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RELEASE AND ECOLOGICAL IMPACT OF ALGICIDAL HYDROLYSABLE POLYPHENOLS IN *MYRIOPHYLLUM SPICATUM*

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Key Word Index—*Myriophyllum spicatum*; Haloragaceae; Eurasian watermilfoil; submerged macrophyte; plant defences; algicidal activity; allelopathy; hydrolysable polyphenol; tellimagrandin II; eugeniin.

Abstract—Aqueous acetone extracts of shoots of Eurasian watermilfoil (*Myriophyllum spicatum*) exhibit a strong inhibitory action against various coccoid and filamentous cyanobacteria and to a slightly less extent against chlorophytes and diatoms. Bioassay-directed fractionation led to the isolation of a hydrolysable polyphenol, tellimagrandin II, which turned out to be the main inhibitory substance. *Myriophyllum spicatum* contains large amounts of this compound (1.5% of dry wt). Part of the inhibitory activity is due to complexation and inactivation of algal extracellular enzymes (e.g. alkaline phosphatase) by hydrolysable polyphenols from *M. spicatum*.

INTRODUCTION

The production and excretion of allelochemicals provides aquatic macrophytes with an effective defence strategy against other photosynthetic organisms competing for light, e.g. other macrophytes, algae and cyanobacteria [1, 2]. Light is one of the most limiting factors in the interaction between submerged macrophytes and epiphyton or phytoplankton. The alternating stable states hypothesis applied to shallow eutrophic lakes [3] predicts that there are only two stable equilibria, one dominated by submerged macrophytes and the other by phytoplankton. Shifts between these equilibria occur rapidly and without intermediate states. Submerged macrophytes can stabilize their dominance through several abiotic and biotic interactions [3, 4] and by releasing algicidal compounds. Until now, only a few allelochemicals from aquatic plants could be structurally elucidated. To learn more about the chemical interactions between aquatic macrophytes and competing algae and other plants, it is necessary to combine natural product analysis with appropriate studies on the biological and ecological impact of the active compounds.

Eurasian watermilfoil (*Myriophyllum spicatum* L., shortnamed milfoil here) is a submerged species exhibiting potent growth and dispersal strategies which enable the plant rapidly to dominate different aquatic systems [5, 6]. Milfoil propagated fast in the eastern United States and Canada after its introduction from Europe during the end of the last century [7]. It may displace the native vegetation and milfoil-dominated lakes usually have low phytoplankton densities. Similar observations have been made in shallow lakes in western Germany (G. Friedrich, pers. commun.). Several morphological and physiological strategies, such as canopy building [8], nutrient removal from open water [9], heavy vegetative spread by autofragmentation and low light- and CO_2-compensation points [5] are proposed for its highly competitive strength. Allelopathically active phenolic compounds from this macrophyte were thought to be involved in the interaction with cyanobacteria and chlorophytes [10], but until now no active compound had been identified.

The production of anticyanobacterial polyphenols was reported from two other species of the genus *Myriophyllum*, namely, *M. brasiliense* [11] and *M. verticillatum* [12]. Considering these results and present data, we propose that hydrolysable polyphenols should not only be regarded as effective herbivore deterrents [13–15], but also as powerful algistatic or algicidal compounds from submerged macrophytes, especially of the Haloragaceae and the genus *Myriophyllum*.

Macrophytes producing allelochemicals, e.g. *M. spicatum*, could be used for biomanipulation of shallow eutrophic lakes that exhibit severe cyanobacterial or algal blooms. Low phytoplankton densities in milfoil-dominated lakes indicate that active compounds are released and can effect the target organisms.

Therefore, we focused not only on the isolation of the main inhibitory compounds, but also on the release by

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exudation or excretion of polyphenols by *M. spicatum* and their mode of action on cyanobacteria and algae. Furthermore, we studied the specificity of the algicidal compounds towards certain groups of cyanobacteria, chlorophytes and diatoms. A modified method for the isolation of active compounds by semi-preparative HPLC, yielding higher amounts than conventional methods, is described.

One of the most prominent actions of polyphenols is their complexation with proteins [13]. Many algae produce exoenzymes to make additional nutrients available (e.g. phosphatases, glucosidases, peptidases). Macrophyte derived polyphenols should be able to inhibit the action of such extracellular or outer-membrane bound enzymes. A new bioassay system for measuring the alkaline phosphatase (APA) activity of target organisms influenced by milfoil polyphenols was established. These investigations provide new evidence on the relationship between structure and function of single hydrolysable polyphenols [16, 17].

RESULTS AND DISCUSSION

Algicidal hydrolysable polyphenols in M. spicatum

Extracts of fresh or lyophilized shoots of Eurasian milfoil with 50% aqueous acetone exhibited a high algicidal activity towards several cyanobacteria, chlorophytes and diatoms. The main inhibitory compounds were purified by bioassay directed fractionation. As a standard bioassay the agar-diffusion assay (ADA) with *Trichormus* var. P-9 [18] was used. Aliquots of the crude extract equivalent to 0.5 mg dry wt caused a clearing area of *ca* 5 mm. Sequential partitioning of the concentrated extract was performed with *tert*-butylmethylether (*t*BME), ethyl acetate and *n*-butanol. The highest algicidal activity was found in the ethyl acetate fraction. No inhibition was observed after treatment of the extract or fractions with polyvinyl pyrrolidone (PVP) [19], which supports the assumption that phenolic compounds were responsible for the algicidal effect. The strength of inhibition in the ADA with *Trichormus* var. P-9 correlated well with the phenolic content of the different fractions tested (Fig. 1, $r^2 = 76\%$, $P < 0.001$).

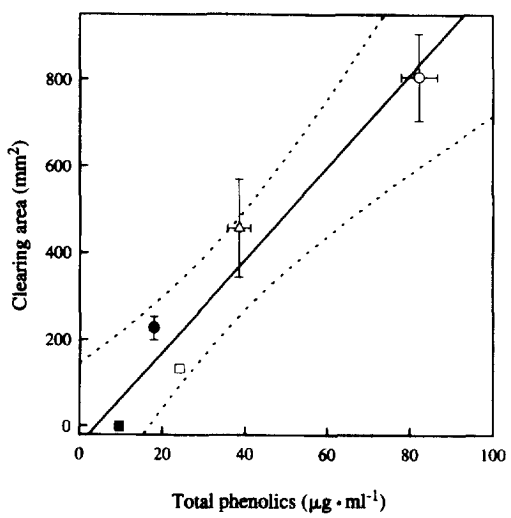
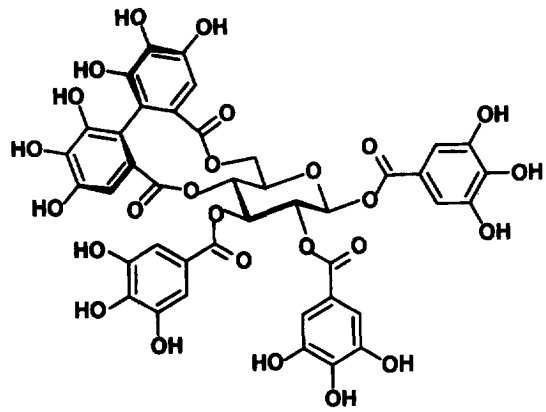


Fig. 1. Correlation between total phenolics and inhibition of *Trichormus* var. P-9 in the ADA of crude extracts and fractions from *Myriophyllum spicatum*. Data are means of triplicates ± s.d., $r^2 = 76\%$, $P < 0.001$, dashed line: 95% C.I. In some cases error bars are smaller than symbol size. ○ Crude 50% aq. Me_2CO extract; ● *t*BME fraction; △, EtOAc fraction; □, *n*-BuOH fraction; ■, H_2O fraction.

Chromatography of the ethyl acetate fraction by reversed-phase HPLC on Kromasil-C18 led to the isolation of several polyphenols. Each active fraction was rechromatographed for purification. The main inhibitory hydrolysable polyphenol (**1**) in milfoil was isolated and its structure determined by FAB mass spectrometry and NMR. Gallic and ellagic acid as well as five to seven other, as yet unidentified, polyphenols are also present in crude extracts. These compounds caused only little inhibition in the bioassay.



1

FAB-mass spectrometry (MS) indicated a M_r of 938. In the negative FAB mode, **1** exhibited a peak at *m/z* 937, corresponding to the $[M - H]^-$ ion. This was confirmed by MS/MS, where *m/z* 937 yielded daughter ions at *m/z* 919, 767 and 599, matching the loss of water, of gallic acid and of hexahydroxydiphenic acid. In the FAB^+ mode the protonated molecular ion at *m/z* 939 was detected in low intensity. Addition of 0.1% sodium acetate in methanol shifted the signal to *m/z* 961 $[(M + Na)^+]$ and increased its intensity. Daughter ions at *m/z* 791 $[(M + Na - C_7H_6O_5)^+]$ and 622 $[(M + Na - H - C_{14}H_{10}O_{10})^+]$ proved the identity of the sodium adduct. All these data corroborate a M_r of 938 for **1**.

The ^1H NMR showed three singlets for the aromatic protons of gallic acid at δ7.12, 7.00 and 6.97 and two singlets of the HHDP group at δ6.66 and 6.46. The sequence of the seven protons of the glucose moiety was determined by ^1H–^1H COSY. The data are in agreement with a fully acylated glucose. The ^{13}C NMR spectrum showed at δ167.99 and 167.57 the CO groups of the

HHDP moiety and at δ166.20, 165.43 and 164.92 three signals for the CO carbons of the galloyl ester groups. A comparison with the literature data [20–22] revealed that the HHDP moiety is coupled with OH-4 and OH-6 of glucose. Thus, all the data obtained for **1** correspond to those of tellimagrandin II (1,2,3-tri-*O*-galloyl-4,6-(*S*)-hexahydroxydiphenoyl-β-D-glucose).

Minimum amounts of **1** needed for a distinct clearing area (> 5 mm) in the ADA were 5 μg (5.3 nmol) with the cyanobacteria *Anabaena* sp. PCC 7120, *Synechococcus* sp. PCC 6911, *Synechocystis* sp. CB-3 and *Trichormus* var. P-9, and the coccoid chlorophyte *Nannochloris* sp. SAG 55-81. The chlorophytes *Scenedesmus falcatus* SAG 2.81 and *Stigeoclonium tenue* SAG 477-2 were less sensitive in this bioassay by one order of magnitude. This slight difference in sensitivity may not be particularly significant. With fischerellin, an allelochemical from the freshwater benthic cyanobacterium *Fischerella muscicola* [18], much greater differences in sensitivity (100 to 1000-fold) were recorded between cyanobacteria and chlorophytes. The minimum amount of gallic and ellagic acid to obtain a comparable inhibition in the ADA were 30 and 50 μg (160 and 166 nmol), respectively, with *Trichormus* var. P-9 and *Anabaena* sp. PCC 7120. Thus, a 10-fold increase in the amount of the simple phenolics was needed to obtain clearing zones comparable to those obtained using **1**.

Tellimagrandin II (**1**, syn. eugeniin, [13]) has also been reported to be the main inhibitory compound in *Myriophyllum brasiliense* [11]. Its isolation from *M. brasiliense* yielded 0.008% of dry wt [11]. The total content of **1** in *M. spicatum* was assessed as 1.5%, and the total phenolic content of the crude extract was *ca* 10%. Preliminary separations with Sephadex LH-20, conventionally used for separation of hydrolysable polyphenols resulted in lower extraction yields of **1**. Sephadex LH-20 is known for poor recoveries of some polyphenols [23]; it had been used for the isolation of eugeniin from *M. brasiliense* [11]. Further, we suggest that the growth form of both milfoil species may influence the plant content of such a potent algicide. *M. brasiliense* usually grows emergent or in near-shore, shallow areas (0.1–1.0 m), where as *M. spicatum* mostly grows in deeper zones (1–5 m). Therefore, *M. spicatum* has to compete more effectively with other shading organisms, which may result in a higher production of algicidal compounds.

For *M. verticillatum* phenylpropanoid-galloyl esters have been described to be the major cyanotoxins [12]. Our investigations of other native milfoil species from northern Germany revealed that both *M. alterniflorum* and *M. verticillatum* do not contain **1**, but several other algicidal hydrolysable polyphenols. In the ADA, raw aqueous methanol or aqueous acetone extracts of shoots of both milfoil species exhibited the same degree of inhibition against *Trichormus* var P-9 as *M. spicatum*. Similar to *M. spicatum* (see Fig. 1), *M. alterniflorum* and *M. verticillatum* showed a good correlation between the phenolic content of the different fractions after sequential partitioning of the crude extract and the clearing area of these fractions in the ADA against *Trichormus* var. P-9 (*M.a.* $r^2 = 87\%$, $P < 0.001$., *M.v.* $r^2 = 63\%$, $P < 0.001$). Similar rates of inhibition were observed with *Proserpinaca palustris*, another member of the Haloragaceae.

Enzymatic hydrolysis of the crude extract with tannase yielded gallic and ellagic acid for *M. alterniflorum*. For crude extracts of *M. verticillatum* and *P. palustris* mainly gallic acid and only traces of ellagic acid could be found after enzymatic hydrolysis. The nature of the main inhibitory polyphenols in *M. alterniflorum* and *P. palustris* is currently being investigated. According to Mole [24], all Haloragaceae species contain polyphenols. These data strongly support the hypothesis that algicidal hydrolysable polyphenols are a common feature of the genus *Myriophyllum* and perhaps even of the entire Haloragaceae.

Release of polyphenols by M. spicatum

The evaluation of the ecological impact of the algicidal hydrolysable polyphenols requires the study of the release of these compounds by intact plants. A suitable test system was developed in order to optimize the performance of experiments under fully controlled conditions. Axenic cultures were prepared from field material by surface sterilization and subsequent sterile cultivation (modified after ref. [25]). They are a necessary prerequisite to avoid interference from epiphytes, i.e. the detection of compounds exuded by attached bacteria or algae, or the modification of macrophyte derived compounds.

Preliminary experiments were conducted to compare the content of polyphenols in extracts of axenic and non-axenic shoots. No qualitative or quantitative differences could be found in comparison of aqueous acetone extracts of both samples (Table 1). Total phenolic content, amount of **1** and the number of UV-detectable (280/254 nm) compounds were very similar in crude extracts of both axenic and non-axenic shoots. This indicates that these polyphenols are present constitutively in milfoil and that their production is not induced or increased due to the presence of other organisms, e.g. epiphytes.

The incubation medium was subjected to solid phase extraction (SPE) and subsequent reversed-phase HPLC

Table 1. Influence of culture type (axenic or non-axenic) on total phenolic content (TP), content of **1** and number of phenolic compounds present in crude aq. 50% acetone extracts of *Myriophyllum spicatum*. Data are given as means ± s.d. Two different plant samples (I and II) were used with each triplicate extraction

	Axenic	Non-axenic
TP (μg mg^{-1} dry wt)	77.3 ± 1.5 (I) 89.4 ± 1.3 (II)	81.5 ± 1.7 (I) 101.5 ± 1.2 (II)
1 (μg mg^{-1} dry wt)	9.85 ± 0.36 (I) 9.50 ± 0.53 (II)	11.38 ± 0.88 (I) 13.03 ± 0.23 (II)
No. of main compounds	14 (I), 15 (II)	15 (I), 15 (II)

analysis. A qualitative analysis of the released compounds revealed at least five to ten UV-absorbing (280/254 nm) peaks when compared with the control (culture medium before incubation). Most of these peaks disappeared after treatment of the sample with PVP. Ultrafiltration showed that all of the compounds have M_rs below 1000, and many even M_rs below 500. Enzymic hydrolysis of samples of SPE-enriched incubation medium yielded mainly gallic and ellagic acid, thus providing evidence that at least part of the released and detectable compounds are hydrolysable polyphenols. Among the identified compounds of the SPE-enriched incubation medium are **1** and ellagic acid. Three of the other compounds are probably HHDP esters based on the results from enzymatic hydrolysis and analysis of the UV scans (see ref. [26]).

Total phenolics in the incubation medium increased with time [27]. Within 10 days, for instance, 2–4 $\mu g\,mg^{-1}$ dry wt of phenolics, measured as tannic acid equivalents with Folin–Ciocalteau reagent, were released. Further studies are in progress to elucidate the structures of the HHDP esters and other polyphenols in the incubation medium. Analysis of the incubation water of non-axenic shoots indicated that the original pattern of released compounds changed with increasing incubation time. This may be due to metabolism of the compounds by accompanying bacteria.

This study showed that milfoil is able to release part of its hydrolysable polyphenols into the surrounding medium. Milfoil possesses secretory trichomes [28, 29]. It has been known since the beginning of this century that these secretory trichomes contain tannin-like compounds [30, 31]. Excretion from these trichomes seems to be one way to release algicidal polyphenols, which may interact with target organisms, especially epiphytes.

Inhibition of algal exoenzymes by milfoil polyphenols

One prominent attribute of polyphenols is their complexation with proteins [13]. Such interactions might be part of the algicidal activity observed with the milfoil polyphenols. Many epiphytes and phytoplanktonic organisms produce extracellular enzymes to make additional nutrients available, e.g. alkaline phosphatase (APA, E.C. 3.1.3.1), which hydrolyses organic phosphorus compounds under inorganic phosphate limitation. A new bioassay was established to measure APA activity of whole cyanobacterial cells under the influence of milfoil polyphenols. Control APA activity with *Trichormus* var. P-9 ranged from 300 to 500 pkat.

First, the influence of **1** on APA activity of *Trichormus* var. P-9 was investigated. The dose–response curve (Fig. 2) shows an exponential relationship between the amount of **1** and the APA inhibition. As little as 0.2 μM of **1** leads to a 10% decrease in enzyme activity. This indicates that even low concentrations, as can be found in the incubation medium or in lake water, are effective in inhibiting exoenzymes from target organisms like APA.

Similar to the observations made in the ADA, **1** was a stronger inhibitor of APA than simple phenolics in comparable concentrations (Fig. 3). An 18 μM concentration of **1** led to an inhibition of APA activity of *Trichormus* var. P-9 of 83%; however, 90 μM gallic acid, providing the same amount of hydroxyl groups and aromatic systems, led to only 28% inhibition. These data strongly support the hypothesis [32, 13] that the specificity of a polyphenol to bind proteins is not only dependent on the number of its functional groups, but also on its molecular size, shape and structure.

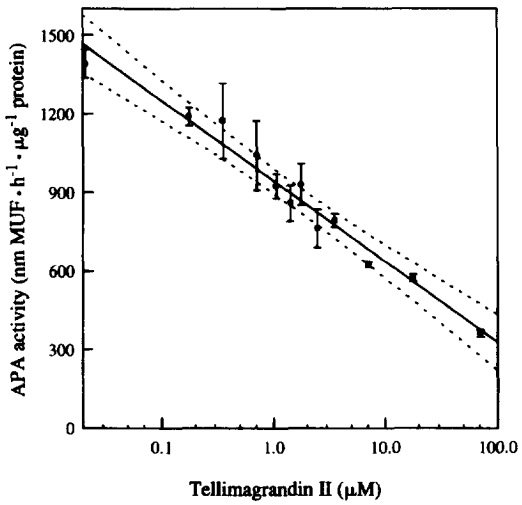


Fig. 2. Effect of increasing concentrations of **1** isolated from *M. spicatum* on the APA activity of *Trichormus* var. P-9. Data are means of triplicates ± s.d., $r^2 = 96\%$, $P < 0.0001$, dashed line: 95% C.I.

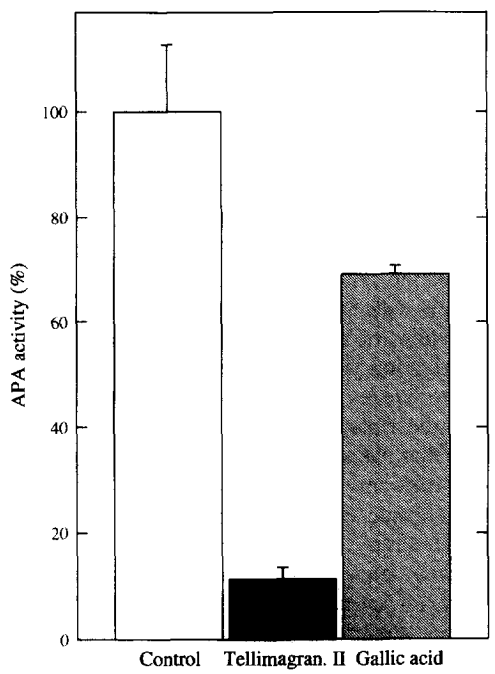


Fig. 3. Effect of **1** (18 μM) and gallic acid (90 μM) on APA activity of *Trichormus* var. P-9. Data are given as percentage of control (EtOH) and are means of triplicates ± s.d.

Polyphenols from the incubation medium of milfoil, when trapped with SPE, also inhibited APA of *Trichormus* var. P-9. Total phenolics as low as 1.6 and 4.4 mg l^{-1} led to an inhibition of 60 and 40%, respectively. These concentrations are comparable to total phenolics or humic like substances in lake waters. Normal values for dissolved organic compounds in lake water are 2–10 mg l^{-1} [33], up to 80% of them being polyphenolic or humic-like substances [34].

These data show clearly that milfoil derived polyphenols are able to complex with extracellular enzymes of target organisms even when tested against intact cyanobacterial cells.

EXPERIMENTAL

Plant material and cultivation. Myriophyllum spicatum L. was collected during the summers of 1991–1994 in Schöhsee, a mesotrophic lake in the Plön Lake area (northern Germany), by snorkelling or SCUBA diving. A voucher was deposited to the herbarium at the Botanical Institute, CAU University, Kiel, F.R.G. Axenic plant material was achieved by a surface-sterilization method modified after [25]. Cultivation of axenic shoots took place in complete medium [25] or in mineral medium [35].

General. Fluorescence was recorded on a Kontron SM 25. NMR spectra was recorded in Me_2CO-d_6. TMS was used as int. standard and chemical shifts are given in δ (ppm).

Mass spectrometry. $FAB^{+/-}$-MS and MS/MS measurements were performed on a 4-sector tandem mass spectrometer at 10 kV accelerating voltage. Resolution of both MS was set to 1:1000. The FAB gun was operated at 6 kV with Xe as the FAB gas. Collision-induced dissociation took place in the third field free region (collision gas He, 30% precursor intensity). FAB-MS/MS spectra (linked scans of MS2 at constant B/E ratio) were recorded at 100 Hz filtering.

Extraction. Fresh, tap-water rinsed shoots were N_2 frozen, homogenized and lyophilized. This material was extracted for 1 h in an ultrasonic bath at 4° with 50% aq. Me_2CO (40 ml g^{-1} dry wt). After filtration, the Me_2CO was evapd and the aq. extract sequentially partitioned with *t*BME, EtOAc and *n*-BuOH [10 ml (×3) g^{-1} dry wt)].

HPLC. Aliquots of the EtOAc fr. were chromatographed on a semi-prep. RP-C18 column (250 × 8 mm, Kromasil, 5 μm) with solvents A: 10 mM H-Pi buffer (pH 2.5) and B: MeOH, and an elution profile 0–20 min 5–60% B, 20–25 min 60–100% B, 25–40 min 100% B. For analyt. purposes, a 250 × 4.6 mm column was used. UV-absorbing compounds were detected at 280 and 254 nm. Purification for NMR analysis was achieved by a second HPLC sepn step with the following elution profile: 0–22.5 min 22–25% B, 22.5–33.5 min 25–58% B, 33.5–35 min 58–100% B. Frs were desalted by SPE on RP-C18-cartridges.

Cultivation of test organisms and bioassays. The cultivation of the test organisms and the prepn of the ADA was performed as described in ref. [18]. Inhibition of diatoms was measured with the radiocarbon primary productivity bioassay as described previously [27]. Inhibition of APA of test organisms was determined by fluorescence spectrometry with MUF-P (methylumbelliferyl-phosphate) as substrate. Algal suspension in 0.1 M Tris buffer, pH 8.5 (3 ml) was mixed with substrate (90 μl 1 mM MUF-P, 30 μM final concn) and 50 μl inhibitor dissolved in EtOH (or solvent control). Buffer strength and pH represent optimum values for the measurements of algal APA activity. The formation of the product was immediately measured using fluorescence spectrometry for more than 30 min.

Folin assay of total phenolic content. The determination of total phenolic content of *Myriophyllum* extracts was performed with the Folin–Ciocalteau assay as described in ref. [36]. Calibration was done with gallic and tannic acids. Total phenolics are given as tannic acid equivalents.

PVP assay. This assay was modified after ref. [19]. Per ml of extract 1 ml PVP suspension (0.1 g ml^{-1} PVP in Millipore® water) was added, vortexed and incubated overnight at 4°. The suspension was centrifuged (4000 *g*) for 5 min, and the supernatant tested for phenolics or algicidal activities.

SPE. Filtered (Whatman GF/F) culture water of *M. spicatum* was passed over a preconditioned 500 mg sorbent RP-C18 cartridge (ICT). The cartridge was washed with Millipore water and the bound compounds were desorbed with methanol. SPE was also used to desalt frs from HPLC.

Ultrafiltration. Ultrafiltration was performed with YC05 and YM1 membranes; M_rs cut off were 500 and 1000, resp., in a model 8200 ultrafiltration cell (Amicon) under N_2.

Enzymatic hydrolysis with tannase. 50 μg 1 or subsamples of extracts or frs dissolved in 350 μl 10 mM OAc^- buffer, pH 5.0, were supplemented with 2 μl tannase (10 mg ml^{-1}, 10 mM OAc^- buffer, pH 5.0) and incubated 24 h at room temp. The reaction was stopped by adding TFA at a final conc of 0.1%. Hydrolysis products were recorded by HPLC (gallic and ellagic acid, other polyphenols) and TLC (glc).

TLC for glc. This was performed on cellulose plates with EtOAC–HOAc–H_2O (3:1:1). Spots were detected with the aniline–phthalic acid reagent [37]. Glc was identified by reference material.

Tellimagrandin II (**1**). Tan amorphous powder. λ_{max}^{EtOH} nm (log ε) 218 (4.97), 279 (4.64), as reported in ref. [20]. FAB^--MS (TEA): *m/z* 937 $[M-H]^-$, *m/z* 919 $[M-H-H_2O]^-$, *m/z* 767 $[M-H-C_7H_6O_5]^-$, *m/z* 599 $[M-H-C_{14}H_{10}O_{10}]^-$. The FAB^+-MS data are the same as reported in ref. [11]. ^{13}C and ^1H NMR data are identical to those reported in refs. [20–22].

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