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SEARCHING FOR BIOLOGICAL ACTIVITIES IN A NORTHERN ADRIATIC RED ALGA *POLYSIPHONIA* SP.

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ABSTRACT

Red alga Polysiphonia *sp. was tested for presence of hemolysins, antibiotics, acetylcholinesterase inhibitors and hemagglutinins. Although we found no activity in this organism, the screening procedure developed in our laboratory proved suitable for rapid detection of other potentially interesting bioactive molecules from different source material.*

Key words: natural marine products, red algae, *Polysiphonia*, antibacterial activity, hemagglutination, hemolysis, acetylcholinesterase

RICERCA DELLE ATTIVITÀ BIOLOGICHE NELL'ALGA ROSSA *POLYSIPHONIA* SP. DEL NORD ADRIATICO

SINTESI

L'alga rossa Polysiphonia *sp. è stata analizzata per la presenza di emolisine, antibiotici, inibitori dell'acetilcolinesterasi ed emagglutinine. Benché gli autori non abbiano trovato alcuna attività in tale organismo, le procedure di screening sviluppate nei loro laboratori si sono dimostrate appropriate per una identificazione rapida di altre molecole bioattive potenzialmente interessanti, provenienti da fonti diverse di materiale.*

Parole chiave: prodotti marini naturali, alghe rosse, *Polysiphonia*, attività antibatterica, emagglutinazione, emolisi, acetilcolinesterasi

INTRODUCTION

About 30% of the current worldwide human therapeutics derive from natural sources (Grabley & Thiericke, 1999). Recent trends in drug discovery emphasize investigation of the marine environment, which has already given some commercially known pharmaceuticals like Aracytine and Vidarabine from the sponge *Cryptotethya crypta*, or Ziconotide from the mollusc *Conus magus*. Marine organisms endow different defense strategies to survive in the highly competitive marine environment, thus resulting in a tremendous diversity of highly active compounds affecting numerous targets involved in eukaryotic cell signaling processes. However, toxic principles often dominate the spectrum of biologically active metabolites, hence in the last 20 years none of the isolated compounds have reached the pharmaceutical marketplace (Faulkner, 2000).

The aim of this study is to present a methodology that has been developed in our laboratory for fast screening of biologically active molecules in marine organisms. It is composed of a hemolytic, an antibacterial, a hemagglutination and an anticholinesterase test. A marine organism containing potential biologically active compounds usually has a clean surface, not fouled by other micro- or/and macroorganisms. Another indication of bioactivity may be the expansive growth of one species over the others during the territorial competition.

In 1996, a large quantity of algal complex with predominating red algae *Polysiphonia* spp. was noted in the sublittoral area of Cape Oštro (Northern Adriatic, Croatia; Fig. 1). Monitoring of its growth for a period of 1 year revealed that in the summertime, when the water temperature averaged about 23°C, the algal complex covered 80% of the area being observed (Arko-Pijevac, 2000), and dominated over different sessile marine organisms. A virtually black color of the sandy sediment surface at a depth of 12.5 to 15 m suggested the presence of anoxic conditions below the algal layer.

Red algae have been already reported to possess compounds exerting hemagglutinating (Hori *et al.*, 1987; Okamoto *et al.*, 1990), antibacterial (Mahasneh *et al.*, 1995; Etahiri *et al.*, 2001; Gao *et al.*, 2001), neurotoxic (Freitas *et al.*, 1995), mitogenic (Hori *et al.*, 1987), hemolytic (Freitas *et al.*, 1995; Igarashi *et al.*, 1998), antimutagenic (Okai *et al.*, 1996), ichthyotoxic (Igarashi *et al.*, 1998), antifungal and molluscicidal (Gao *et al.*, 2001), antimalarial (Etahiri *et al.*, 2001), antiviral (Carlucci *et al.*, 1997; Duarte *et al.*, 2001), anticoagulant (Carlucci *et al.*, 1997), cytotoxic and antialgal (König *et al.*, 1999), and fatty acid-oxidizing activity (Kajiwara *et al.*, 2000).

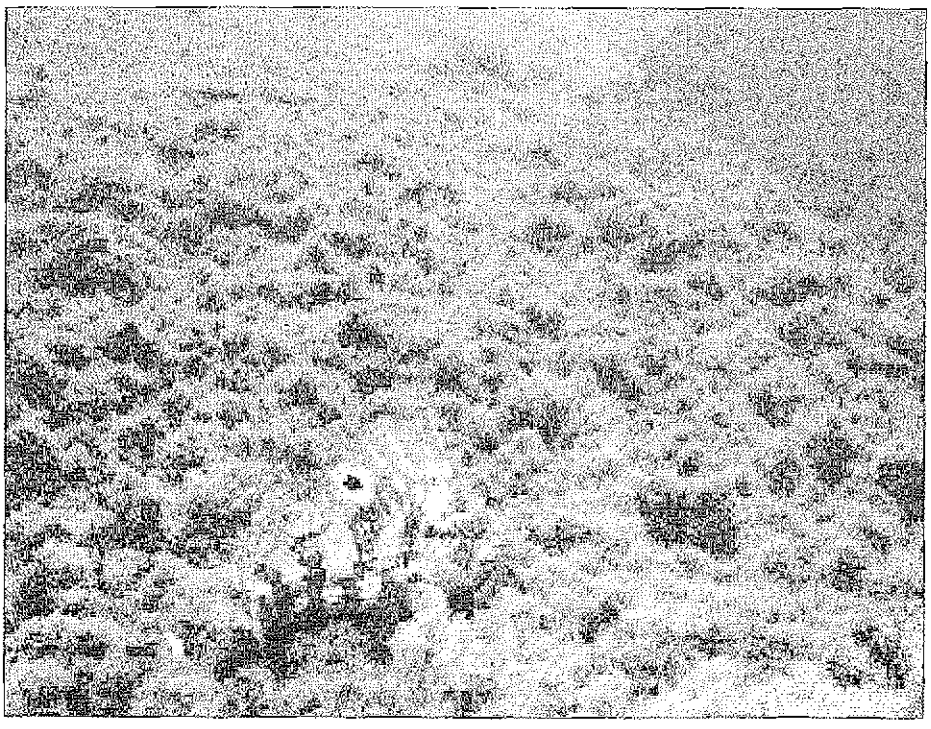

Fig. 1: A large quantity of algal complex with predominating red algae** Polysiphonia **spp. in the sublittoral area of Cape Oštro, covering 80% of the area. (Photo: M. Kovačić)
Sl. 1: Gosta prevleka alg s prevladujočimi vrstami iz rodu** Polysiphonia **v infralitoralu rta Oštro je prekrivala 80% obravnavanega območja. (Foto: M. Kovačić)

Concerning *Polysiphonia*, it has been shown that the extracts from some species of this genus are antiviral (Serkedjieva, 2000; Serkedjieva *et al.*, 2000), antibacterial (De Rosa *et al.*, 2001; Hellio *et al.*, 2001), antifungal and toxic to *Artemia salina* (De Rosa *et al.*, 2001). To examine if Northern Adriatic species of *Polysiphonia* contains certain biologically active compounds, leading to the observed overgrowth, we have tested its extracts using a developed methodology.

MATERIAL AND METHODS

Algal material

The *Polysiphonia* samples were collected at Cape Oštro (Northern Adriatic, Croatia) in 1998. They were kept at a temperature of -20°C until the start of the experiments.

Extraction procedure

Frozen algal tissue was lyophilized and powdered. Equal amounts (10 g each) of the obtained powder were suspended in eight different solvents (150 ml each) that would extract different substances contained in the plant according to the level of their polarity. The used solvents were water, methanol, absolute ethanol, acetone, 1-butanol, n-hexane, chloroform and aether-petrolei. Samples were shaken for 24 hours on the orbital shaker at 100 rpm and then filtered through a paper filter. The obtained filtrates were concentrated under reduced pressure. The dry weight of the concentrated extracts was determined by evaporating the solvent from 1 ml of the filtrate at 80°C.

Biological activity tests

For monitoring the possible presence of biologically active molecules in *Polysiphonia*, we tested different algal extracts for antibacterial, hemolytic, hemagglutinating and antiacetylcholinesterase activities. Pure solvents were used as controls for all tests.

Evaluation of antibacterial activity

For detecting possible antibacterial activities, a standard agar diffusion test was applied. Two Gram negative *(Shigella sonnei, Escherichia coli)* and two Gram positive *(Micrococcus luteus, Staphylococcus aureus)* bacterial strains were used. Bacteria were obtained from the bacterial collection at the Department of Biology, University of Ljubljana. They were allowed to grow overnight in Lauria Broth Base (Sigma, U.S.A.) and their concentrations were then determined. Bacterial cultures were incorporated into the agar-supplemented Lauria Broth that was cooled to 42°C beforehand. The final concentration of bacteria was approximately $5x10^5$ of colony-forming units per milliliter (CFU/ml). Twenty ml of inoculated medium were poured into Petri dishes and kept at a temperature of 4°C, then circles (Ø =1 cm) of agar were cut from the medium. Hundred µl of each extract or pure solvent were put into a circle of the Petri dish with different types of bacteria. The system was then kept overnight at 37°. Antibacterial activity was evaluated by measuring the diameter of inhibition zones.

Evaluation of hemolytic activity

Hemolysis was measured with a turbidimetric method according to Maček & Lebez (1981). Typically, 10-50 µl of different *Polysiphonia* extracts or pure solvents were added to a cuvette containing 3 ml of bovine erythrocyte suspension in 0.13 M NaCl, 0.02 M TRIS/HCl, pH 7.4, having an apparent absorbance of 0.5 at 700 nm. The decrease of apparent absorbance, deriving from hemolysis, was recorded using a UV/VIS spectrophotometer (Shimadzu 2100, Japan). The suspension in the cuvette was magnetically stirred. The experiment was performed at 25°C.

Evaluation of hemagglutination

Bovine erythrocytes were washed three times with 0.9% saline and twice with 0.14 M NaCl, 0.013 M TRIS/HCl, pH 7.4. Two-percent suspensions of washed erythrocytes were prepared in the same buffer, and pipetted onto microtiter plates (Nunc, Denmark). To the 100 µl of erythrocyte suspension in each well, 50-10 µl of different extracts or pure solvents were added. Following a 1-hr incubation at room temperature, hemagglutination was scored (Sepčić *et al.*, 1997).

Determination of acetylcholinesterase inhibition

The inhibition of acetylcholinesterase (AChE) was measured colorimetrically as described by Ellman *et al.* (1961). Briefly, to 3 ml of 0.1 M phosphate buffer, pH 8.0, 20 µl of 0.075 M acetylthiocholine iodide was added, followed by 100 µl of 0.1 M 5'-5'-dithiobisnitrobenzoic acid, and usually 10-100 µl of the sample to be tested (different extracts or pure solvents). After the absorption in reference and sample cuvettes had been auto-zeroed, the reaction was started by the addition of 20 µl AChE from electric eel (5 U/ml, Sigma, U.S.A.). The reaction was monitored for 10 minutes.

RESULTS AND DISCUSSION

Antibacterial test

Eight different extracts of red alga *Polysiphonia* sp.

were tested against 4 strains of bacteria and no extract showed antibacterial activity. All bacterial strains showed some growth inhibition by algal chloroform extract, which was proved as a solvent effect. The absence of antibacterial activity was somehow surprising, as different *Polysiphonia* species had already been reported to inhibit the bacterial growth. Organic extracts of two *Polysiphonia* species, a widely spread *P. denudata* and an endemic Black Sea species *P. denudata* f. *fragilis* showed considerable antibacterial activity against *Staphylococcus aureus* (De Rosa *et al.*, 2000). Hellio *et al.* (2001) have recently demonstrated that the antibacterial activity of marine algae is very selective. They tested 90 algal extracts against 35 strains of marine bacteria and found that only 18 extracts showed some activity. Among them, there was also an organic extract from *Polysiphonia lanosa*, exhibiting specific inhibitory activity against Gram positive marine bacteria.

Hemagglutination test

Compounds exerting hemagglutinating activities are usually glycoproteins and are therefore expected in water extracts. Although there are some reports on hemagglutination activities from marine red algae *Carpopeltis flabellata* (Hori *et al.*, 1987) and *Gracillaria bursa-pastoris* (Okamoto *et al.*, 1990), we did not detect any in our *Polysiphonia* extracts.

Hemolytic activity

The testing of *Polysiphonia* extracts did not reveal any presence of hemolytic substances. This is not surprising in view of very few reports of hemolytic activity of red algae (Freitas *et al.*, 1995; Igarashi *et al.*, 1998).

Acetylcholinesterase inhibition

In this study, we also report on the testing for acetylcholinesterase inhibition. If tested positive, it would indicate the presence of a neurotoxin. Although the test was negative, this is, as far as we know, the first attempt to detect AChE inhibition in red algae. A sole other example of a neurotoxic activity in red algae was found in *Liagora farinosa*, whose polar extract induced a dose-dependent inhibition of action potentials in the isolated crustacean nerve (Freitas *et al.*, 1995).

CONCLUSIONS

The results of this study suggest that the extracts from the *Polysiphonia* material collected in the Northern Adriatic do not possess biological activities that could be detected with the used tests. It is therefore possible that the observed overgrowth of this alga is due to some undetected activities, or rather to different environmental effects (light, turbidity, nutrients, temperature). It is also possible that the anoxic conditions below the algal layer had caused the mortality of other organisms living there, or had simply prevented them to settle there. Nevertheless, the presented screening procedure, consisting of four rather economical biological tests, can be further used for rapid (3-4 days) detection of other potentially interesting bioactive molecules deriving from different source material.

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RAZISKAVA BIOLOŠKIH AKTIVNOSTI V SEVERNOJADRANSKI RDEČI ALGI *POLYSIPHONIA* SP.

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POVZETEK

Morski organizmi so bogat vir strukturno še nepoznanih in biološko aktivnih molekul. V tej raziskavi so predstavljene nekatere metode za detekcijo takšnih spojin. Kot testni organizem je bila izbrana rdeča alga Polysiphonia *sp. Leta 1996 se je ta alga zelo razrasla v severnem Jadranu, kar bi lahko bil kazalec možnih prisotnih bioaktivnih spojin. Hemolitična, protibakterijska, hemaglutinacijska in protiholinesterazna aktivnost je bila testirana na različnih ekstraktih alge. Kljub temu da nobena od teh aktivnosti ni bila zaznana v preučevani algi, so se uporabljene metode pokazale kot uporabne za hitro določitev potencialno zanimivih bioloških molekul iz različnih virov. Še več, protiholinesterazni test je bil v tej raziskavi prvič uporabljen na rdečih algah.*

Ključne besede: naravni produkti iz morskih organizmov, rdeče alge, *Polysiphonia*, protibakterijska aktivnost, hemaglutinacija, hemoliza, acetilholinesteraza

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