position of the sulfate groups, the molecular weight and composition of the sugar[8]. The haemostasis, in addition to representing the mechanism by which the body regulates blood circulation, consists of a series of complex cascading events (sequential and/or synergistic biochemical reactions) that occur after the injury of a blood vessel, aimed at stopping or restricting the blood spill[9]. Heparin, among the various drugs, is used to threat disorder like laminitis or podoflemmatite in *Equus caballus* for its anticoagulant action. In this regard, many studies have reported that polysaccharides extracted from seaweeds can be a viable alternative in the production of new anticoagulant drugs[10,11]. We aimed to test polysaccharides extracts from *U. pinnatifida* on equine red blood cells (RBCs) to evaluate potential haemolytic effects on cell membranes through the trypan blue test and the haemolysis test[12].

2. Materials and methods

U. pinnatifida samples were collected in the Venice lagoon in May 2011, cleaned of the epiphytic component, air dried for several days and crushed. Crude polysaccharides were extracted in distilled water at 70 °C, after pretreatments in absolute ethanol and acetone (delipidation and depigmentation). The algal extracts were precipitated by ethanol, air dried, and dissolved in a buffered saline solution (125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgSO_4$, 32 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 5 mmol/L D-(+)-glucose, 1 mmol/L $CaCl_2$; pH 7.4) at two different concentrations 10 mg/mL and 20 mg/mL. Blood samples were collected from five healthy horses by jugular venipuncture. Venous blood was drawn into 3.6 mL vacutainer tubes containing 3.8% sodium citrate (Terumo Corporation, Tokyo, Japan). Two blood aliquots were drawn from each horse to assess the algal extracts toxicity and clottingparameters, respectively.

In all studies, using horse RBCs, control experiments were carried out without extract. Statistical analyses were performed using the student's *t*-test.

2.1. Trypan blue test

In order to define cell viability, the horse whole blood samples were incubated for 30 min at room temperature in the saline solution described above containing both concentrations of algal extract (10 mg/mL and 20 mg/mL). Afterwards, 20 μL of trypan blue stock solution sample was added to 20 μL of RBCs, hence they were loaded on a haemocytometer and examined immediately under a microscope at low magnification. The numbers of blue staining and total cells were counted. The percentage cell viability was calculated by the formula:

$$\text{Cell viability (\%)} = \frac{\text{No. of viable cells (unstained cells)}}{\text{Total No. of cells (stained and unstained)}} \times 100$$

2.2. Haemolysis test

Horse whole blood samples collected were incubated in saline solution containing each algal extract under study at 10 mg/mL and 20 mg/mL concentration. After 30 min incubation at 37 °C, the samples were centrifuged (5 min at 1 500 r/min), and the supernatants were obtained. As a measure of haemolysis, hemoglobin (Hb) concentration of the supernatants was determined photometrically at 540 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% haemolysis.

2.3. Anticoagulant action measured by activated partial thromboplastin time (APTT)

APTT carried out with the standard kit (Helena/BioSciences Europe), on equine blood samples were collected from five healthy donators, were drawn into syringes filled with sodium citrate as an anticoagulant. In these assay, platelet-poor plasma sample (0.1 mL) was mixed with the different concentration of algal extracts (0.01 mL) in saline solution and warmed for 60 seconds at 37 °C and then 0.1 mL prewarmed APTT reagent was added and allowed to incubate for 3 min at 37 °C. Prewarmed 0.025 mol/L calcium chloride (0.1 mL) was then added, and the APTT was recorded as the time for clot formation in a coagulant.

2.4. Anticoagulant action measured by prothrombin time (PT)

PT was examined through the standard kit (Helena/BioSciences Europe). Briefly, the reaction mixture containing both different concentration of algal extract (0.01 mL) in saline solution was incubate with 0.1 mL plasma for 3 min at 37 °C, the added 0.2 mL prewarmed PT reagent and recorded the time for clot formation in a coagulant.

2.5. Anticoagulant action measured by fibrinogen test

Quantitative of fibrinogen was determinate through the standard kit (Helena/BioSciences Europe). A total of 0.2 mL plasma in a buffer was mixed with the different concentration of algal extracts (0.01 mL) and warmed for 2 min at 37 °C and then was added 0.1 mL of thrombin reagent and recorded the time for clot formation.

3. Results

Algal extracts (10 mg/mL and 20 mg/mL) did not influence RBC membrane integrity as shown by trypan blue test. The haemolysis test showed no difference in horse RBC samples

added with *U. pinnatifida* compared to control horse RBC samples, therefore no toxic effect of the algal extract was found on equine RBC (Table 1).

Table 1
Percentage of hemolysis in control and after the additional of *U. pinnatifida* polysaccharide extract.

U. pinnatifida extract	300 mOsm	200 mOsm	150 mOsm	100 mOsm	0 mOsm
Control	0%	0%	9%	85%	100%
Extract (10 mg/mL)	0%	0%	10%	84%	100%
Extract (20 mg/mL)	0%	0%	9%	81%	100%

Significant differences were found on studied clotting parameters between experimental and control samples. Plasma added with the algal extract showed an abnormal response when tested for PT, APTT and fibrinogen (Table 2). The values of PT, APTT and fibrinogen were significantly changed in the presence of the extract. Both concentration tested (10 mg/mL and 20 mg/mL) showed values of PT, over 20 seconds; APTT values, exceed 100 seconds, and finally, the values of fibrinogen were lower than the minimum reference value.

Table 2
Anticoagulant activity of *U. pinnatifida* extract by PT and APTT assays and fibrinogen.

U. pinnatifida extract	PT (second)	APTT (second)	Fibrinogeno (mg/dL)
Normal values	9–15	31–61	100–400
Control	10.38	36.16	173.26
Extract (10 mg/mL)	24.04	>100	9.42
Extract (20 mg/mL)	27.40	>100	<8

4. Discussion

The presence of *U. pinnatifida* extract seems to interfere with both the extrinsic and intrinsic pathways of coagulation inhibiting clot formation.

The most recognized and studied the activities of sulfonated polysaccharides of brown algae is the anticoagulant properties, which has an action similar to that carried out by heparin. The development of antithrombotic algal polysaccharides would be advantageous since their use would avoid the potential for contamination with prions or viruses in commercial heparins, which are obtained from pig and bovine intestine. Moreover, with more specific activities and/or targets, the algal sulfated polysaccharides could find applications complementary to heparin[2].

Currently in the lagoon of Venice, *U. pinnatifida* is removed and treated as waste stored in landfills and incinerated. According to a recent law, its use as a fertilizer in agriculture is permitted, but is not actually practiced[3].

In conclusion, this study suggests a possible exploitation of *U. pinnatifida*, thriving in the lagoon of Venice, as a source of anticoagulant drug, with the aim of transforming waste into a valuable biomass.

Conflict of interest statement

We declare that we have no conflict of interest.

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