

Potential Use of Polysaccharides from the Brown Alga *Undaria pinnatifida* as Anticoagulants

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ABSTRACT

Undaria pinnatifida (*U. pinnatifida*) is a highly invasive species and has caused concern all over the world because it has invaded coastal environments, has the potential to displace native species, significantly alters habitat for associated fauna, and disturbs navigation. Any attempt to eradicate it would be futile, owing to the elusive, microscopic gametophyte, and because the alga thrives in sites rich in anthropic activities. Venice Lagoon is the largest Mediterranean transitional environment and the spot of the highest introduction of non-indigenous species, including *U. pinnatifida*, which is removed as a waste. We demonstrated that polysaccharide extracts from *U. pinnatifida* have an anticoagulant effect on human blood *in vitro* and are not cytotoxic. The results obtained by PT (normal values 70-120%) and APTT (normal values 28-40s) assays were significantly prolonged by the polysaccharide extracts of *U. pinnatifida*, therefore algal extracts are ideal candidates as antithrombotic agents.

Key words: Algal extract, anticoagulant activity, cytotoxicity, fucoidans, human blood cells, *Undaria pinnatifida*

INTRODUCTION

Venice Lagoon is located along the northwest coast of the Adriatic Sea and is the largest Mediterranean transitional environment (about 550 km²) with its unique ecology. Because of the intense human activities it is also the spot of the highest introduction of non-indigenous species. Among these, the brown alga *Undaria pinnatifida* (Harvey) Suringar (Phaeophyceae) was detected for the first time in the 1990s (Rismondo et al. 1993) and have gradually colonized most of the hard substrates, becoming the most important species for cover and biomass on the banks of canals (Curiel and Marzocchi 2010).

U. pinnatifida is a highly invasive species, classified as one of the top 100 global invasive species, and has caused concern all over the world

as it has invaded the coastal environment in parts of Europe, Australia, New Zealand, Argentina and the USA (Hunt et al. 2009). Although published evidences of ecological impacts are equivocal (Forrest and Taylor 2002), recent studies suggest that *Undaria* has the potential to displace native seaweed species and significantly alters habitat for associated fauna including commercial species like abalone (*Haliotis iris*) and sea urchins (*Evechinus chloroticus*) (Curiel et al. 2002, Silva et al. 2002, Valintine and Johnson 2003, Casas et al. 2004).

There are concerns that *Undaria* could have also economic impacts on the aquaculture industry, because of the potential fouling risks to mussel and fish farms resulting in increased harvesting costs and farm maintenance (Hunt et al. 2009).

In European countries (France, England) and North America (Washington State, California), the

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problem of invasive algae threatening biodiversity has been addressed with a multidisciplinary approach based on a series of operations for the eradication, including mechanical (with tractors or pontoons) or manual, chemical (herbicides) or biological (herbivores) treatments, without success (Gray and Jones 1977, Lewey and Jones 1977, Loraine 1989, Belsher 1991, Davison 1996).

The eradication in Venice Lagoon does not appear easy to implement mainly because the colonization is established over several years and also due to the complexity of the sites, which are strongly related to anthropic activities and economic structures or public utilities (docks, piers, pipes and cables buried under piles of wood or concrete) (Curiel and Marzocchi 2010).

Moreover, there are observations on Australian established populations that any attempt to eradicate it would be futile (Brown and Lamare 1994), owing to the elusive, microscopic gametophyte stage of the alga (Sanderson 1990).

However, in original Indo-Pacific regions, *Undaria* plays an important role in the local economy and its biomass is particularly exploited on a global industry.

It is a popular food source in Japan, Korea and China where the market is worth about \$ 400 million (Barratt-Boyes 2012). Scientists are researching the commercial applications of *Undaria* as nutraceutical, drug, fertilizer or fish food, and are also exploring new market opportunities.

Undaria pinnatifida is one of the most utilized brown alga in macrobiotic diet (Kolb et al. 2004) and for the extraction of sulphated polysaccharides, which represent a group of hydrocolloids from marine macroalgae (Camara et al. 2011, Jiao et al. 2011). They are structural components of the cell walls, partly responsible for the flexibility of the thalli (Laurienzo 2010). The most typical in brown algae are alginate, a polysaccharide of guluronic and mannuronic acids and fucans, a polysaccharide family rich in sulphated L-fucose. They differ in structure among algal species and even within the same species (Jiao et al. 2011) and may be present in the form of homopolymers or heteropolymers (Li et al. 2008).

Recently, there has been increasing interest in the screening of bioactive compounds from natural sources, such as marine organisms (Vishchuk et al. 2011, Genovese et al. 2013). The literature reports a large diversity of biomedical activities associated

with sulphated polysaccharides (Lahaye and Robic 2007, Assreuy et al. 2008, Pomin and Mourao 2008, Costa et al. 2010, Jiao et al. 2011, Wijesekara et al. 2011), such as antiviral (Ponce et al. 2003, Lee et al. 2004, Chattopadhyay et al. 2007), antioxidant (Rupérez et al. 2002), anticancer, anti-inflammation (Noda et al. 1990, Berteau and Mulloy 2003, Na et al. 2010, Magalhaes et al. 2011), and anticoagulant (Li et al. 2008, Faggio et al. 2014). After the investigation of blood anticoagulant properties from marine brown algae (Killing 1913), it has been reported that sulphated polysaccharides derived from seaweeds are alternative sources for the manufacture of novel anticoagulants (Church et al. 1989, Carlucci et al. 1997, Mao et al. 2006, Barroso et al. 2008, Nishino et al. 2000, Matsubara 2004, Mao et al. 2009, Costa et al. 2010, Camara et al. 2011, Mestechkina and Shcherbukhin 2010, Magalhaes et al. 2011).

The coagulation of blood is the third phase of haemostasis and is a process that intervenes to stop the bleeding in the event of major damage of the vessel wall. The primary pathway involves activation of platelets, the recruitment of von Willebrand factor (VWF) to promote platelet attachment to the site of injury, and the engagement of secondary haemostasis through various procoagulant proteins including fibrinogen, and factors (F) V and VIII (Lippi et al. 2009). Secondary haemostasis is also initiated directly by damage to the vasculature, primarily via the tissue factor pathway involving FVII, but also via the contact pathway, and as amplified by the primary pathway. *In vitro*, the tissue factor pathway and the contact pathway are respectively represented by the prothrombin time (PT) and activated partial thromboplastin time (APTT).

Various natural anticoagulants act moderating the secondary haemostasis pathway and preventing excessive procoagulant activity that may lead to a thrombosis or vascular occlusion. Therefore, primary haemostasis dysfunction leading to arterial thrombosis is usually managed using antiplatelet therapy (e.g., aspirin, glycoprotein IIb/IIIa inhibitors and ADP receptor/ P2Y12 inhibitors), whereas secondary haemostasis dysfunction leading to venous thrombosis is typically managed by anticoagulant therapy, classically unfractionated heparin (UH) or low molecular weight heparin (LMWH) (Casella et al. 2011, Favaloro et al. 2011, Casella et al. 2013). They are the drugs of choice in current clinical use

when rapid effect is desired such as in intensive care setting, during surgery and for patients with renal failure. The current sources of heparin are animals, as it is obtained only from pig intestine or bovine lung. It is the second most frequently used natural drug but it has some disadvantages and it exhibits haemorrhagic-like side effects. Consequently, new research trends for the discovery of novel anticoagulant agents or of alternative sources of heparin are now an urgent need (Mourão and Pereira 1999, Shanmugam and Mody 2000).

Blood anticoagulant and antithrombotic properties have been reported for about 150 species of marine algae (Shanmugam and Mody 2000), mostly belonging to Phaeophyceae (McLellan and Jurd 1992, Irhimeh et al. 2009) in some cases superior to that of heparin (Springer et al. 1957, Kusaykin et al. 2008, Li et al. 2008, Pomin and Mourao 2008, Irhimeh et al. 2009). Unlike heparin, which produces a rapid but transient antithrombotic effect, the *in vivo* action of this sulphated galactofucan progressed slowly, showing maximal effectiveness about eight hours post injection.

The purpose of the present study was to suggest a possible use of *U. pinnatifida*, collected in Venice Lagoon (Italy), an invasive species of Indo-Pacific origin, as a potential source of bioactive molecules. In this topic we evaluated *in vitro* the anticoagulant activity of sulphated polysaccharides extracted from *U. pinnatifida* on human blood by means of the PT and APTT assays.

MATERIAL AND METHODS

Polysaccharides extraction

Undaria pinnatifida was collected in Venice Lagoon (Italy). Thalli were manually cleaned from debris and epiphytes using tap water, sun dried and milled to powder.

Powdered algae (5 g) were stirred with absolute ethanol for 1 h at 70°C. The mixture was centrifuged at 4000×g for 10 min at room temperature. The pellet was treated with acetone for 1 h at room temperature, and the mixture was centrifuged at 4000×g for 10 min at room temperature. Then, the pellet was dissolved in distilled water and allowed to stand for 24 h at 70°C. After centrifugation (4000×g for 10 minutes), polysaccharides were precipitated by adding 96% ethanol to the supernatant. The

precipitate was collected and dried at room temperature. Algal extracts were dissolved in physiological solution (0.9% NaCl) at 10 µg/mL and 20 µg/mL.

Haematological parameters

Normal pooled blood from 15 individual healthy donors, without history of bleeding or thrombosis was collected. Blood samples were taken for haematological analysis. Plasma samples were obtained by centrifugation (1500×g for 15 min at room temperature). Haematological parameters included haemoglobin concentration, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), white blood cell count (WBC), red blood cell count (RBC), and were measured at the Blood Chemistry Laboratory Lo Piano in Villafranca Tirrena (Sicily, Italy) (Table 1).

Trypan blue test

In order to define cell viability, the whole blood samples were incubated for 30 min at room temperature in the saline solution described above containing both concentrations of algal extract (10 µg/mL and 20 µg/mL). Afterwards, 20 µL of Trypan blue stock solution were 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:

$$\text{Cell viability (\%)} = \frac{\text{Number of viable cells (unstained cells)} \times 100}{\text{Total number of cells (stained and unstained)}}$$

Anticoagulant activities

The anticoagulant activities of all samples were investigated by the classical coagulation assays APTT and PT, using heparin as a reference.

Anticoagulant action measured by activated partial thromboplastin time (APTT). Activated partial thromboplastin time carried out with the standard kit (SGM Italy), on human blood samples were collected from fifteen healthy donators, were drawn into syringes filled with sodium citrate as an anticoagulant. In this assay, platelet-poor plasma sample (0.1 mL) were mixed with the different concentration of algal extracts (0.01 mL) in saline solution and warmed for 60s at 37°C and then 0.1 mL pre-warmed APTT reagent was added and allowed to incubate for 3 min at 37°C. Pre-

warmed 0.025M calcium chloride (0.1 mL) was then added, and the APTT was recorded as the time for clot formation in a coagulant. All assays were performed in triplicate.

Anticoagulant action measured by prothrombin time (PT). Prothrombin time was examined through the standard kit for prothrombin time determination (SGM Italy). Briefly, the reaction mixture containing both different concentration of algal extract (0.01 mL) in saline solution was incubated with 0.1 mL of plasma for 3 min at 37°C, then 0.2 mL of pre-warmed PT reagent was added and the time for clot formation in a coagulant was recorded. All assays were performed in triplicate.

The prothrombin time (PT) and activated partial thromboplastin time (APTT) coagulation assays were performed with a semiautomatic coagulometer (SEAC Clot 2) and measured using 3.2% citrate treated human plasma.

Statistical analysis

Statistical analyses were performed using Student's t-test. All calculations were carried out using Prism version 4.00 statistical software (GraphPad Software Inc., USA).

RESULTS AND DISCUSSION

No cytotoxic effect was observed on human red blood cells. Fucoidan administration caused no significant difference in haematological parameters (Table 1).

Table 1 - Complete blood count (CBC).

Blood parameters	Men n=7	Women n=8
Erythrocytes (RBC) ($\times 10^6/\mu\text{l}$ blood)	5.11 \pm 10.0	4.51 \pm 10.0
Haemoglobin (Hb) (g/100ml blood)	16 \pm 0.01	14 \pm 0.01
Haematocrit (Hct) (%)	46.0 \pm 1.0	40.9 \pm 1.0
Mean cell volume (MCV) (μm^3 / erythrocytes)	90.1 \pm 1.0	90.4 \pm 2.0
Mean cell haemoglobin (MCH) (pg/ erythrocytes)	30.2 \pm 0.01	30.2 \pm 0.01
Mean corpuscular haemoglobin concentration (MCHC) (g/100ml blood)	34 \pm 0.01	34 \pm 0.01
Thrombocyte (Plt) ($\times 10^3/\mu\text{l}$ blood)	295 \pm 10.9	295 \pm 10.8
White Blood Cell Count (WBC) ($\times 10^3/\mu\text{l}$ blood)	7.25 \pm 7.0	7.28 \pm 7.8
Lymphocytes (%)	32.5 \pm 1.5	32.5 \pm 1.0
Neutrophil granulocytes (%)	60 \pm 1.0	60 \pm 1.2
Eosinophil granulocytes (%)	5 \pm 1.1	5 \pm 1.5
Basophil granulocytes (%)	0.5 \pm 1.0	0.5 \pm 1.0
Monocytes (%)	2 \pm 1.0	2 \pm 1.0

The results were showed in mean \pm standard deviation.

PT and APTT were significantly prolonged by the polysaccharide extracts of *U. pinnatifida*. Both tested concentrations (10 $\mu\text{g}/\text{mL}$ and 20 $\mu\text{g}/\text{mL}$) showed PT = 1% and APTT > 100(s).

The anticoagulant properties of the polysaccharide extracts are summarized in Table 2.

Table 2 - Anticoagulant activity of *Undaria pinnatifida* extract by PT and APTT assays.

	PT (s)	APTT (s)
Normal values	9-15	31-61
Heparin	100	36.16
<i>Undaria pinnatifida</i> extract (10 mg/ml)	1	>100
<i>Undaria pinnatifida</i> extract (20 mg/ml)	1	>100

Brown seaweeds are known to be not only important resources of food, feed and energy, but as also rich sources of polysaccharides with valuable biological activities, including alginate and sulphated polysaccharides (fucoidans) (Vishchuk et al. 2011). The composition of algal polysaccharides varies according to species, extraction procedure, season of harvest and local climatic conditions. Natural polysaccharides play a relevant role in biomedical and pharmaceutical applications, particularly in the field of drug delivery, for their intrinsic biocompatibility and potential low cost (Laurienzo 2010).

Probably, the most widely recognized and investigated bioactivity of marine sulphated polysaccharides is the heparin-like anticoagulant activity exhibited by fucans of brown seaweeds.

Heparin is the drug of the choice in the prevention of thromboembolic disorders, but recently alternative drugs for heparin are in high demand due to its bad and long-term side effects.

To overcome the obvious potential side-effect of bleeding, researchers have investigated means of reducing the anticoagulant activities of heparin while enhancing its anti-thrombotic activities including chemical modification and fractionation of native heparin to lower molecular forms (Jiao et al. 2011).

As an alternative source of anticoagulants, seaweed polysaccharides gained much attention in the pharmaceutical industry to develop better and safe drugs with low or less side effects (Athukorala et al. 2007, Laurienzo 2010, Mestechkina and Shcherbukhin 2010, Fitton 2011, Mayer et al. 2011, Wijesekara et al. 2011). The

development of antithrombotic algal extracts would be advantageous also because their use would avoid the potential for contamination with prions or viruses in commercial heparins, which are obtained from animals. Moreover, with more specific activities and/or targets, the algal extracts having sulphated polysaccharides could find applications complementary to heparin.

In this study, we demonstrated that polysaccharide extracts of *U. pinnatifida* collected in Venice Lagoon have an anticoagulant effect prolonging coagulation time with respect to heparin. The results showed that prothrombin time (PT) and activated partial thromboplastin time (APTT) were effectively prolonged by the algal extracts. The prolongation of PT suggests that the extrinsic pathway of coagulation was inhibited, whereas the prolongation of APTT indicates the inhibition of the intrinsic and/or common pathway.

CONCLUSIONS

Over the last 15-20 years, numerous non-indigenous species have been found in Venice Lagoon (Curiel et al. 2006). Among these, *U. pinnatifida* produces a high biomass annually. Density, biomass and size of thalli of *U. pinnatifida* vary in different areas of Venice Lagoon depending on the chemical-physical and trophic characteristics of the water column. On average, density can be up to 400-450 thalli/m² with biomass values of 5-10 kg fresh weight/m² (Curiel et al. 2002, Curiel et al. 2004), and fronds of about 1-2 m. An eradication intervention would not be applicable because the settlement is established over several years and also because the alga thrives in sites rich in anthropic activities (Curiel and Marzocchi 2010).

In New Zealand, conversely, the Ministry of Agriculture and Fisheries revised its policy to allow for greater commercial use of *Undaria* (Barratt-Boyes 2012). The general scope of the new regime includes: allowing *Undaria* to be farmed in certain heavily infested areas, and allowing it to be harvested when it is growing on artificial surfaces, when it has been cast ashore onto the beach or when part of a programme specifically designed to control *Undaria*.

At present, in Venice Lagoon *U. pinnatifida* is removed and processed as a waste, stocked in

dumps and incinerated. According to a recent law (decree-law 217/2009), its use as a fertilizer in agriculture was allowed, but it is actually not practiced.

In conclusion, we propose a possible exploitation of *U. pinnatifida* thriving in Venice Lagoon as a source of anticoagulant drug with the aim of converting a waste into a valuable biomass.

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