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# COMPOUNDS WITH ANTIBACTERIAL ACTIVITY FROM THE FRESHWATER ALGA *Spirogyra crassa* (L.) Kutz

***Ivanova A.[1*], J. Nechev[2], I. Tsvetkova[3], H. Najdenski[3], K. Stefanov[2], S. Popov[2]***

[1]*Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

[2]*Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

[3]*Institute of Microbiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria*

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**Summary.** The alga *Spirogyra crassa* (L.) Kutz present in two lakes near Sofia (Bulgaria) has an antibacterial effect on both Gram-positive and Gram-negative bacteria. The activity was concentrated mainly in the n-butanol algal extracts. As compounds with activity against *Staphylococcus aureus 209* were identified different fatty acids in a mixture, in which palmitic and linolenic acid as well as ethyl- and propyl gallates predominated. The composition of the compounds with activity against *Echerichia coli WF+* was not identified.

***Key words*:** *Spirogyr*; *Chlorophyta*; freshwater algae; antibacterial activity.

## INTRODUCTION

The freshwater algae are a promising source of bioactive natural products (Ozturk et al., 2006). The green freshwater alga *Spirogyra crassa* (L.) Kutz is widespread all around Europe. It is known that *Spirogyra* possesses antibacterial activity (Stefanov et al., 1999), but the data on the composition of the active compounds are limited (Cannel et al., 1998). The aim of the present work was to provide some data on the composition of the compounds with antibacterial activity extracted from *Spirogyra crassa* growing in two different lakes in Bulgaria.

## MATERIALS AND METHODS

### Algal material

Samples of *Spirogyra crassa* (L.) Kutz (Chlorophyta) were collected from two lakes in Simeonovo. This is a cascade of artificial lakes with running water, situated in a mountain near Sofia. *S. crassa* is growing only in two of them,

*Corresponding author: albena55@yahoo.com

noted hereafter as "upper" and "lower".

For comparison, from one of the lakes ("upper lake"), samples were collected in two consecutive years (2003 and 2004). All samples were taken in July. Voucher specimens were deposited in the Pharmacy Faculty, Sofia 1000, Bulgaria, and were identified by Dr. S. Dimitrova-Konaklieva.

**Isolation of the total lipophylic and n-butanol extracts**

The fresh algal biomass (about 150 g) was homogenized with 100 ml methanol and refluxed for 10 min in order to inactivate the enzymes. An equal volume of chloroform was added, the mixture was filtered and an equal amount of water was added. The lower layer contained the total lipophylic extract. The upper phase was extracted with n-butanol (2 x 50 ml). The total lipophylic extract and the combined butanolic extracts were evaporated under vacuum.

**Hydrolysis of the total lipophylic extract**

About 40 mg sample was hydrolyzed with 10 ml 1N ethanolic KOH. The mixture stood overnight and after the addition of an equal volume of water the unsaponifiables were extracted with hexane (2 x 10 ml). After acidification with 2N sulfuric acid the saponifiable compounds (mainly fatty acids) were extracted with hexane (2 x 10 ml).

**Column chromatography**

Parts (1.5 g each) of the total lipophylic and the butanol extracts were applied on a 50 g silica gel S (Merck, Darmstadt) column and separated chromatographically with chloroform-methanol mixtures with increasing polarity. Fractions of 50 ml were collected and the amount of the components was determined gravimetrically.

**Test for antibacterial activity**

The antibacterial activity was determined according to Spooner and Sykes (1972). As test organisms *Staphylococcus aureus* 209 and *Escherichia coli WF*+ were used and 0.5 mg from the total lipophylic and n-butanol extracts were tested in each experiments. The antibacterial activity was measured by the diameter of the inhibitory zones in the soft agar layer after 48-72 h incubation at 37°C. An inhibitory zone with a diameter less than 12 mm indicated lack of activity.

**Statistical analysis**

All the experimental values are means of three independent experiments. The significant differences were calculated using Student's *t*- test.

## RESULTS AND DISCUSSION

The effects of lipophylic and butanol extracts of *S. crassa* on Gram positive and Gram negative bacteria are summarized in Table 1. The lipophylic extracts of all samples from Simeonovo lakes have antibacterial activity against *Staphylococcus aureus*, but only those from the "upper" lake showed activity against *E. coli*.

Part of the lipophylic extract of sample 2 (Simeonovo 2004, "upper" lake, Table 1) was hydrolyzed leading to saponifiable (52.5%) and unsaponifiable (47.5%) fractions. The antibacterial activity remained only in the saponifiable fraction (32 mm against *S. aureus* and

Table 1. Antibacterial activity of extracts from *Spirogyra crassa.*

| № | Sample | Extract [mg $g^{-1}$ DW] | Inhibitory zone* [mm] | |
|---|---|---|---|---|
| | | | *S. aureus 209* | *E.coli WF* |
| 1 | Simeonovo, July 2003 Upper lake | | | |
| | Lipophylic extract | 50 ± 4.0 | 17 | 14 |
| | n-butanol extract | 9.8 ± 0.8 | 29 | 14 |
| 2 | Simeonovo, July 2004 Upper lake | | | |
| | Lipophylic extract | 56 ± 4.0 | 20 | 0 |
| | n-butanol extract | 6.6 ± 0.5 | 32 | 20 |
| 3 | Simeonovo, July 2004 Lower lake | | | |
| | Lipophylic extract | 51 ± 4.0 | 12 | 0 |
| | n-butanol extract | 9.1 ± 0.7 | 22 | 16 |

*The results are mean values from 3 parallel determinations.

16 mm against *E. coli*) and was even higher than in the initial sample, while the unsaponifiable fraction had no activity. It was evident that the alkaline hydrolysis resulted in changes in the biologically active compounds. For this reason, the other algal samples were not hydrolyzed.

In order to isolate the compound (or compounds) possessing this activity, we separated part of the lipophylic extract of sample 1 (Simeonovo, July 2003, "upper" lake, Table 1) on a Silica gel column. The results are presented in Table 2. Although the initial sample possessed activity against *E. coli*, this activity was lost after the fractionation. This can be due to a synergistic effect of some compounds divided by the chromatographic procedure. The activity against *S. aureus* was concentrated in fractions 3, 4, 7 and 8. These fractions were analyzed further by GC-MS. Fractions 3 and 4 were identified as a mixture of ethyl- and propyl gallates. It is well known that polyphenols and tannic acid from the freshwater macrophyte *Myriophyllum spicatum* are active inhibitors of microalgal exoenzymes ( Leu et al. 2002). Dodecyl gallate was found to possess antibacterial activity specifically against Gram-positive bacteria. The activity of this compound comes in part from its ability to inhibit the membrane respiratory chain (Kubo et al., 2002). Catechin gallates are active against *S. aureus* (Gibbons et al., 2004). It was established that the algae from the order *Zygnematales* contain gallates and tannins (Nakabayashi and Nada, 1954; Nishizawa et al., 1988), but there no data are available on their antibacterial activity.

Fractions 7 and 8 were combined, analyzed by GC-MS and identified as a mixture of fatty acids. It is known that fatty acids from freshwater diatoms (Juttner, 2001) and micro- and macroalgae (Chiang et al., 2004; Herrero et al., 2006; Chazala and Shameel, 2005; Ozturk et al., 2006) posses antibacterial activity and cause reduced growth of the zooplankton (Spruell, 1984).

Table 2. Column chromatography of the total lipophylic extract of the sample from Simeonovo 2003, "upper" lake (Sample 1, Table 1).

| Fraction № | Eluent | Fraction % of total | Inhibitory zone* [mm] *S. aureus 209* |
|---|---|---|---|
| 1 | chloroform | 38.8 | 0 |
| 2 | chloroform-methanol (95:5) | 5.2 | 0 |
| 3 | chloroform-methanol (90:10) | 18.8 | 16 |
| 4 | chloroform-methanol (80:20) | 5.4 | 18 |
| 5 | chloroform-methanol (60:40) | 5.8 | 0 |
| 6 | chloroform-methanol (50:50) | 1.4 | 0 |
| 7 | chloroform-methanol (40:60) | 0.4 | 14 |
| 8 | chloroform-methanol (30:70) | 1.3 | 14 |
| 9 | chloroform-methanol (20:80) | 12.1 | 0 |
| 10 | chloroform-methanol (10:90) | 7.3 | 0 |
| 11 | methanol | 3.3 | 0 |

*The results are obtained from at least 2 determinations in 3 replicates.

The relationship fatty acid structures – antibacterial activity of plant extracts has been reviewed (McGaw et al., 2002). These results correlate with our investigation of the antibacterial activity of saponifiables of sample 2 (Table 1). This fraction showed very high antibacterial activity, probably due to the fatty acids, which are the main constituents.

The fatty acid composition of *S. crassa* is relatively simple (Stefanov et al., 1996). The main acids are palmitic, linoleic and linolenic, but a lipophylic extract of the algae can find application as a stabilizing agent in some cosmetical and food products.

Similar to the column fractionation of the lipophylic extracts of sample 1 (Table 1) we tried to separate the n-butanol extract of the same sample. The results are shown in Table 3. Most of the polar compounds are biogenetically connected to the lipophylic compounds and often possess biological activity (Kubo et al., 2002; Herrero et al., 2006). Contrary to the lipophylic extract where the antibacterial activity was concentrated in few fractions, in the butanol extract the activity was divided among all fractions. Similarly to the lipophylic fraction, the fractions had lost their activity against the Gram-negative bacteria. The loss of physiological activity due to fractionation of the total extracts is a well-known phenomenon (Lu et al., 2006). To avoid this problem, we analyzed the whole butanol extracts of samples 1 (Table 1) directly by GC-MS. The results showed that the main components of this extract represented a mixture of hydroxy fatty acids, derivatives of hydroxy benzoic acids and furanone.

Table 3. Column chromatography of the n-butanol extract of the sample from Simeonovo 2003, "upper" lake (Sample 1, Table 1).

| Fraction № | Eluent | Fraction % of total | Inhibitory zone* [mm] *S. aureus 209* |
|---|---|---|---|
| 1 | chloroform | 17.1 | 22 |
| 2 | chloroform-methanol (95:5) | 1.5 | 16 |
| 3 | chloroform-methanol (90:10) | 2.7 | 18 |
| 4 | chloroform-methanol (80:20) | 18.3 | 16 |
| 5 | chloroform-methanol (70:30) | 5.6 | 12 |
| 6 | chloroform-methanol (60:40) | 11.2 | 18 |
| 7 | chloroform-methanol (50:50) | 26.7 | 19 |
| 8 | chloroform-methanol (30:70) | 5.0 | 12 |
| 9 | methanol | 11.7 | 10 |

*The results are obtained from at least 2 determinations in 3 replicates.

The fatty acids serve as energetic substrates and allelophatic agents and their antibacterial effect is well known (McGaw et al., 2002). This effect could be increased if the fatty acid possess a hydroxy group in 2-position (Ben Jannet et al., 2006). The antibacterial effect of benzoic acids and their derivatives was discussed above. The antibacterial effect of furans is well-known. Many furanone derivatives are isolated or synthesized as biologically active products (Lorimer et al., 1995; Mendling and Mailland, 2002; Ravicumar et al., 2005).

The compounds in *S. crassa* could have an ecological role. They protect the biological systems in the lakes and probably affect the concentration of bacteria in the region. It is evident that extracts of *S. crassa* could find practical application, mainly as additives for cosmetic preparations and foods.

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