The protozoan toxin climacostol and its derivatives: Cytotoxicity studies on 10 species of free-living ciliates

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Abstract: Climacostol (5-(Z)-non-2-enyl-benzene-1,3-diol) is a natural toxin isolated from the freshwater ciliated protozoan *Climacostomum virens* and belongs to resorcinolic lipids, a group of compounds that show antimicrobial, antiparasitic, and anticancer activities. We investigated the cytotoxic activity of the chemically synthesized toxin and its alkyl and alkynyl derivatives on *C. virens* and nine other common species of free-living freshwater ciliates. Our results show that the cytotoxic potency of climacostol can be modulated by the substitution of the double bond present in the aliphatic chain of the toxin with a single or a triple one that was previously obtained during the synthesis of the unsaturated and saturated derivatives of the parent molecule. We demonstrated that the cytotoxicity level of the molecules considered in this study appears to be inversely correlated to the unsaturation level of their aliphatic chains, and that the potency of their action is also related to the target organism.

Key words: Climacostol; *Climacostomum*; resorcinols; resorcinolic lipids; cytotoxicity; extrusomes

Introduction

Climacostomum virens Ehrenberg, 1933 is a flattened heterotrich ciliate, usually green due to the presence of endosymbiotic algae in the cytoplasm, and commonly found in the benthos of lakes, ponds, marshes and canals. Like other heterotrichs, *C. virens* uses a chemical weapon against unicellular and multicellular predators, represented by the colourless toxin climacostol (5-(Z)-non-2-enyl-benzene-1,3-diol) (Masaki et al. 1999, 2004; Miyake et al. 2003). The toxin is stored in cortical granules that are membrane-bound extrusive organelles, usually denoted as “extrusomes”, which can be discharged in response to a variety of environmental stimuli (Hausmann 1978).

Climacostol can be structurally assigned to the large group of natural compounds known as resorcinolic lipids (also called alkylresorcinols, or 5-alkylresorcinols), widely detected in the kingdoms of Plantae, Fungi and Monera (Kozubek & Tyman 1999). To date, several compounds that show antimicrobial, antiparasitic, antitumoral and genotoxic activities have been identified among the alkylresorcinols (Picardo et al. 1987; Lytollis et al. 1995; Singh et al. 1995; Kozubek et al. 2001). In addition, their potential use in the therapy and/or prevention of specific classes of diseases has attracted particular attention (Itokawa et al. 1989; Suresh & Ray 1990; Gasiorowsky et al. 1996; Kozubek et al. 2001; Filip et al. 2002; Barbini et al. 2006). Up to the present time, the cytotoxic activity of climacostol was assayed on a panel of protozoa, flatworms and crus-

tacea, as well as on human cancer and non-cancer cell lines (Miyake et al. 2003; Buonanno 2005; Buonanno et al. 2008). Among protozoa, *Dileptus margaritifer* Ehrenberg, 1833, *Didinium nasutum* Müller, 1786 and *Paramecium caudatum* Ehrenberg, 1838 appeared to be highly sensitive to climacostol, whereas *Blepharisma japonicum* Suzuki, 1954 and *Stentor coerules* Ehrenberg, 1830 seemed to be less sensitive to it. Among invertebrates, a copepod (*Cyclops* sp.) and a cephalocarid (*Daphnia* sp.) seemed to be very sensitive to the toxin (Pucciarelli et al. 2008), whereas the turbellarian flatworm *Stenostomum sphagnetorum* Luther, 1960 appeared immune to it (Buonanno 2005). The cytotoxic activity of climacostol was also recently investigated on human cancer and non-cancer cell lines (Buonanno et al. 2008). The results showed that climacostol effectively inhibited the growth of cancer cell lines in a dose-dependent manner by inducing a mitochondria-mediated apoptosis, with non-cancer cells proving to be significantly more resistant to the toxin. However, very little is known about the mechanism of action of climacostol on free-living organisms (Miyake et al. 2003; Buonanno et al. 2008).

In recent years climacostol was chemically synthesized by two alternative pathways (Masaki et al. 1999, 2004; Mori & Abe 2001). In the approach adopted by Masaki et al. (2004) the alkynyl (5-Non-2-ynyl-benzene-1,3-diol) and alkyl (5-Nonyl-benzene-1,3-diol) derivatives of climacostol were also synthesized, in order to evaluate their biological activity. In the present study we focused on the evaluation of the structure-related

cytotoxicity of climacostol and its derivatives on 10 species of free-living ciliated protozoa.

Material and methods

Ciliates and cultures

Climacostomum virens stock W-24, kindly provided by Dr. Tavrovskaya (Institute of Cytology, Russian Academy of Sciences, St. Petersburg), was cultured in a balanced salt solution called SMB (Miyake 1981) and fed with the flagellate *Chlorogonium elongatum* Dangeard, 1899 grown as described by Buonanno et al. (2005). *Euplotes aediculatus* Pierson, 1943 (Colorado strain), kindly provided by Dr. J. Kloetzel, (University of Baltimore, Maryland, USA), *Stentor coeruleus* stock Mün1 (Miyake et al. 2001) and *Stentor niger* O.F. Muller, 1773 stock Ch-2 (collected in Chieti, Italy), were cultured in SMB and fed with *C. elongatum*. *Dileptus margaritifer*, formerly *D. anser*, stock SHL-BB (Buonanno et al. 2005), was cultured in SMB and fed with a small ciliate, *Sathrophilus* sp., grown in boiled lettuce medium inoculated with *Enterobacter aerogenes* 2 days before use (Miyake & Beyer 1973). *Blepharisma japonicum* Suzuki, 1954 stock R1072 (Harumoto et al. 1998), *Paramecium tetraurelia* Sonneborn, 1975 stock 51 (Miyake et al. 2003), *Spirostomum ambiguum* Ehrenberg, 1835 stock Pol-5 (collected in Policoro, Italy) and *Spirostomum teres* Claparede et Lachmann, 1858 stock Pol-1 (Buonanno 2005), were cultured in bacterized culture medium. *Didinium nasutum* stock 7771 (derived from the strain 30399 purchased from the American Type Culture Collection) was cultured in SMB and fed with *P. tetraurelia*. All ciliates were cultured and handled at room temperature ($23 \pm 1^\circ\text{C}$) and used for testing their response to the toxin one day after they had consumed food.

Climacostol and its derivatives

Chemically-synthesized climacostol (5-(Z)-non-2-enyl-benzene-1,3-diol) and its two derivatives (5-nonylbenzene-1,3-diol and 5-non-2-ynyl-benzene-1,3-diol) were kindly provided by Dr. Iio (see "Acknowledgements"). The compounds were dissolved in ethanol (1 mM) and stored in the dark at -20°C . The solutions were diluted with SMB at the time of the experiment.

Toxicity test

Triplicate samples of ten ciliate cells each were placed in depression slides containing 250 μl SMB and increasing concentrations of each toxin, and kept in a dark moist chamber. The number of surviving cells was counted, after 1 h or after 24 h, unless otherwise specified. The LC_{50} concentrations were estimated based on a concentration-survival curve, essentially according to the procedure described by Buonanno (2009).

Data analysis

A concentration-survival curve describes the relationship between the concentrations of a toxin and the response to toxin treatment in order to evaluate the reduction in viability. In this work, the values of LC_{50} , defined as the concentration of a toxin that caused a 50% reduction in viability compared to an identical culture without the toxin after 1 h or 24 h of incubation, were computed by nonlinear regression analysis using GraphPad Prism 4 software (GraphPad Software, San Diego, CA). The differences between the fitted midpoints ($\log_{10} \text{LC}_{50}$) were assessed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test, and toxicity values were

Fig. 1. Chemical structures of climacostol (5-(Z)-non-2-enyl-benzene-1,3-diol) (A) and its alkyl (5-nonylbenzene-1,3-diol) (B) and alkynyl (5-non-2-ynyl-benzene-1,3-diol) (C) derivatives.

reported as $1/\text{LC}_{50}$. Data are presented here as the mean $\pm$ standard error (SE) of 3 independent determinations, with the minimum level of significance set at $P < 0.05$.

Results

A set of dose-response experiments was performed to evaluate the effects of climacostol and its two derivatives (Fig. 1) on the viability (normal morphology and locomotion) of *C. virens* and nine other species of free-living ciliates. Cells were incubated in the presence of each compound at concentrations ranging from 0.25 to 60 μM , and the number of viable cells was determined by light microscopy. Values of LC_{50} were then computed and used to compare the cytotoxicity of the three alkyl-resorcinols as described in the "Materials and Methods" section.

The data indicated that the three compounds studied exerted an appreciable cytotoxic effect on all the species examined in a concentration- and exposure time-dependent manner (Fig. 2). Climacostol and its derivatives showed medium to high levels of toxicity against *S. teres*, *S. ambiguum*, *E. aediculatus*, *D. nasutum*, *P. tetraurelia* and *D. margaritifer*, and moderate levels of toxicity against *S. coeruleus*, *S. niger*, *B. japonicum* and *C. virens*, at both 1 h and 24 h of incubation.

Also, the cytotoxic effect of the three compounds appeared strictly related to their molecular structures as well. In fact, the substitution of the double bond in the side chain of climacostol with a triple bond to obtain the alkynyl derivative conferred to this compound the lowest cytotoxic potency ($0.05 \mu\text{M}^{-1} < 1\text{h-1}/\text{LC}_{50} < 0.19 \mu\text{M}^{-1}$; $0.06 \mu\text{M}^{-1} < 24\text{h-1}/\text{LC}_{50} < 0.52 \mu\text{M}^{-1}$), with the exception of *C. virens*, against which the lowest cytotoxicity was exerted by climacostol ($1\text{h-1}/\text{LC}_{50} =$

Fig. 2. Cytotoxic effect of climacostol (A) and its alkyl (B) and alkylnyl (C) derivatives on ten ciliate species. Cell viability was assessed after 1 h (panel A) and after 24 h (panel B) as described in Materials and Methods section. Each bar represents the mean ($\pm$ SE) of 3 independent experiments. * $P < 0.05$ and ** $P < 0.001$.

$0.04 \mu\text{M}^{-1}$; $24\text{h-1/LC}_{50} = 0.1 \mu\text{M}^{-1}$). On the other hand, the alkyl derivative without the double bond in its side chain, exhibited the highest cytotoxicity against all the species ($0.06 \mu\text{M}^{-1} < 1\text{h-1/LC}_{50} < 0.52 \mu\text{M}^{-1}$; $0.18 \mu\text{M}^{-1} < 24\text{h-1/LC}_{50} < 1.81 \mu\text{M}^{-1}$), showing 1.5- to 2.7-fold a more cytotoxic activity than climacostol. Climacostol, which maintains a double bond in its side chain, showed a cytotoxic potency against nine species between those of the alkyl and alkylnyl derivatives ($0.04 \mu\text{M}^{-1} < 1\text{h-1/LC}_{50} < 0.28 \mu\text{M}^{-1}$; $0.1 \mu\text{M}^{-1} < 24\text{h-1/LC}_{50} < 0.67 \mu\text{M}^{-1}$).

To better explore the unique situation of *C. virens*, which constitutively produces climacostol, we further investigated the early effects of this toxin and its derivatives on this species, by measuring the LC₅₀ values of each compound within 30 minutes. As reported in Fig. 3, climacostol demonstrated the lowest cytotoxic potency against *C. virens*, showing values of LC₅₀ that

are from 2.1- to 24-fold higher than those of its derivatives.

Discussion

Alkylresorcinols have a dual, hydrophilic and hydrophobic character, due to the presence of a hydroxylated aromatic ring and a straight hydrocarbon chain. Apart from simple 5-alkylresorcinols, the occurrence of various derivatives (ring or chain modified) has also been demonstrated in plants and bacteria. The side chain of these compounds can be saturated or it carries one to four double bonds in a *cis* configuration, with the localization of double bonds usually related to the chain length (Kozubek & Tyman 1999). The same authors observed, in addition, that in the case of C₁₅ homologues the most frequent position of double bonds is at the C₈, C₁₁, and C₁₄ carbon atoms, whereas

Fig. 3. Cytotoxic effect of climacostol (A) and its alkyl (B) and alkynyl (C) derivatives on *C. virens* after 10, 15 and 30 minutes. Each bar represents the mean ($\pm$ SE) of 3 independent experiments. * $P < 0.05$ and ** $P < 0.001$.

in homologues with longer side chains, double bonds were detected at other carbon atoms (Suzuki et al. 1997). Interestingly, the double bond that climacostol (a C₁₅ 5-alkylresorcinol) carries at the C₈ position is also conserved in the two less represented C₁₇ homologues extracted from *C. virens*, the 5-(Z,Z)-undeca-2,5-dienyl-benzene-1,3-diol and the 5-(Z,Z,Z)-undeca-2,5,8-trienyl-benzene-1,3-diol, which unexpectedly presents one or two additional double bonds in C₁₁ and C₁₄ positions, respectively (Masaki et al. 2004). To date, nothing is known about the biological activity of these C₁₇ homologues. On the contrary, due to the possibility of chemically synthesizing climacostol, some investigations were performed on the ecological role of this toxin (Miyake et al. 2003; Buonanno 2005), whereas only one study on its potential use as anticancer agent is available (Buonanno et al. 2008). In addition, despite the availability of synthetic climacostol, the spectrum of the biological activity of the toxin has not yet been fully investigated, especially with regard to its mechanism of action.

In this study we demonstrate that the cytotoxic activity of climacostol against a panel of ten species of ciliates can be modulated by the substitution of the double bond present in the aliphatic chain of the toxin with a single or a triple one. This was easily achieved through the synthesis of the alkyl and alkynyl derivatives of the parent molecule (Masaki et al. 1999, 2004; Mori & Abe 2001).

The analysis of the data clearly demonstrated an appreciable concentration- and time-dependent cytotoxic effect of the three compounds against all the examined species.

In addition, the cytotoxic potential of the three compounds appeared strictly related to their molecular structures, with particular regard to the presence of a single, double or triple bond at the C₈ position of the side chain. It emerged that the alkyl derivative, which carries a single bond in the side chain, showed the highest cytotoxicity against all the species studied. The alkynyl derivative, which carries a triple bond in the side chain, showed the lowest cytotoxic potency against nine species, but an intermediate cytotoxicity against *C. virens*. Climacostol, which maintains a double bond at the C₈ position, showed an intermediate cytotoxicity level against nine species, but the lowest cytotoxicity against *C. virens*. Summarizing, it appears that the cytotoxic potential of climacostol and its two derivatives is such that the more saturated the side chain is, the more toxic the molecule is.

The effect of the length and degree of unsaturated side chains of a number of 5-alkylresorcinols extracted from different organisms has been extensively investigated by numerous researchers. For example, in studies concerning the antibacterial activity of short-chain (<C₁₃) 5-alkylresorcinols with different degrees of the aliphatic chain unsaturation isolated from *Anacardium occidentale* (cashew tree) (Himejima & Kubo 1991), it was observed that the homologues with unsaturated chains resulted in the most active compounds, showing a minimal inhibition concentration (MIC) spanning from 0.78 to 1.56 $\mu\text{g ml}^{-1}$, whereas the saturated homologue was the least active (MIC = 50 $\mu\text{g ml}^{-1}$). On the other hand, according to the results reported here, when long-chain (>C₁₃) 5-alkylresorcinol homologues isolated from *A. occidentale* were used against *Biomphalaria glabrata* Say, 1818, the intermediate host for the human blood parasite *Schistosoma mansoni* Sambon, 1907 an inverse correlation between the antiparasitic activity of the resorcinolic lipids and the unsaturation degree of their side chains was revealed (Suresh & Ray 1990). Furthermore, it has been reported that the introduction of double bonds in the side chain of 5-alkylresorcinols extracted from the medicinal plant *Lysimachia japonica* can increase their cytotoxic action against P-338 leukemia cells by four times (Kozubek & Tyman 1999).

The above-listed observations and the data presented in this work, confirm the close relationship between the structure of the side chain of 5-alkylresorcinols and their biological activity, the level of which can be either increased or decreased in relation to the specific biological system considered. In fact, the cytotoxic potency of climacostol and its derivatives is not equally distributed among the different organisms subjected to the action of the toxins, with activity levels that appear to be species-specific, and span from moderate (0.09–0.26 μM^{-1}) to high (0.31–1.81 μM^{-1}) toxicity values, at both 1 h and 24 h of incubation.

It is also evident that the first four ciliated species (*C. virens*, *B. japonicum*, *S. niger*, *S. coeruleus*), among those reported in Fig. 2, are appreciably less affected by the toxins than the others (*D. margaritifera*, *P. tetraurelia*, *D. nasutum*, *E. aediculatus*, *S. ambiguum*, *S. teres*). A possible explanation for the low cytotoxicity that climacostol and its derivatives exert against the first group of species can be related to the similar biology that these heterotrichs share, with particular regard to the chemical weapons adopted against predators. Similar to *C. virens*, also *B. japonicum*, *S. niger*, and *S. coeruleus* are in fact equipped with extrusomes containing the toxins blepharismine (from *Blepharisma*) and stentorin (from *Stentor*), that can be used in response to a variety of environmental stimuli. Despite the differences in chemical structures of climacostol, blepharismine and stentorin, a close relation between the hypothesized biosynthesis of the toxins of *Climacostomum* and those of *Blepharisma* and *Stentor* (Masaki et al. 1999, 2004) was observed. According to the biosynthesis suggested for long-chain 5-alkylresorcinols (Kozubek & Tyman 1999), some authors have recently proposed that climacostol and its C₁₇ homologues, as well as stentorin and blepharismine, would be synthesized also from a common polyketide pathway (Miyake et al. 2003; Masaki et al. 2004; Terazima & Harumoto 2004; Buonanno 2005; Mukherjee et al. 2006; Pucciarelli et al. 2008). We can assume, as a working hypothesis, that the adoption of a common biosynthetic pathway for the assembly of defense toxins has been accompanied, in the four species of heterotrichs, by the evolution of similar self-protection systems, which confer the ciliates a good level of resistance against the content of extrusomes of taxonomically near organisms. Therefore, in addition to the expected "immunity" showed by *C. virens* against climacostol, the evidence that this species also exert a strong resistance against the alkyl and alkenyl derivatives of the parent molecule is not surprising.

Taken as a whole, the experimental data reported in the current study indicate, on one hand, that the unsaturation rate of the aliphatic chain of climacostol represents a key element for the effectiveness of its biological activity, and, on the other hand, that the potency of its action is also related to the molecule's efficacy in harming the target organisms.

Interestingly, it was recently reported that climacostol can exert a specific cytotoxic effect on HL60 and A431 human cancer cells by triggering a mitochondrion-dependent apoptotic program (Buonanno et al. 2008). In our opinion, the availability of synthetic climacostol and its derivatives could therefore be very useful not only to fully understand their mechanism of action on single-celled and on multicellular organisms, but more specifically to evaluate their potential use in cancer chemotherapy.

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References

- Barbini L., Lopez P., Ruffa J., Martino V., Ferraro G., Campos R. & Cavallaro L. 2006. Induction of apoptosis on human hepatocarcinoma cell lines by an alkyl resorcinol isolated from *Lithraea molleoides*. *World J. Gastroenterol.* **7**: 5959–5963.
- Buonanno F. 2005. Variations in the efficiency of ciliate extrusomal toxins against a common ciliate predator, the catenulid *Stenostomum sphagnetorum*. *Ital. J. Zool.* **72**: 293–295.
- Buonanno F. 2009. Antipredator behavior of freshwater microturbellarian *Stenostomum sphagnetorum* against the predatory ciliate *Dileptus margaritifer*. *Zool. Sci.* **26**: 443–447.
- Buonanno F., Quassinti L., Bramucci M., Amantini C., Lucciarini R., Santoni G., Iio H. & Ortenzi C. 2008. The protozoan toxin climacostol inhibits growth and induces apoptosis of human tumor cell lines. *Chem.-Biol. Interact.* **176**: 151–164. DOI 10.1016/j.cbi.2008.07.007
- Buonanno F., Saltalamacchia P. & Miyake A. 2005. Defence function of pigmentocysts in the karyorelictid ciliate *Loxodes striatus*. *Eur. J. Protistol.* **41**: 151–158. DOI 10.1016/j.ejop.2005.01.001
- Deszcz L. & Kozubek A. 2000. Higher cardol homologs (5-alkylresorcinols) in rye seedlings. *Biochim. Biophys. Acta* **1483**: 241–250.
- Filip P., Anke T. & Sterner O. 2002. 5-(2'-oxoheptadecyl)-resorcinol and 5-(2'-oxononadecyl)-resorcinol, cytotoxic metabolites from a wood-inhabiting basidiomycete. *Z. Naturforsch.* **57**: 1004–1008.
- Gasiorowski K., Szyba K., Brokos B. & Kozubek A. 1996. Antimutagenic activity of alkylresorcinols from cereal grains. *Cancer Letters* **106**: 109–115. DOI 10.1016/0304-3835(96)04294-2
- Harumoto T., Miyake A., Ishikawa N., Sugibayashi R., Zenfuku K. & Iio H. 1998. Chemical defense by means of pigmented extrusomes in the ciliate *Blepharisma japonicum*. *Eur. J. Protistol.* **34**: 458–470.
- Hausmann K. 1978. Extrusive organelles in protists. *Int. Rev. Cytol.* **52**: 197–276.
- Himejima M. & Kubo J. 1991. Antibacterial agents from the cashew *Anacardium occidentale* (Anacardiaceae) nut shell oil. *J. Agric. Food Chem.* **39** (2): 418–421. DOI 10.1021/jf00002a039
- Itokawa H., Totsuka N., Nakamura K., Maezuru M., Takeya K., Kondo M., Inamatsu M. & Morita H. 1989. A quantitative structure-activity relationship for antitumor activity of long-chain phenols from *Ginkgo biloba* L. *Chem. Pharm. Bull.* **37**: 1619–1621.
- Kozubek A. & Tyman J.H.P. 1999. Resorcinolic lipids, the natural non-isoprenoid phenolic amphiphiles and their biological activity. *Chem. Rev.* **99**: 1–25.
- Kozubek A., Zarnowski R., Stasiuk M. & Gubernator J. 2001. Natural amphiphilic phenols as bioactive compounds. *Cell. Mol. Biol. Lett.* **6**: 351–355.
- Lytollis W., Scannel R.T., An H., Murty V.S., Reddy K.S., Barr J.R. & Hecht S.M. 1995. 5-Alkylresorcinols from *Hakea trifurcata* that cleave DNA. *J. Am. Soc.* **117**: 12683–12690. DOI 10.1021/ja00156a004
- Masaki M.E., Harumoto T., Terazima M.N., Miyake A., Usuki Y. & Iio H. 1999. Climacostol, a defense toxin of the heterotrich ciliate *Climacostomum virens* against predators. *Tetrahedron Lett.* **40**: 8227–8229. DOI 10.1016/S0040-4039(99)01722-0
- Masaki M.E., Hiro S., Usuki Y., Harumoto T., Terazima M.N., Buonanno F., Miyake A. & Iio H. 2004. Climacostol, a

- defense toxin of *Climacostomum virens* (Protozoa, Ciliata), and its congeners. *Tetrahedron* **60**: 7041–7048. DOI 10.1016/j.tet.2003.09.105
- Miyake A. 1981. Cell interaction by gamones in *Blepharisma*, pp. 95–129. In: O'Day D.H. & Horgen P.A. (eds), *Sexual Interaction in Eukaryotic Microbes*, Academic Press, New York.
- Miyake A. & Beyer J. 1973. Cell interaction by means of soluble factors (gamones) in conjugation of *Blepharisma intermedium*. *Exp. Cell Res.* **76**: 15–24. DOI 10.1016/0014-4827(73)90413-8
- Miyake A., Buonanno F., Saltalamacchia P., Masaki M.E. & Iio H. 2003. Chemical defence by means of extrusive cortical granules in the heterotrich ciliate *Climacostomum virens*. *Eur. J. Protistol.* **39**: 25–36. DOI 10.1078/0932-4739-00900
- Miyake A., Harumoto T. & Iio H. 2001. Defense function of pigment granules in *Stentor coeruleus*. *Eur. J. Protistol.* **37**: 77–88. DOI 10.1078/0932-4739-00809
- Mori K. & Abe Y. 2001. Simple synthesis of climacostol, a defensive secretion by the ciliate *Climacostomum virens*. *Biosci. Biotechnol. Biochem.* **65**: 2110–2112.
- Mukherjee P., Fulton D.B., Halder M., Han X., Armstrong D.W., Petrich J.W. & Lobban C.S. 2006. Maristentorin, a novel pigment from the positively phototactic marine ciliate *Maristentor dinoferus*, is structurally related to hypericin and stentorin. *J. Phys. Chem.* **110**: 6359–6364.
- Picardo M., Passi S., Nazzaro-Porro M., Breathnach A., Zompetta C., Faggioni A. & P. Riley. 1987. Mechanism of antitumoral activity of catechols in culture. *Biochem. Pharmacol.* **36** (4): 417–425. DOI 10.1016/0006-2952(87)90345-5
- Pucciarelli S., Buonanno F., Pellegrini G., Ballarini P. & Miceli C. 2008. Biomonitoring of Lake Garda: Identification of ciliate species and symbiotic algae responsible for the “black-spot” bloom during the summer of 2004. *Environ. Res.* **107** (2): 194–200. DOI 10.1016/j.envres.2008.02.001
- Singh U.S., Scannel R.T., An H., Carter B.J. & Hecht S.M. 1995. DNA cleavage by di- and trihydroxyalkylbenzenes. Characterization of products and the roles of O₂, Cu(II), and alkali. *J. Am. Soc.* **117**: 12691–12699. DOI 10.1021/ja00156a005
- Suresh M. & Ray R.K. 1990. Cardol: the antifilarial principle from *Anacardium occidentale*. *Curr. Sci.* **59**: 477–479.
- Suzuki, Y., Esumi Y., Uramoto M., Kono Y. & Sakurai A. 1997. Structural analyses of carbon chains in 5-alk(en)ylresorcinols of rye and wheat whole flour by tandem mass spectrometry. *Biosci. Biotech. Biochem.* **61**: 480–486.
- Terazima M.N. & Harumoto T. 2004. Defense function of pigment granules in the ciliate *Blepharisma japonicum* against two predatory protists, *Amoeba proteus* (Rhizopodea) and *Climacostomum virens* (Ciliata). *Zool. Sci.* **21**: 823–828.

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