SUPPLEMENTARY MATERIAL

Evaluation of anticoagulant activity of two algal polysaccharides

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Marine algae are important sources of phycocolloids like agar, carrageenans and alginates used in industrial applications. Algal polysaccharides have emerged as an important class of bioactive products showing interesting properties. The aim of our study was to evaluate the potential uses as anticoagulant drugs of algal sulphate polysaccharides extracted from *Ulva fasciata* (Chlorophyta) and *Agardhiella subulata* (Rhodophyta) collected in Ganzirri Lake (Cape Peloro Lagoon, north eastern Sicily, Italy). Toxicity of algal extracts through Trypan blue test and anticoagulant action measured by Activated partial thromboplastin time (APTT), Prothrombin time (PT) test has been evaluated. Algal extracts showed to prolong the PT and APTT during the coagulation cascade and to avoid the blood coagulation of samples. Furthermore, the algal extracts lack toxic effects toward cellular metabolism and their productions are relatively at low cost. This permits to consider the algae as the biological source of the future.

Keywords: Algal extract; anticoagulant activity; cytotoxicity; fucoidans; human red blood cells.

Algal sources.

Ulva fasciata (POL044.1) and *Agardhiella subulata* (POL037.2) samples were collected in Ganzirri Lake (north-eastern Sicily, Italy; 38°15'0"N; 15°36'0"E) in June 2011.

Polysaccharides extraction.

The first step of preparation of extracts from collected algal samples was to wash the biomass in sea water to remove epiphytes, particles of sand and impurities and then to rinse the samples in fresh water to remove the salt precipitations. After air shade drying of the samples, polysaccharides were obtained by soaking in hot water (70°C) over night. Then, residual thalli have been removed and three volume of absolute ethanol were added to the supernatant to obtain the precipitation of crude polysaccharides. Pellet was finally dried at 40°C and stored at room temperature.

Haematological parameters.

The algal extracts have been dissolved in a saline buffer at pH 7.4. and tested at two different concentrations: 10 mg/ml and 20 mg/ml. In all tests control experiments have been carried out without algal extract.

Normal pooled blood from 15 individual healthy donors, without history of bleeding or thrombosis, has been collected into syringes filled with sodium citrate as an anticoagulant. Blood samples have been taken for haematological analysis. Plasma samples have been obtained by centrifugation (1500xg for 15 minutes at room temperature). Haematological parameters measured included haemoglobin concentration, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell distribution with (RDW), white blood cell count (WBC), red blood cell count (RBC) (Tab. 2).

Table 2. Complete Blood Count.

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±1.0	2±1.0
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Trypan blue test.

In order to define cell viability, the whole blood samples were incubated for 30 minutes at room temperature in saline buffer containing both concentrations of algal extract (10 mg/ml and 20 mg/ml). Afterwards, Trypan blue stock solution (0.4%) were added (v:v), hence samples 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 (1).

$$\text{Cell viability (\%)} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100 \quad (1)$$

Anticoagulant activities.

The anticoagulant activities of all samples were investigated by the classical coagulation assays Activated Partial Thromboplastin Time (APTT) and Prothrombin Time (PT), using heparin as a reference. Activated partial thromboplastin time assay has been carried out by the standard kit (Giese Diagnostics- Rome, Italy) on human blood samples. In this assay, 3.2% citrate treated platelet-poor plasma samples (0.1 ml) were mixed with the different concentrations of algal extracts (0.01 ml) in saline solution and incubated for 60 s at 37°C. APTT reagent (0.1 ml) was added and samples incubated for 3 minutes at 37°C. 0.025M calcium chloride (0.1 ml) was added, and the APTT has been recorded as the time for clot formation in a coagulant. All assays were performed in triplicate.

Prothrombin time was examined by the standard kit for prothrombin time determination (Giese Diagnostics- Rome, Italy). Reaction mixtures containing algal extracts (0.01 ml) at different concentrations in saline solution were incubated with plasma (0.1 ml) for 3 min at 37°C. 0.2 ml PT reagent was added and the time for clot formation in a coagulant was recorded. All assays were performed in triplicate. PT and APTT coagulation assays were performed with a semiautomatic coagulometer (C.G.A. Strumenti scientifici S.P.A.).

Statistical analysis.

Statistical analyses were performed using Student's t-test ($p < 0.05$). All calculations were carried out using Prism version 5.00 statistical software (GraphPad Software Inc., USA).