# Antiinfluenza Virus Effect of Extracts from Marine Algae and Invertebrates

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Sixty products, derived from marine organisms, typical of the Bulgarian Black Sea coast, were examined for inhibitory activity on the reproduction of influenza viruses in tissue cultures. The antiviral effect was investigated by the reduction of virus infectivity. Using representative strains of influenza virus it was shown that apparently the inhibitory effect was strain-specific. The most effective products were further studied in fertile hen's eggs and in experimental influenza infection in white mice.

## Introduction

Marine organisms represent a promising source of antiviral compounds (Cannell, 1993; Che, 1991). The inhibitory effect on a range of viruses has been studied (Ballesteros *et al.*, 1992; Ehresmann *et al.*, 1977; Hayashi *et al.*, 1993; Premanathan *et al.*, 1994; Witvrouw and De Clercq, 1997). A limited number of studies have been performed with influenza viruses (Gerber *et al.*, 1958; Ivanova *et al.*, 1991, 1994; Kathan, 1965).

The objective of our study was to investigate a number of marine plants and organisms, typical of the Bulgarian Black Sea coast and to evaluate their potential as a source of biologically active substances with antiviral properties. Sixty products were tested for antiinfluenza virus activity in tissue cultures. The most effective products were further studied in fertile hen's eggs and in experimental influenza infection in mice.

## Materials and Methods

### *Substances*

The investigated marine algae and invertebrates were collected in the Black Sea in summer. The fresh organisms were cleaned from other organisms, immediately dipped in ethanol and transported to the laboratory. About 100–120 g of the fresh material, corresponding to 10–15 g of dry material, was homogenized with chloroform – methanol (500 ml, 1:1, v/v) and refluxed for a few minutes in order to inactivate the enzymes. The extractions were repeated three times, the combined extracts were concentrated to about 200 ml and 200 ml water was added. Two layers were formed – the upper one – water (W) contains polar compounds, while the lower layer – chloroform, contains lipophilic compounds (L). The lipophilic layers contain about 1 g material, while the water layers contain about 2–3 g material. In some cases total MeOH extracts (about 3 g), *n*-BuOH or $CH_3Cl$ extracts (about 1 g) have been prepared by extraction of 100 g fresh material with $3 \times 400$ ml of the solvent *n*-butanol (see Table I). The most effective extracts (*Ceramium rubrum, Phyllophora nervosa, Polysiphonia denudata*) were subjected to further separation by consecutive extraction of the water extract (150 mg in water:ethanol 1:3, v/v 100 ml) with equal volumes of petroleum ether, dichloroethane, ethyl acetate and *n*-butanol. The most active product, water extract from *Polysiphonia denudata* (Rhodophyta) was subjected to chromatography with charcoal and eluted by MeOH/$H_2O$ mixtures with increasing concentrations of MeOH, pure MeOH and acetone:MeOH 2:1, v/v. The water extract was subjected to acidic hydrolysis with 5 ml 15% HCl. After refluxing for two hours the reaction mixture was cooled and subsequently extracted with equal

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volumes of methylene chloride and *n*-butanol. For the antiviral experiments 10% stock solutions of the preparations were made in distilled sterile water and the working dilutions further were made in cell culture medium *ex tempore.*

*Viruses*

Avian influenza virus A/chicken/Germany/27, strain Weibridge (H7N7) (A/H7N7) and human influenza viruses – A/PR/8/34 (H1N1) (A/H1N1), A/Krasnodar/101/59 (H2N2) (A/H2N2), A/Hong Kong/1/68 (H3N2) (A/H3N2), B/Lee/40 were grown in tissue cultures of chorioallantoic membranes (CAM) or in fertile hen's eggs. A/Aichi/2/68 (H3N2) had been adapted to the lung of white mice. The viruses were maintained by passages in 11 days old hen's fertile eggs and were used as allantoic fluids. The virus stock was stored at – 70 °C. The virus infectious titers were determined by hemagglutinin(HA) titration endpoint calculated according to Reed and Muench (1938) and expressed in 50% infectious doses per ml (log $ID_{50}$/ml).

*Test systems*

The antiviral studies were carried out in the following test systems:

1. Roller cultures of CAM from 11–12 days old fertile hen's eggs were prepared a/ according to Zakstelskaya (1975) for toxicity and b/ according to Horvath (1954) for the antiviral experiments. The cultures were maintained in glass tubes on a roller apparatus. The culture medium contained 4% phosphate buffer, 4% glucose, 1% ovoalbumin and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin G).
2. Stationary cultures of CAM were prepared after Maltzeva *et al.* (1973). The culture medium was as described above.
3. 10–11 days old fertile hen's eggs. The eggs were kept at 37 °C.
4. White mice, strain ICR, male and female, 18–20 g.

*Toxicity*

In CAM the toxicity was monitored following the effect of the preparations on tissue morphology and viability. Roller CAM (1a) in glass tubes were cultivated in the presence of serial two-fold dilutions of the substances and checked daily for 72 h for morphological changes. The toxicity was scored (score 0 = 0% changed CAM, score 1 = 0–25% changed CAM, score 2 = 25–50% changed CAM, score 3 = 50–75% changed CAM, score 4 = 75–100% changed CAM). The maximal tolerated concentration (MTC) was determined as the dose that caused no detectable morphological changes in CAM. The concentration required to cause visible changes in 50% of intact CAM with respect to control, 50% toxic concentration ($TC_{50}$), was evaluated from graphic plots. Fertile hen's eggs were inoculated with serial two-fold dilutions of the substances; early and late toxicity was estimated (Ilyenko, 1977). The toxicity for mice was determined according to Ilyenko (1977). 50% lethal dose ($LD_{50}$) was determined as the dose that caused death in 50% of intact fertile eggs and animals.

*Hemagglutination (HA) assay*

Fifty μl of allantoic virus suspensions were diluted 8 times, two-fold dilution each time, and incubated with an equal volume of 1% hen erythrocyte suspension for 30 min at the room temperature. The hemagglutination titre was estimated as the reciprocal value of the last dilution in which hemagglutination was observed.

*Virucidal activity*

The direct inactivating effect was tested in a contact assay. Ten-fold diluted allantoic fluids of the viruses were treated with equal volumes of two-fold serial dilutions of the substances for 1 h at 37 °C. The difference in the biological activities of the control and treated viruses was determined on the base of HA-production (HA assay).

*Antiviral assays*

*In vitro* and *in ovo* the virus-inhibitory effect was followed by the reduction of virus infectivity. In CAM a modification of EPTT (50% endpoint titration technique) described by Vanden Berghe *et al.* (1986) for cell cultures was used. In roller (1b) or stationary cultures of CAM serial ten-fold dilutions of the viruses ($1–10^4$ $ID_{50}$/ml) were inoculated simultaneously with two-fold dilutions of

the preparations. The antiviral experiments lasted for 48 h (72 h for B/Lee) at 37 °C (33 °C for B/Lee). At the end of the incubation period the viral growth was evaluated by hemagglutination of the supernatants of the tissue cultures *per se* with 1% solution of hen's erythrocytes and the infectious titres were calculated. The minimal inhibitory concentration (MIC) was determined as the minimal dose that caused significant reduction of viral reproduction in CAM ($\delta \log ID_{50}/ml = 1$). The significance of the difference between infectious titres of control and treated viruses was estimated by Student's *t* test. The dose that caused 0.5 log (50%) reduction of infectious titres, 50% effective concentration ($EC_{50}$), was estimated from graphic plots. The selectivity index (SI) was calculated from the ratio $TC_{50}/EC_{50}$.

In fertile eggs two-fold dilutions of the substances were inoculated 1 h before the addition of serial ten-fold dilutions of the viruses ($1-10^4$ $ID_{50}$/ml). At the end of 48 h incubation the viral growth was estimated as described above for CAM. The antiviral effect of the preparations was determined by the differences in the virus infectious titres ($\delta \log ID_{50}$/ml) in the absence and in the presence of the preparations.

*In vivo* the antiinfluenza effect was determined in white mice infected nasally with 1–10 $LD_{50}$ of A/Aichi (H3N2). The substances were inoculated according to a prophylactic-therapeutic schedule before and after infection (–24, –2, +2, +24, +48 h). The mortality rate was followed for 14 days. The inhibitory effect was determined on the basis of the reduction of mortality and the prolongation of survival times (Ilyenko, 1977). The protective index (PI) was determined from the equation PI% = PR-1/PR, where PR = $M_{control}/M_{experiment}$ and M is% of mortality. The results are the mean values of 2–4 experiments.

The *antiviral effect* was scored with respect to the inhibition of the reproduction of one or more viral strains caused by the preparations applied in a 1/4 MTC dose as follows: score – =non-significant inhibition, score + = 50% inhibition, score ++ = 90% inhibition, score +++ = 99% inhibition, score ++++ = >99% inhibition.

In all antiviral experiments non-drug treated, mock-infected cultures were used as tissue culture control and non-drug treated, virus-infected cultures – as virus control. Rimantadine hydrochloride (Hoffmann – La Roche Inc., Nutley, NJ), (1 μg/ml) was used as a positive control.

## Results and Discussion

Many natural products have been found to possess virus-inhibitory activity (Vanden Berghe *et al.*, 1986). Recent biochemical and pharmacological investigations on marine organisms evaluate them as an interesting source of potential antiviral compounds (Cannell, 1993; Che, 1991; Molinski, 1993; Rinehart, 1989; Vozzinskaya *et al.* 1993; Witvrouw and De Clercq, 1997). The information about the antiinfluenza virus activity of marine products is scarce. Gerber *et al.* (1958) established a protective effect of seaweed extracts for chicken embryos infected with influenza B virus (but not influenza A). Kathan (1965) reported inhibitory effect on influenza virus neuraminidase for kelp extracts. A six-component antibiotic complex, isolated from *Ulva lactuca* reduced the infectivity of human influenza virus A/PR8 (Ivanova *et al.*, 1991), some polysaccharides inhibited the reproduction of influenza viruses types A and B (Ivanova *et al.*, 1994, Witvrouw and De Clercq, 1997). Antiinfluenza virus activity of marine microalgae (Michailov *et al.*, 1994) and marine invertebrates has also been detected (Bichurina *et al.*, 1993).

As a part of a project on the biological effects of marine products sixty extracts, fractions and synthetic compounds from marine organisms (sea plants and invertebrates) were tested for antiinfluenza virus activity (Table I). 24 of the products (40%) showed a significant antiviral effect, among them 5 markedly inhibited viral reproduction and 2 exhibited notable activity. This result is a much higher figure than that, reported by Ballesteros *et al.* (1992) for a screening experiment of 65 preparations (21%) and closer to the outcome of screening experiment of 73 extracts, reported by Premanathan *et al.* (1992) (50%). Virus-inhibitory effect was found mainly among the water extracts – *Cystoseira barbata* (Heterocontophyta), *Ulva rigida* (Chlorophyta), *Phyllophora nervosa* (Rhodophyta), *Ceramium rubrum* (Rhodophyta) and *Polysiphonia denudata* (Rhodophyta). The lipophilic extracts from *Gelidium latifolium* and *Polysiphonia denudata* and two synthetic marine sponge alkaloids (aplysinopsine and its N-methyl derivative) were also active. The virus-inhibitory effect

Table I. Antiinfluenza virus effects of marine products.

| N | Marine organism | Preparation | MTC[a] [mg/ml] | MIC[b] [mg/ml] | Antiviral effect |
|---|---|---|---|---|---|
| | **Heterocontophyta – Phaeophycaceae** | | | | |
| **1** | *Cystoseira barbata* C. Agardh | lipophilic extract | 1.0 | 0.25 | + |
| 2 | *Cystoseira barbata* | water extract | 5.0 | 1.0–2.0 | ++ |
| 3 | *Cystoseira crinita* (Desf.) Bory | lipophilic extract | 0.12 | >0.12 | – |
| 4 | *Cystoseira crinita* | water extract | 0.5 | 0.25 | + |
| 5 | *Punctaria plantaginea* (Roth) Grev. | lipophilic extract | >500 | >500 | – |
| | **Chlorophyta – Cladophorophycaceae** | | | | |
| 6 | *Chaetomorpha aerea* (Dilwyn) Kutz | MeOH extract – I fr | 2.0 | >2 | – |
| 7 | *Chaetomorpha aerea* | MeOH extract – II fr. | 2.0 | >2 | – |
| 8 | *Cladophora vagabunda* (L.) Hoek | lipophilic extract | 3.3 | 0.8 | + |
| 9 | *Cladophora vagabunda* | water extract | 2.0 | >2 | – |
| | **Chlorophyta – Ulvophycaceae** | | | | |
| 10 | *Ulva rigida* C. Agardh | lipophilic extract | 1.0 | >1.0 | – |
| 11 | *Ulva rigida* | water extract | 10.0 | 0.7 | +++ |
| 12 | *Ulva rigida+Chaetomorpha aerea* | MeOH extract – I fr. | 2.0 | 0.5 | + |
| 13 | *Ulva rigida+Chaetomorpha aerea* | MeOH extract – II fr. | 2.0 | 0.5 | + |
| 14 | *Ulva rigida+Chaetomorpha aerea* | MeOH extract – III fr. | 2.0 | 0.5 | + |
| | **Rhodophyta – Bangiophycaceae** | | | | |
| 15 | *Bangia fuscopurpurea* (Dillwyn) (Yutz.) | lipophilic extract | 0.15 | >0.1 | – |
| | **Rhodophyta – Florideophycaceae** | | | | |
| 17 | *Ceramium rubrum* (Huds.) Ag. | lipophilic extract | 5.0 | >5 | – |
| 18 | *Ceramium rubrum* | water extract | 10.0 | 0.12–1.0 | +++ |
| 19 | *Ceramium rubrum* | $CH_3Cl$ | 0.3 | 0.05 | + |
| | **Fractionation of *C. rubrum* (W)** | | | | |
| 20 | –"- | petroleum ether fr. | 0.1 | >0.1 | + |
| 21 | –"– | dichloroethane fr. | 0.1 | >0.1 | – |
| 22 | –"– | ethylacetic fr. | 0.1 | >0.1 | – |
| 23 | –"– | *n*-butanol fr. | 0.1 | >0.1 | – |
| 24 | –"– | $H_2O$ residue | 0.1 | >0.1 | – |
| 25 | *Corallina officinalis* Linnaeus | lipophilic extract | 1.0 | >1 | – |
| 26 | *Corallina officinalis* | water extract | 10.0 | 2.5 | + |
| 27 | *Gelidium latifolium* (Grev.) Born et Thur. | lipophilic extract | 10.0 | 0.25 | ++ |
| 28 | *Gelidium latifolium* | water extract | 10.0 | 2.5 | + |
| 29 | *Polysiphonia denudata* (Dillwyn) Kutz. | lipophilic extract | 8.0 | 0.5–1.6 | ++ |
| 30 | *Polysiphonia denudata* | water extract | 10.0 | 0.03–0.25 | ++++ |
| 31 | *Polysiphonia denudata* | BuOH extract | 0.5 | >0.5 | – |
| | **Fractionation of *P. denudata* (W)** | | | | |
| 32 | –"- | petroleum ether fr. | 0.1 | >0.1 | – |
| 33 | –"– | dichloroethane fr. | 0.1 | >0.1 | – |
| 34 | –"– | ethylacetic fr. | 0.1 | 0.02 | ++ |
| 35 | –"– | *n*-butanol fr. | 0.1 | >0.1 | – |
| 36 | –"– | $H_2O$ residue | 0.1 | >0.1 | – |
| | **Chromatography on charcoal of *P. denudata* (W)** | | | | |
| 37 | –"– | $H_20$:MeOH 9:1 | 0.01 | >0.01 | – |
| 38 | –"– | $H_20$:MeOH 1:1 | 0.2 | 0.05 | ++ |
| 39 | –"– | MeOH | 0.1 | >0.1 | – |
| 40 | –"– | acetone:MeOH 2:1 | 0.2 | >0.2 | – |
| | **Acidic hydrolysis of *P. denudata* (W)** | | | | |
| 41 | –"– | $(CH_2Cl)_2$ | 1.0 | >1 | – |
| 43 | –"– | $H_20$ residue | 1.0 | >1 | – |
| 44 | *Phyllophora nervosa* (DC.) Grev. | lipophilic extract | 1.0 | 0.25 | + |
| 45 | *Phyllophora nervosa* | water extract | 10.0 | 0.25–1.8 | +++ |

Table I. (cont).

| N | Marine organism | Preparation | MTC[a] [mg/ml] | MIC[b] [mg/ml] | Antiviral effect |
|---|---|---|---|---|---|
| | **Fractionation of *P. nervosa* (W)** | | | | |
| 46 | –"- | petroleum ether fr. | 0.1 | >0.1 | – |
| 47 | –"– | dichloroethane fr. | 0.1 | >0.1 | – |
| 48 | –"– | ethylacetic fr. | 0.1 | >0.1 | – |
| 49 | –"– | *n*-butanol fr. | 0.1 | >0.1 | – |
| 50 | –"– | residue | 0.1 | >0.1 | – |
| | **Marine invertebrates** | | | | |
| 51 | *Botryllus schlosseri* (Tunicate) | lipophilic extract | 0.5 | >0.5 | – |
| 52 | *Botryllus schlosseri* | water extract | 0.5 | 0.03 | ++ |
| 53 | *Dysidea fragilis* (Sponge) | lipophilic extract | 0.02 | >0.02 | – |
| 54 | *Dysidea fragilis* | $CH_3Cl$ extract | 0.02 | >0.02 | – |
| 55 | *Dysidea fragilis* | MeOH extract | 0.2 | >0.2 | – |
| 56 | *Dysidea fragilis* | water extract | 0.2 | >0.2 | – |
| 57 | Synthetic sponge alkaloid | aplysinopsin | 0.01 | 0.002 | ++ |
| 58 | Synthetic sponge alkaloid | N-methyl aplysinopsin | 0.01 | 0.002 | ++ |
| 59 | Synthetic sponge alkaloid | desmethyl aplysinopsin | 0.01 | >0.01 | – |
| 60 | *Rapana thomasiana* (Snail) | water extract | 0.12 | >0.12 | – |

[a] Maximal tolerated concentration, the dose that caused no detectable morphological changes in intact CAM.
[b] Minimal inhibitory, the minimal dose that caused significant reduction of viral reproduction in CAM.

was dose-dependent (Tables I, II). The strain-specificity of the virus-inhibitory effect (Table II) was consistent with its selectivity. The toxicity of the active extracts varied and their selectivity index ($SI = TC_{50}/EC_{50}$) was between 5 to 403. The active preparations did not exhibit any direct virus-inactivating activity when applied in doses up to 5 mg/ml.

The same algae extracts were tested for antimicrobial activity (Serkedjieva *et al.*, 1998); it was shown that some of them reduced the growth of *St. aureus* and *B. subtilis*, but there was limited coincidence between the antiviral and antimicrobial activities. Lack of direct virus-inactivating and antimicrobial activities in the effective preparations suggested that the virus-inhibitory effect was specific.

Further the most active extracts *Ceramium rubrum* (W), *Polysiphonia denudata* (W) and *Phyllophora nervosa* (W) were fractionated with solvents

Table II. Drug susceptibility of influenza viruses to marine products.

| N | Marine organism (Preparation) | | A/H7N7 | | A/H1N1 | | A/H2N2 | | A/H3N2 | | B/Lee | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $TC_{50}$[a] [mg/ml] | $EC_{50}$[b] [mg/ml] | δ log $ID_{50}$/ml[c] | $EC_{50}$[b] [mg/ml] | δ log $ID_{50}$/ml[c] | $EC_{50}$[b] [mg/ml] | δ log $ID_{50}$/ml[c] | $EC_{50}$[b] [mg/ml] | δ log $ID_{50}$/ml[c] | $EC_{50}$[b] [mg/ml] | δ log $ID_{50}$/ml[c] |
| 2 | *Cystoseira barbata* (W) | 5.6 | n.d. | | 1.2 | 2.1 | 1.1 | 1.67 | 1.6 | 2.33 | 2.0 | n.s. |
| 10 | *Ulva rigida* (W) | 10.5 | n.d. | | 0.5 | 2.23 | 0.4 | 1.4 | 0.5 | 2.67 | 0.5 | 1.6 |
| 18 | *Ceramium rubrum* (W) | 10.2 | 0.12 | 2.3 | 0.5 | 1.5 | 0.25 | 1.6 | 0.12 | 2.6 | 1.1 | 1.5 |
| 27 | *Gelidium latifolium* (L) | 10.1 | n.d. | | 0.25 | 1.63 | 0.25 | n.s. | 0.25 | 1.8 | 0.25 | n.s. |
| 29 | *Polysiphonia denudata* (L) | 8.2 | 0.5 | 2.5 | 0.5 | 1.5 | 1.4 | n.s. | 0.25 | 1.6 | 1.6 | n.s. |
| 30 | *Polysiphonia denudata* (W) | 12.1 | 0.1 | 2.8 | 0.12 | 3.23 | 0.25 | 1.5 | 0.03 | 3.67 | 1.3 | 2.1 |
| 45 | *Phyllophora nervosa* (W) | 10.8 | 0.8 | 1.2 | 0.25 | 2.5 | 1.8 | 2.33 | 0.25 | 2.8 | 0.25 | 2.33 |
| 57 | Aplysinopsin | 0.01 | n.d | | 0.002 | 3.5 | n.d | | 0.002 | 2.67 | 0.002 | 3.23 |
| 58 | N-Methyl aplysinopsin | 0.01 | n.d | | 0.01 | 1.8 | n.d | | 0.01 | 1.6 | 0.01 | 2.0 |

[a] 50% toxic concentration, the dose required to cause visible changes in 50% of intact cultures.
[b] 50% effective concentration, the dose that caused 0.5 log (50%) reduction of infectious titres.
[c] Inhibition exhibited by the preparations in a 1/4 $LD_{50}$ dose.
n.s. – not significant ($P<0.05$).
n.d. – not determined.
W: Water phase.
L: Lipophilie phase, see Materials and Methods.

of increasing polarity. Only the ethyl acetate and water/methanol fractions of *Polysiphonia denudata* (W) reduced significantly influenza virus replication (Table I).

The water extracts from *Polysiphonia denudata, Cystoseira barbata, Ulva rigida, Phyllophora nervosa* and *Ceramium rubrum* inhibited significantly the reproduction of influenza virus also in fertile eggs (Table III). The water extracts from *Polysiphonia denudata* and *Ulva rigida* reduced the mortality rate of white mice in experimental influenza infection with A/Aichi (H3N2) when applied orally and extended the time of survival (Table IV). The inhibitory effect of the aqueous extract from *Polysiphonia denudata* was further studied in detail. The preparation reduced selectively and in a dose-related manner the virus-induced cytopathic effect, the infectious virus yields, the expression of viral proteins on the infected cell surface in ELISA, the virus-specific protein synthesis in chicken embryo fibroblast cells, infected with A/FPV (H7N1) (unpubl.).

The presented results do not offer an explanation about the mode of the inhibitory activity of the extracts from marine organisms. There are data that many marine bioactive products have affinities for certain cellular receptors in the mammalian cell (Molinski, 1993) and thus can interfere with the initial steps of viral replication. Ehresmann *et al.*, 1997 suggested that herpes virus inhibition by algal extracts was due to a block at viral adsorption. An algal extract inhibited herpes virus penetration in a dose-dependent manner, did not inhibit virus attachment to cells and did not affect host protein synthesis (Hayashi *et al.*, 1993). We have studied in detail the virus-inhibitory effect of the water extract from *Polysiphonia denudata* and it was found that the preparation reduced the reproduction of A/H3N2 by inhibition of viral adsorption to susceptible cells and had an effect on viral synthetic stages (unpubl.).

At this stage of our investigations we can not define the biologically active components in the extracts. However the presented results support the view that marine organisms are an interesting source of potential antiviral compounds (Cannell, 1993; Che, 1991; Rinehart, 1989; Vozzinskaya *et al.* 1993; Witvrouw and De Clercq, 1997). Projects on

Table III. Inhibitory effect of effective marine products on the reproduction of influenza virus in embryonated hen's eggs.

| N | Marine organism (Preparation) | $LD_{50}$[a] [mg/ml] | A/H1N1 δ log $ID_{50}$/ml[b] | A/H3N2 δ log $ID_{50}$/ml[b] |
|---|---|---|---|---|
| 2 | *Cystoseira barbata* (W) | 12.2 | n.s. | 1.17 |
| 10 | *Ulva rigida* (W) | 10.1 | 1.87 | 2.67 |
| 18 | *Ceramium rubrum* (W) | 12.4 | 2.1 | 2.33 |
| 30 | *Polysiphonia denudata* (W) | 16.2 | 2.37 | 2.77 |
| 45 | *Phyllophora nervosa* (W) | 12.0 | 1.25 | 1.63 |

[a] 50% lethal dose, the dose that caused death in 50% of intact embryonated eggs.
[b] Inhibition exhibited by the preparations in a 1/4 $LD_{50}$ dose.
* Not significant ($P<0.05$).

Table IV. Protective effect of marine algae extracts on experimental influenza infection in mice.

| N | Marine organism (Preparation)# | Inoculation | $LD_{50}$[a] [g/kg] | Dosage[b] [g/kg] | PI[c] % | MST[d] [days] |
|---|---|---|---|---|---|---|
| 10 | *Ulva rigida* (W) | oral | >10.0 | 1.0 | 46.1 | +2.6 |
| 30 | *Polysiphonia denudata* (W) | oral | >10.0 | 1.0 | 63.7 | +4.8 |

[a] 50% lethal dose, the dose that caused death in 50% of intact animals.
[b] The indicated dose x 5.
[c] Protective index = PR-1/PR, where PR (protective ratio) = $M_{control}/M_{experiment}$, and M is% of mortality (Ilyenko, 1977).
[d] Mean survival time.
# Application according to the schedule −24, −2, +2, +24, +48 h with respect to viral infection.

the virus – inhibitory activity start with a screening of extracts (Ballesteros *et al.*, 1992; Ehresmann *et al.*, 1997; Premanathan *et al.*, 1992), followed by the isolation of biologically active substances. Some of the constituents of marine algae, such as carageenans and alginates have no analogues (Vozzinskayaa *et al.*, 1993). Sulfated polysaccharides attracted considerable attention as potential antiviral agents (for review see Witvrouw and De Clercq, 1997).

In conclusion it can be stated that the Bulgarian Black Sea coast offers marine plants and invertebrates possessing pronounced antiviral activity. The study of their biologically active constituents is in progress.

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