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Mimics of natural phenylpropanoids – *In vitro* antialgal activity

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ABSTRACT

Twenty-two compounds, mimics of natural phenylpropanoids were tested in broth against *Selenastrum capricornutum* and the influence of hydroxyl and methoxyl groups on their activity has been evaluated. Selected phenylpropanoids were also tested on different freshwater algal strains and selective activity of these compounds has been reported.

Key words : Antialgal activity, *In vitro*, microalgae, phenylpropanoids, *Selenastrum capricornutum*

INTRODUCTION

Shikimate metabolites as phenolic acids or coumarins represent some of the most active allelochemicals (9, 12) and their toxic effects on germination (1), seedlings growth (8), photosynthesis and respiration in higher plants (10) have been extensively investigated. Shikimate metabolites as phenylpropanoids have also been found in several aquatic weeds such as *Acorus gramineus* Soland (5), *Pistia stratiotes* L. (2) and *Myriophyllum verticillatum* L. (3) and may have a role in the control of microalgal growth. *In vitro* preliminary tests (5) showed that compounds such as α - asarone (**I**), β–asarone (**II**) and the corresponding epoxides **III** and **IV** were active against several algal strains. A study of the effects of natural and synthetic material on the growth of *S. capricornutum* showed some relationship between the structure and activity of phenylpropanoids (6).

Compounds with different C_3 chains and variously substituted with one, two or three methoxyl groups in the ring showed that activity was greatly influenced by the number of the methoxyl groups, by their position and by the nature of the side chain. The activity increases from the monomethoxy to the trimethoxy derivatives and methoxyl groups at the positions *ortho* and *para* in the side chain provide the highest toxicity. Furthermore, compounds with an E-propenylic side chain were more active than those with a Z-propenylic and an allylic chain.

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The possible use of phenylpropanoids as selective algal inhibitors in natural environments affected by algal blooms prompted us to study synthetic compounds, mimics of the natural phenylpropanoids, with hydroxyl groups in place of one or more methoxyl groups to have a larger hydrophile character in the compounds.

MATERIALS AND METHODS

The analysis was run on *S. capricornutum* strain 'T 1648' and on 17 strains from Cyanochloronta, Rhodophycophyta, Chrysophycophyta and Chlorophycophyta.

Synthesis of Phenylpropanoids

(i) *2-Allylphenol* (**V**), *4-allyl-2-methoxyphenol* (**XVII**), *4-allyl-2,6-dimethoxyphenol* (**XXIII**) and all the commercially available precursors were purchased from Aldrich. The purity of the samples used in the bioassays was found higher than 99.5% by HPLC.

(ii) *3-Allylphenol* (**VI**) : 3-Hydroxycinnamic acid (2 g) in dry ethyl ether (50 ml) was refluxed with $LiAlH_4$ (800 mg) to give the 3-(4-hydroxy-phenyl)-propanol which was directly acetylated with acetic anhydride (1.5 ml) in dry pyridine (2 ml). Usual work-up of the reaction mixture gave the crude diacetate (300 mg) which was chromatographed on silica gel (hexane-ethyl acetate 17 : 3). The diacetate (100 mg) in dry THF (10 ml) was kept at 65°C under N_2 and Pd $[(C_6H_5)_3\ P]_4$ (50 mg), $(C_6H_5)_3P$ (80 mg) and $NaBH_3CN$ (60 mg) were added. After 16 h saturated NaCl and ethyl ether were added and the material extracted into the organic layer was hydrolyzed with ethanolic 10% KOH to give. **VI** (50 mg).

(iii) *4-Allylphenol* (**VII**) : 4-Allylanisol (300 mg) in dry CH_2Cl_2 (10 ml) was treated at 70°C with 1 M BBr_3 in CH_2Cl_2 (1 ml). After 3 h at 0°C the reaction mixture was neutralized with 1 M $NaHCO_3$. The organic layer was chromatographed on silica gel (benzene-ethyl ether 9 : 1) to give pure **VII** (210 mg).

(iv) *2-Allyl-6-hydroxyphenol* (**VIII**), *2-allyl-5-hydroxyphenol* (**IX**), *2-allyl-4-hydroxyphenol* (**X**), *2-allyl-3-hydroxyphenol* (**XI**) and *4-allyl-2-hydroxyphenol* (**XII**) : Catechol (1 ml) in anhydrous acetone (8 ml) was refluxed with K_2CO_3 (1.5 g) and allyl bromide (1 ml). After 8 h ethyl ether and water were added and the organic layer was washed with aqueous 10% NaOH, neutralized with water to give the monoallyl ether (800 mg) which was isomerized at 50°C for 1 h to give a mixture (7 : 3) of **VIII** and **XII**. Chromatography on silica gel (hexane-ethyl ether 9 : 1) gave pure **VIII** and **XII**. The mixture of **IX** and **XI** was prepared from resorcinol as described for the above compounds. The mixture was separated by silica gel

described for the above compounds. The mixture was separated by silica gel chromatography (hexane-ethyl ether 9 : 1). Compound **X** was prepared from hydroquinone in the same way.

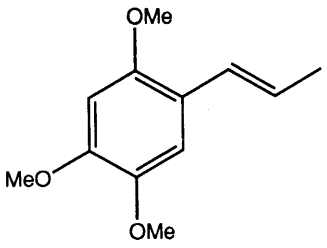
(v) *2-Allyl-6-methoxyphenol* (**XIII**), *2-allyl-5-methoxyphenol* (**XIV**), *2-allyl-4-methoxyphenol* (**XV**) *and 2-allyl-3-methoxyphenol* (**XVI**) : 2-Allyl-6-methoxyphenol (**XIII**) was obtained by treatment of 2-methoxyphenol with allyl bromide and subsequent Claisen isomerization as previously reported. In the same way 3-methoxyphenol was converted into a mixture (1 : 4) of 2-allyl-5-methoxyphenol (**XIV**) and 2-allyl-3-methoxyphenol (**XVI**) which were separated by preparative TLC, and 2-allyl-4-methoxyphenol (**XV**) was obtained from 4-methoxyphenol.

(vi) *6-Allyl-2,3–dimethoxyphenol* (**XVIII**), *2-allyl-4,5-dimethoxyphenol* (**XIX**), *2-allyl-3,5-dimethoxyphenol* (**XX**), *2-allyl-3,4-dimethoxyphenol* (**XXI**) *and 2-allyl-4,6-dimethoxy-phenol* (**XXII**) : **XVIII** was obtained from 2,3–dimethoxyphenol by treatment with allylic bromide and Claisen isomerization. With the same procedure **XIX** and **XXI** in a 7 : 3 ratio were obtained from 3,4-dimethoxyphenol, **XX** from 3,5-dimethoxyphenol and **XXII** from 2,4–dimethoxyphenol.

(vii) *6-Allyl-2-hydroxy-3-methoxyphenol* (**XXIV**), *6-allyl-3-hydroxy-2-methoxyphenol* (**XXV**) *and 6-allyl-3-hydroxy-4-methoxyphenol* (**XXVI**) : **XXIV** and **XXV** in a 4 : 1 ratio were obtained by BBr_3 treatment of **XVIII** as described for **VII**. In the same conditions **XIX** gave **XXVI**.

Bioassays

The activity of the compounds was tested against the algal strains (Table 2). All the algae were preliminarily cultured on Bold basal medium (BBM) (11) and the growth of each strain was followed by a colorimeter at 550 nm (14). In the late exponential phase (0.7 unit of absorbance), it was aseptically inoculated on a Petri dish (10 cm) containing BBM (30 ml) solidified with agar (1.5%). An aliquot of each compound (1 µmol), dissolved in acetone (80 µl/mg), was impregnated on a paper disk (sterile blanks, Difco Bacto Concentration Disks, 6 mm) and after evaporation of the solvent the disk was placed on each inoculated Petri dish. The plates were incubated at 24°C and illuminated under a photoperiod of 16 h light-8 h dark by a fluorescent lamp. The inhibition was calculated as diameter of the non-growth zone including the paper disk and the reported values were the average of three experiments. The phytotoxicity was compared with that of $CuSO_4$ (1 µmol).



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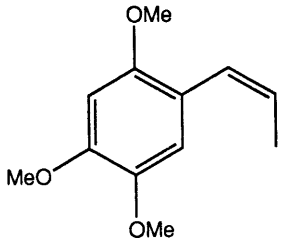

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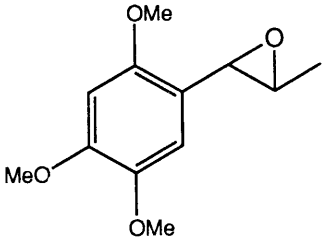

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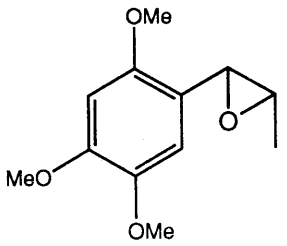
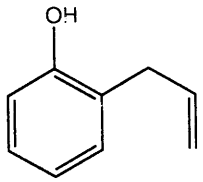
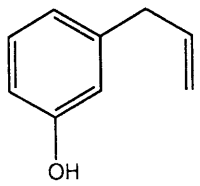


IV 2,4,5-Trimethoxy-Z-3'-methyloxiranylbenzene



V 2-Hydroxyallylbenzene



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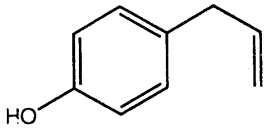
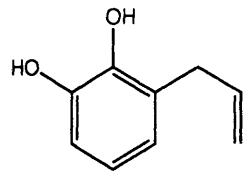


VII 4-Hydroxyallylbenzene



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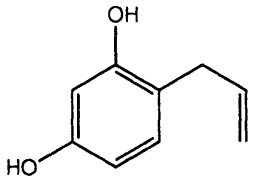

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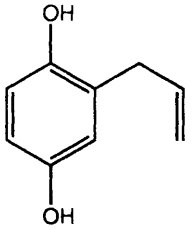

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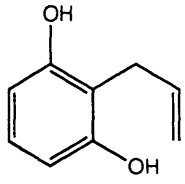

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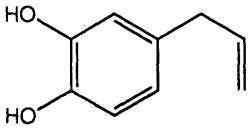
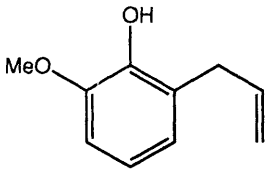
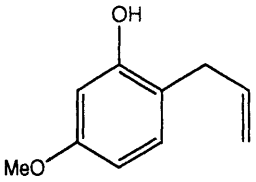


XII 3,4-Dihydroxyallylbenzene



XIII 2-Hydroxy-3-methoxyallylbenzene



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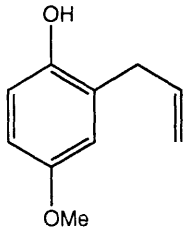
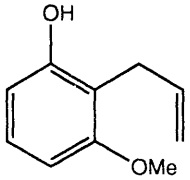
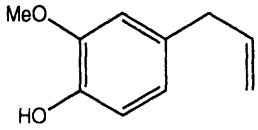


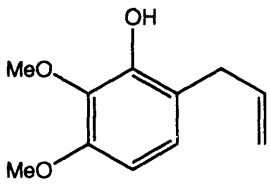
XV 2-Hydroxy-5-methoxyallylbenzene



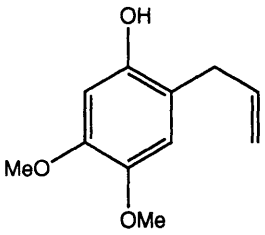
XVI 2-Hydroxy-6-methoxyallylbenzene



XVII 4-Hydroxy-3-methoxyallylbenzene



XVIII 2-Hydroxy-3,4-dimethoxy allylbenzene



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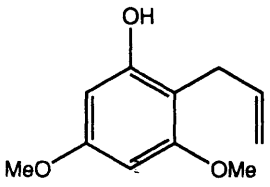

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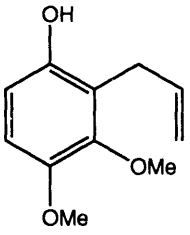

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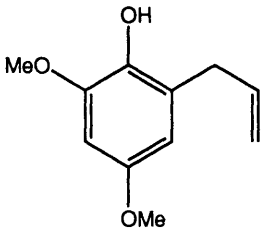

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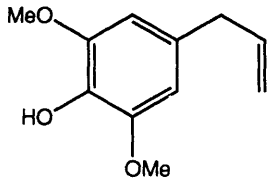

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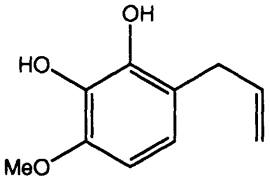

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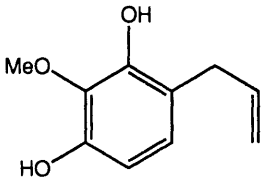

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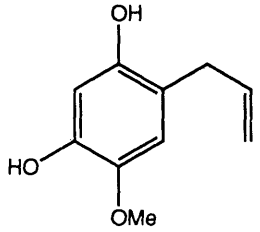

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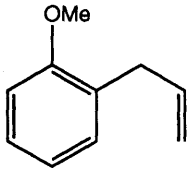
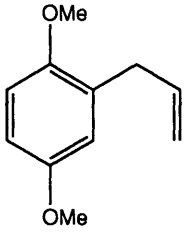
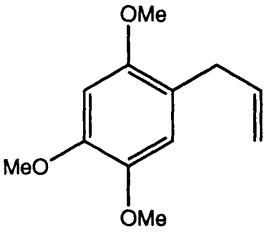


XXVII 2-Methoxyallylbenzene



XXVIII 2,5-Dimethoxyallylbenzene



XXIX 2,4,5-Trimethoxyallylbenzene

Fig. 1. Structures of phenylpropanoids studied in bioassays.

The bioassay against *S. capricornutum* was run in broth. The phenylpropanoids were dissolved in ethanol. Each solution (20 ml) was added to the test tubes containing the inoculated medium (6 ml) to have final concentrations of 5 x 10^{-4}, 2.5 x 10^{-4}, 10^{-4} and 5 x 10^{-5} M. The cell number of the initial inocula ranged from 10^{-6} to 1.5 x 10^{-6} ml^{-1}. The test tubes were incubated at 23°C on a shaking apparatus (13) and irradiated as in the previous assay. The inhibition on the algal growth was calculated according to Blankley (4).

RESULTS AND DISCUSSION

2-Allylphenol (**V**) and 4-allyl-2-methoxyphenol (**XVII**) are commercially available products, while all the remaining phenylpropanoids were synthesized *ad hoc* (Fig. 1).

3-Allylphenol (**VI**) was synthesized starting from 3-hydroxycinnamic acid through reduction, acetylation and elimination of acetic acid in the side chain. 4-Allylphenol (**VII**) was prepared from 4-allylanisol by BBr_3 demethylation.

All the dihydroxyallylbenzenes **VIII-XII** were synthesized through the mono allylethers, obtained by treatment of catechol, resorcinol or hydroquinone with allylbromide in alkaline conditions. The subsequent Claisen isomerization of the ethers gave the targets. In the same way, the isomeric methoxyphenols were converted into **XIII-XVI** and allyldimethoxyphenols **XVIII-XXII** were obtained from the isomeric dimethoxyphenols. BBr_3 demethylation of **XVIII** gave **XXIV** and **XXV**, while the same treatment of **XIX** gave **XXVI**. All the attempts to obtain the isomeric trihydroxyallylbenzenes with this procedure from the corresponding methylderivatives failed owing to the fast oxidation of the products to quinones in the reaction conditions.

The phenylpropanoids were tested in broth on *S.capricornutum* at concentrations from 5 x 10^{-4} to 5 x 10^{-5} M using algal inocula from 10^{-6} to 1.5 x 10^{-6} ml^{-1} (Table 1). All the compounds caused growth rate inhibition at 5 x 10^{-4} M and at 2.5 x 10^{-4} M, while at the lowest concentration 5 x 10^{-5} M only 2-allyl-5-methoxyphenol (**XIV**) and the isomeric allyldimethoxyphenols **XVIII-XXIII** showed significant activity.

The allylphenols **5-7** were more active than the corresponding methoxyderivatives (5) and the *ortho* and *para* isomers had higher effects than the *meta* substituted.

The activity slightly decreased the dihydroxyallylbenzenes **VIII-XII** bearing a second hydroxyl group in the ring, while the presence of a methoxyl group as in **XIII-XVII** caused appreciable increase in the activity.

The same influence was found in the trisubstitute derivatives as the isomeric dimethoxyallylphenols **XVIII-XXIII** were more active than the dihydroxymethoxyallylbenzenes **XXIV-XXVI.**

TABLE 1. Activity of phenylpropanoids on the alga *Selenastrum capricornutum* 'T 76'

Compounds	5×10^{-4} M	2.5×10^{-4} M	10^{-4} M	5×10^{-5} M
2-Allylphenol (V)	+++	++	+	-
3-Allylphenol (VI)	++	+	-	-
4-Allylphenol (VII)	+++	++	+	-
2-Allyl-6-hydroxyphenol (VIII)	++	+	-	-
2-Allyl-5-hydroxyphenol (IX)	+++	++	+	-
2-Allyl-4-hydroxyphenol (X)	++	+	-	-
2-Allyl-3-hydroxyphenol (XI)	++	+	-	-
4-Allyl-2-hydroxyphenol (XII)	++	+	-	-
2-Allyl-6-methoxyphenol (XIII)	++++	+++	+	-
2-Allyl-5-methoxyphenol (XIV)	++++	++++	++	+
2-Allyl-4-methoxyphenol (XV)	++++	+++	+	-
2-Allyl-3-methoxyphenol (XVI)	++++	+++	+	-
4-Allyl-2-methoxyphenol (XVII)	++++	++	-	-
6-Allyl-2,3-dimethoxyphenol (XVIII)	++++	++++	+++	++
2-Allyl-4,5-dimethoxyphenol (XIX)	++++	++++	+++	++
2-Allyl-3,5-dimethoxyphenol (XX)	++++	++++	++++	+++
2-Allyl-3,4-dimethoxyphenol (XXI)	++++	++++	+++	++
2-Allyl-4,6-dimethoxyphenol (XXII)	++++	++++	++	+
4-Allyl-2,6-dimethoxyphenol (XXIII)	++++	++++	++	+
6-Allyl-2-hydroxy-3-methoxyphenol (XXIV)	+++	++	+	-
6-Allyl-3-hydroxy-2-methoxyphenol (XXV)	++	++	+	-
6-Allyl-3-hydroxy-4-methoxyphenol (XXVI)	++	++	+	-

% Inhibition of the growth rate of the alga : 0 (-), 1-25 (+), 26–50 (++), 51-75 (+++), 76-100 (++++).

These results show that the activity of phenylpropanoids was greatly influenced by the number of the methoxyl or hydroxyl groups present in the molecule. The presence of a hydroxyl group ensured activity of the molecule and the introduction of one or two methoxyl groups enhanced this activity. These results are comparable to those previously reported for compounds with only methoxyl groups (6). On the other hand, the introduction of two hydroxyl groups reduced the activity, probably owing to an excessive hydrophylic character of the molecules.

To verify if the structure-activity relationships observed in these tests with *S. capricornutum* were confirmed in other algal strains, selected compounds from those examined in this paper and in the previous report (5) were tested against different microalgae. 2-Allylphenol (**V**) and its methylderivative **XXVII**, 2-allyl-4-hydroxyphenol (**X**) and its mono **XV** and dimethylderivative **XXVIII**, 2-allyl-4,5-dimethoxyphenol (**XIX**) and its derivative γ – asarone (**XXIX**) were tested with the paper disk bioassay on nine Cyanochloronta, six Chlorophycophyta, a Rodophycophyta and a Chrysophycophyta (Table 2).

Phenylpropanoids affected mainly the growth of blue-green algae. On the other hand, the blue-green alga *Nostoc commune* `T 584' was insensitive to all the compounds tested, probably due to the thick gelatinous layer enclosing the cell. Besides, *Plectonema boryanum* `T 485' showed a specific sensitivity only to compound **XIX**.

Among the strains belonging to green algae, the most sensitive were *Closterium acerosum* `T 1075' and *S. capricornutum* `T 1648', which confirmed the strong sensitivity to phenylpropanoids observed in broth tests. The trimethoxy substituted phenylpropanoid **XXIX** was significantly active against all the green algae selected. Moreover, the strains *Ankistrodesmus braunii* `C202.7a' and *Scenedesmus quadricauda* `T 76' were particularly sensitive to compound **V**. In addition, two strains, *Porphyridium aerugineum* `T 755' and *Navicula minima* `T 656', respectively, belonging to Rhodophycophyta and to Chrysophycophyta were assayed. The resistance of these two strains to phenylpropanoids was quite different. In fact, *P.aerugineum* is one of the most sensitive strains tested, being severely inhibited by compounds **X**, **XIX** and **XXIX**, whereas *N. minima* showed a marked sensitivity only to compound **XV**.

Among the aquatic plants that we have analyzed in previous studies (2, 5) α and β–asarone are the most active natural occurring phenylpropanoids, however, they have shown a little selectivity as inhibitors of algal growth. This is a problem which frequently limits the use of allelochemicals isolated from higher plants (7). The response of microalgae to compounds mimic of natural phenylpropanoids may be more selective and in some cases strain-specific.

TABLE 2. Inhibition of algal growth by phenylpropanoids

Phylum	Strain	Species	Phenylpropanoids						
			V	XXVII	X	XV	XXVIII	XIX	XXIX
Cyanochloronta	T 485	*Plectonema boryanum*	+	-	+	+	-	+++	-
	T 584	*Nostoc commune*	-	-	-	-	-	-	-
	T 625	*Synechococcus leopoliensis*	+	-	-	+++	-	+++	+++
	T 1444	*Anabaena flos-aquae*	++	-	-	+	+	+++	+++
	T 1547	*Lyngbia kuetzingii*	+	-	+	+	-	+++	+++
	T 1580	*Phormidium autumnale*	-	-	-	+	-	-	+++
	T 1816	*Porphyrosiphon notarisii*	+	-	+	+++	-	++	+++
	T 1824	*Aulosira terrestre*	+	-	-	-	-	+	+++
	T 2349	*Scytonema hofmanni*	+	-	-	-	-	-	+++
Rhodophycophyta	T 755	*Porphyridium aeurugineum*	+	-	+++	++	-	+++	+++
Chrysophycophyta	T 656	*Navicula minima*	-	-	+	+++	-	-	+
Chlorophycophyta	A 489	*Chlorella saccharophila*	+	-	-	-	-	-	++
	C 202.7a	*Ankistrodesmus braunii*	++	-	+	-	-	-	+++
	C 249.1	*Muriella aurantiaca*	-	-	-	+	-	+	++
	T 76	*Scenedesmus quadricauda*	+++	-	-	+	-	+	++
	T 88	*Asterococcus superbus*	-	-	+	+	-	-	+++
	T 1075	*Closterium acerosum*	-	-	++	++	+	++	+++
	T 1648	*Selenastrum capricornutum*	+	-	-	++	++	++	+++

Diameter of the no growth zone (mm) : (-) no inhibition, (+) 7 - 14 mm, (++) 15 - 23 mm, (+++) > 23 mm.
A= Culture Collection of Algae, Charles University, Praha, Czechoslovakia; C = Culture Centre of Algae and Protozoa, Ambleside, U. K.,
T = Culture Collection of Algae, Austin University of Texas, USA.

The possibility of using properly designed molecules of phenylpropanoids seems to be a promising approach in the control of undesired microalgal growth and field experiments are in progress in this regard.

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