

# The Search for Exudates from Eurasian Watermilfoil and Hydrilla

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## ABSTRACT

Secondary metabolites are produced by aquatic plants, and in some instances, exudation of these metabolites into the surrounding water has been detected. To determine whether infestations of Eurasian watermilfoil or hydrilla produce such exudates, plant tissues and water samples were collected from laboratory cultures and pond populations and were analyzed using solid phase extraction, HPLC, and various methods of mass spectrometry including electrospray ionization, GC/MS, electron impact and chemical ionization. Previously reported compounds such as tellimagrandin II (from Eurasian watermilfoil) and a caffeic acid ester (from hydrilla), along with a newly discovered flavonoid, cyanidin 3-dimalonyl glucoside (from hydrilla), were readily detected in plant tissues used in this research but were not detected in any of the water samples. If compounds are being released, as suggested by researchers using axenic cultures, we hypothesize that they may be rapidly degraded by bacteria and therefore undetectable.

*Key words:* tellimagrandin II, cyanidin 3-dimalonyl glucoside, bacterial degradation, gallic acid, phenolic compounds.

## INTRODUCTION

Secondary metabolites are compounds produced by a plant that are not absolutely necessary to sustain life. They have a variety of functions in the plant and can include phenols, terpenoids, and alkaloids. Some of these functions include the attraction of pollinators (Rosenthal 1986), protection against UV light (Stapleton and Walbot 1994, Dixon and Paiva 1995) and pathogens (Dixon and Paiva 1995, Hipkind et al. 1996), and prevention of herbivory (Scalbert 1991). Secondary metabolites can also be used to prevent the growth of other plants, a phenomenon referred to as allelopathy (Rice 1984).

Eurasian watermilfoil (*Myriophyllum spicatum* L.) and hydrilla (*Hydrilla verticillata* L.f. Royle) plant extracts are allelopathic. Eurasian watermilfoil extract is toxic toward cyanobacteria, green algae, duckweed (*Lemna* spp.), mosquitoes, and midges (Planas et al. 1981, Dhillon et al. 1982, Elakovich and Wooten 1989), and hydrilla extract is allelopathic to duck-

weed and *Ceratophyllum* species (Kulshreshtha and Gopal 1983, Elakovich and Wooten 1989). A caffeic acid ester in hydrilla extract inhibits the germination of *Fusarium culmorum*, a potential fungal pathogen (Hipkind et al. 1992).

None of the above mentioned studies, however, have shown allelopathic compounds being released by either of these plants into the surrounding water. Only Gross et al. (1996) identified an allelopathic compound released by Eurasian watermilfoil into culture medium. This compound, tellimagrandin II (Figure 1A), is a hydrolysable tannin which inhibits the growth of cyanobacteria and green algae. Gross (1999) predicted that 1-6 mg/L might be found in the water surrounding dense stands of Eurasian watermilfoil.

Although the release of allelochemicals into the water has ecological importance, there has been some concern that these or other compounds may be carcinogenic or mutagenic.<sup>3</sup> Humans could be exposed to these compounds through dermal contact or potable water. Given the significance of secondary metabolites to affect other organisms, the objective of this study was to determine if measurable amounts of compounds are being released by Eurasian watermilfoil and hydrilla.

## MATERIALS AND METHODS

*Culture and sampling.* Cultures of Eurasian watermilfoil were maintained in 3-L round bottom flasks that contained 2 L of sterile modified Gerloff's medium (Selim et al. 1989). Cultures of hydrilla were maintained in 2-L Florence flasks that contained 1.5 L of sterile 10% Hoaglands medium (Hoagland and Arnon 1950). The cultures were grown in a growth chamber under the following conditions: a photosynthetic photon flux density of 230 (Eurasian watermilfoil) or 150 (hydrilla)  $\mu\text{mol photons/m}^2/\text{s}$ , 16:8 h light: dark photoperiod, and 25C. Every 30 d, 4-cm sections of the stem apices were cut and transferred to new medium to maintain optimal growth.

Samples consisted of 5 g of tissue harvested from one-month old cultures. The tissue was cut into 1 cm segments and placed in 60 mL HPLC-grade methanol overnight at 4C. The extract was removed and concentrated by flash evaporation at 35C down to 3 mL. The concentrated extract was then centrifuged at 10,000 *g* for 10 min in eppendorf tubes to remove chlorophyll. The chlorophyll adhered to the wall of the tubes allowing the MeOH extract to be removed with a

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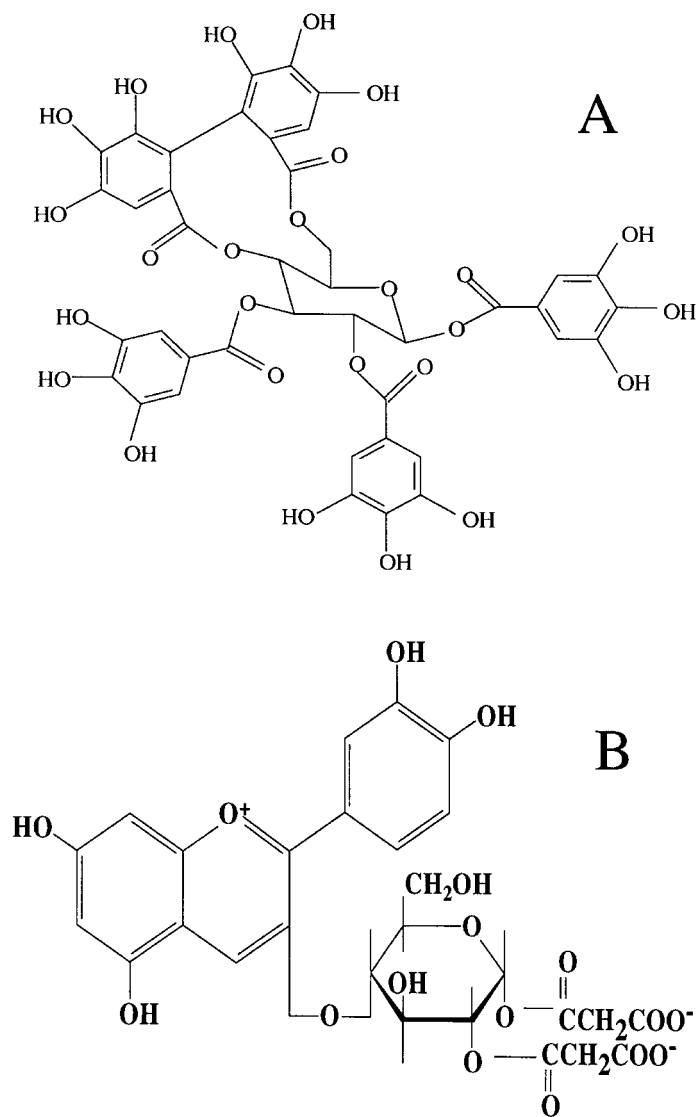


Figure 1. Structure of (A) tellimagrandin II and (B) cyanidin 3-dimalonyl glucoside, compounds found in Eurasian watermilfoil and hydrilla tissue extracts, respectively.

pipet. Plant extracts were then analyzed by high performance liquid chromatography (HPLC) and mass spectrometry. The mass spectrometry used on the Eurasian watermilfoil extract was electrospray ionization (ESI), whereas matrix assisted laser desorption ionization—time of flight (MALDI-TOF) was used to analyze the hydrilla extract.

Eurasian watermilfoil, hydrilla, and unvegetated ponds were maintained at the USAERDC-Lewisville Aquatic Ecosystem Research Facility in Lewisville, Texas, and water samples were collected once a month during the summer of 1999. Samples were taken just below the water surface, packed in a cooler with ice packs, and shipped overnight to Purdue University in West Lafayette, Indiana for analysis. The water was not frozen.

Three Eurasian watermilfoil plants were placed into either flasks containing 6  $\mu\text{L}$  diquat/L water or flasks containing 1 L of water. The plants were maintained in a growth chamber at 25C, 100  $\mu\text{mol photons/m}^2/\text{s}$ , and a 16:8 h light:dark

photoperiod. The plants remained in the chamber until the diquat-treated plants turned necrotic.

Water samples were divided into two categories: water from living Eurasian watermilfoil and hydrilla plants, and water from senescing Eurasian watermilfoil plants. Water from living plants included culture medium (collected after plant growth for 1 month) and pond water, and water from senescing Eurasian watermilfoil included pond water after the Eurasian watermilfoil population at Lewisville senesced in early August and also water from the diquat experiment. All water was filtered through a Whatman filter, a glass fiber prefilter, and a 0.2  $\mu\text{m}$  Millipore filter prior to analysis. The water was then subjected to solid phase extraction using a Sep-Pak® C18 cartridge. The cartridge was preconditioned with 5 mL 100% MeOH followed by 10 mL 4% MeOH. Then 50 mL to 2 L of water were passed through and compounds of interest were eluted with 10 mL 100% HPLC-grade MeOH. The eluted sample was then concentrated to 0.5 mL by flash evaporation at 25C and analyzed by HPLC and mass spectrometry.

**High Performance Liquid Chromatography.** The HPLC analysis used for the Eurasian watermilfoil extract and water analysis was a modification of a method for the detection of phenylpropanoids (Graham 1991). Solvent A was 0.6% perchloric acid and solvent B was 100% acetonitrile. The elution profile was a linear gradient from 0 to 55% B over 30 min, 55 to 100% B over 1 min, 100% B maintained for 2 min, and 100 to 0% B for the remaining 5 min. The flow rate was 1.3 ml/min. The hydrilla extract was analyzed by a modified method (Hipskind et al. 1992) used for the detection of phenylpropanoids and phenylpropanoid derivatives. The compounds of interest were eluted in a solvent system in which solvent A was MeOH and solvent B was 0.6% perchloric acid. The elution profile was 90 to 70% B over 5 min, 70% B maintained for 25 min, and 70 to 90% B over 5 min. The flow rate was 1 ml/min. Separations for both methods were carried out on two reverse-phase C-18 Beckman Ultrasphere columns (15 and 25 cm, respectively) connected in tandem. The compounds that absorbed in the range 200 to 600 nm were detected with a Beckman 168-photodiode-array detector connected to an IBM PC running the Beckman Gold Nouveau® HPLC analysis software.

**Mass Spectrometry.** The electrospray ionization analyses, in the positive and negative ion mode, were carried out on a FinniganMAT LCQ (ThermoQuest Corp./FinniganMAT; San Jose, CA) mass spectrometer system. Matrix-assisted laser desorption ionization (MALDI) coupled with time-of-flight (TOF) mass analysis was performed as described by Sugui et al. (1998). Samples were analyzed using a PerSeptive Biosystems (Framingham, MA) Voyager MALDI-TOF instrument. GC/MS and direct insertion probe analyses were performed on a FinniganMAT GCQ (Thermoquest Corp. San Jose, CA) mass spectrometer system utilizing both electron impact and chemical ionization.

**Bacteria experiment.** To each of six 1 L flasks, 500 mL Volvox medium (Provasoli and Pintner 1959) was added. Gallic acid was added to make a 1 mM solution. The medium was then sterilized by filtration through a 0.2  $\mu\text{m}$  Millipore filter. To three of the flasks, 0.5 mL of Eurasian watermilfoil pond water was added. To confirm the presence of bacteria in the pond water, drops of pond water were plated on potato dex-

trose agar. Colonies that formed on the agar were analyzed by light microscopy to ensure that they were bacterial. The absorbance of gallic acid (258 nm) was measured daily until no gallic acid was detected in the bacteria treated flasks.

## RESULTS AND DISCUSSION

**Plant extracts.** Plant extracts were analyzed in this study to ensure that we could identify compounds in both hydrilla and Eurasian watermilfoil. Tellimagrandin II (Figure 1A) was detected in the Eurasian watermilfoil plant extract along with other flavonoid compounds by HPLC (Figure 2) and ESI. The previously identified caffeic acid ester and a new compound, cyanidin 3-dimalonyl glucoside (Figure 1B), were detected in the hydrilla plant extract by HPLC (Figure 3) and MALDI-TOF. Cyanidin 3-dimalonyl glucoside has been identified in maize (*Zea mays* L.) and sorghum (*Sorghum bicolor* [L.] Moench.) where it accumulates as a response to fungal infection (Hipskind et al. 1996, Wharton and Nicholson 2000). It is also found in a member of the Asteraceae (*Coleostephus myconis* [L.] Rchb. f.) where it is one of the pigments found in the petals (Takeda et al. 1986). The function of cyanidin 3-dimalonyl glucoside in hydrilla, however, has not yet been determined.

**Water analysis.** No compounds were detected in either the Eurasian watermilfoil or hydrilla culture medium by HPLC (Figure 4). Three peaks were detected in both the Eurasian watermilfoil and hydrilla pond water (Figures 5A and B); however, these same three peaks were also detected in the unvegetated pond water (Figure 5C). To determine whether compounds would be exuded from plants treated with an herbicide, Eurasian watermilfoil was exposed to diquat. Results from the diquat experiment showed no differences between the treated and untreated samples by HPLC (data not shown). All HPLC results were confirmed by mass spectrometry (ESI, GC/MS, EI, and CI).

Although no compounds were detected in the water, it is possible that compounds are being released but are immediately degraded by microbes. Gross (1999), working with axenic cultures, suggested that tellimagrandin II and other hydrolysable tannins released by Eurasian watermilfoil may be rapidly degraded by bacteria. A number of different aerobic and anaerobic bacteria and fungi are known to degrade hydrolysable tannins found in industrial effluents. Aerobic bacteria can completely degrade hydrolysable tannins in as little as 6 h, but in some cases this can take up to 4 d (Field and Lettinga 1992). In this study, we determined that a 1 mM solution of gallic acid could be degraded in five days by bacteria present in Eurasian watermilfoil pond water (Figure 6). Gallic acid was used in this experiment because tellimagrandin II is not commercially available and gallic acid is one of the subunits of tellimagrandin II. Degradation of hydrolysable tannin occurs through the release of tannase, an enzyme found in many microbes. When tellimagrandin is exposed to tannase, glucose and gallic acid are released upon hydrolysis. The gallic acid is then oxidized to simple aliphatic acids that can be used in the citric acid cycle (Bhat et al. 1998).

Bacteria are known to degrade almost all natural and synthetic organic compounds, and plants such as hydrilla have been shown to have a rich microbial flora associated with

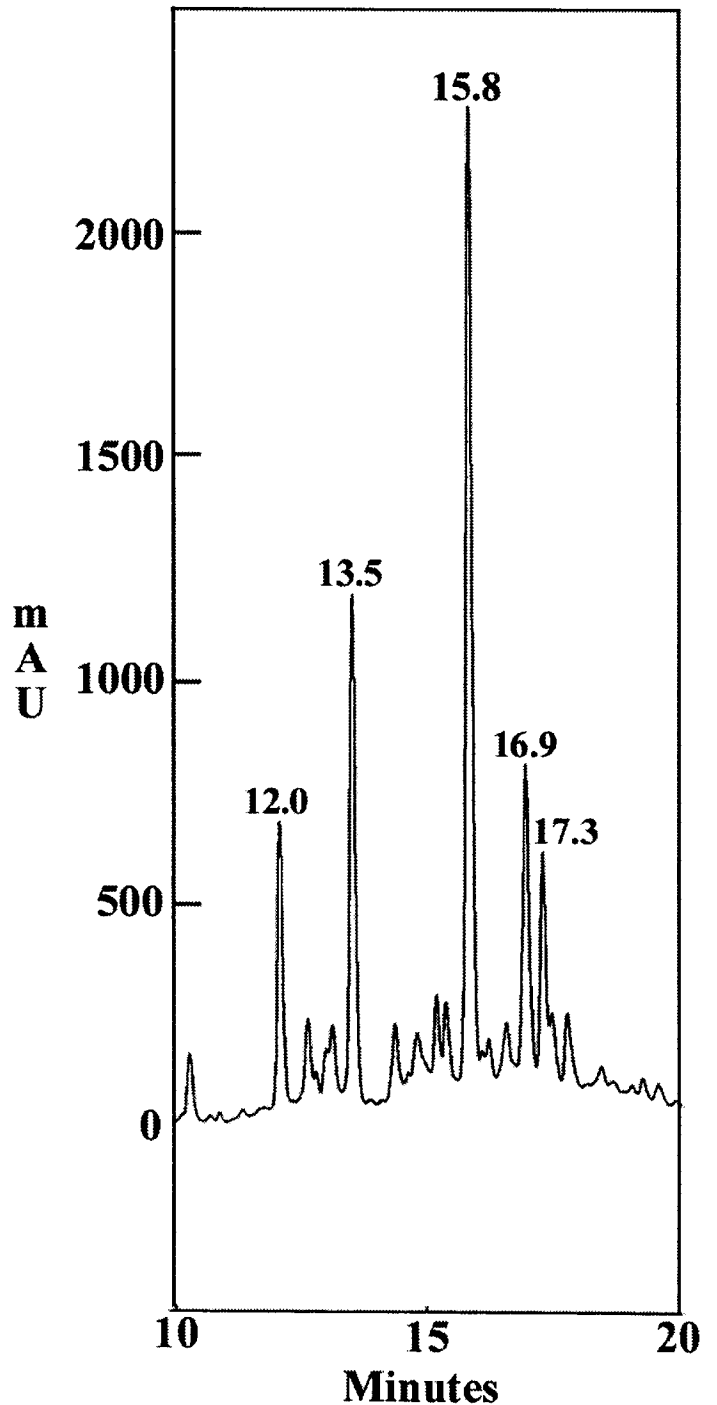


Figure 2. HPLC elution of components in a crude methanol extract from *Myriophyllum spicatum*. Extract components were analyzed at 280 nm. The peak that eluted at 15.8 minutes is consistent with tellimagrandin II.

them (Shabana and Charudattan 1996). Leachates of the emergent aquatic macrophytes *Juncus effusus* L. and *Typha latifolia* L. have been shown to support bacterial growth (Mann and Wetzel 1996). Godmaire and Nalewjkó (1990) suggested that the trichome exudates (polysaccharides and lipophilic substances) of Eurasian watermilfoil are rapidly

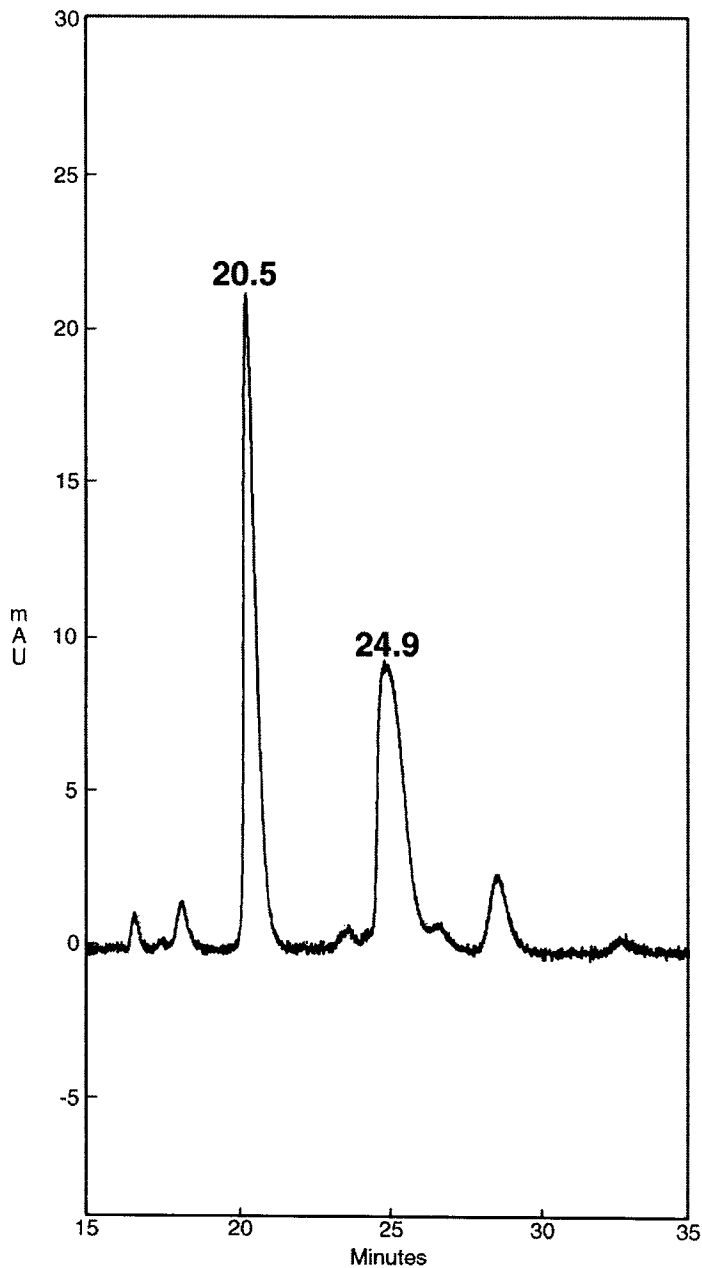


Figure 3. HPLC indicating an anthocyanin eluting at 20.5 minutes with a maximum absorbance of 520 nm. This anthocyanin was identified as cyanidin 3-dimalonyl glucoside. A previously identified caffeic acid ester eluted at 24.9 minutes.

used and recycled within epiphytic communities. They also hypothesized that exudates and released dissolved organic carbon can contribute to the productivity of epiphytic communities as long as noxious compounds are not present. Thus, it is possible that exudates of Eurasian watermilfoil and hydrilla are being degraded by microbes and therefore not detectable by our methods of analysis.

Although it is tempting to think that plants such as hydrilla and Eurasian watermilfoil are successful competitors because of their exudation of allelochemicals, our data suggest that this may not be the case to any significant degree. Sec-

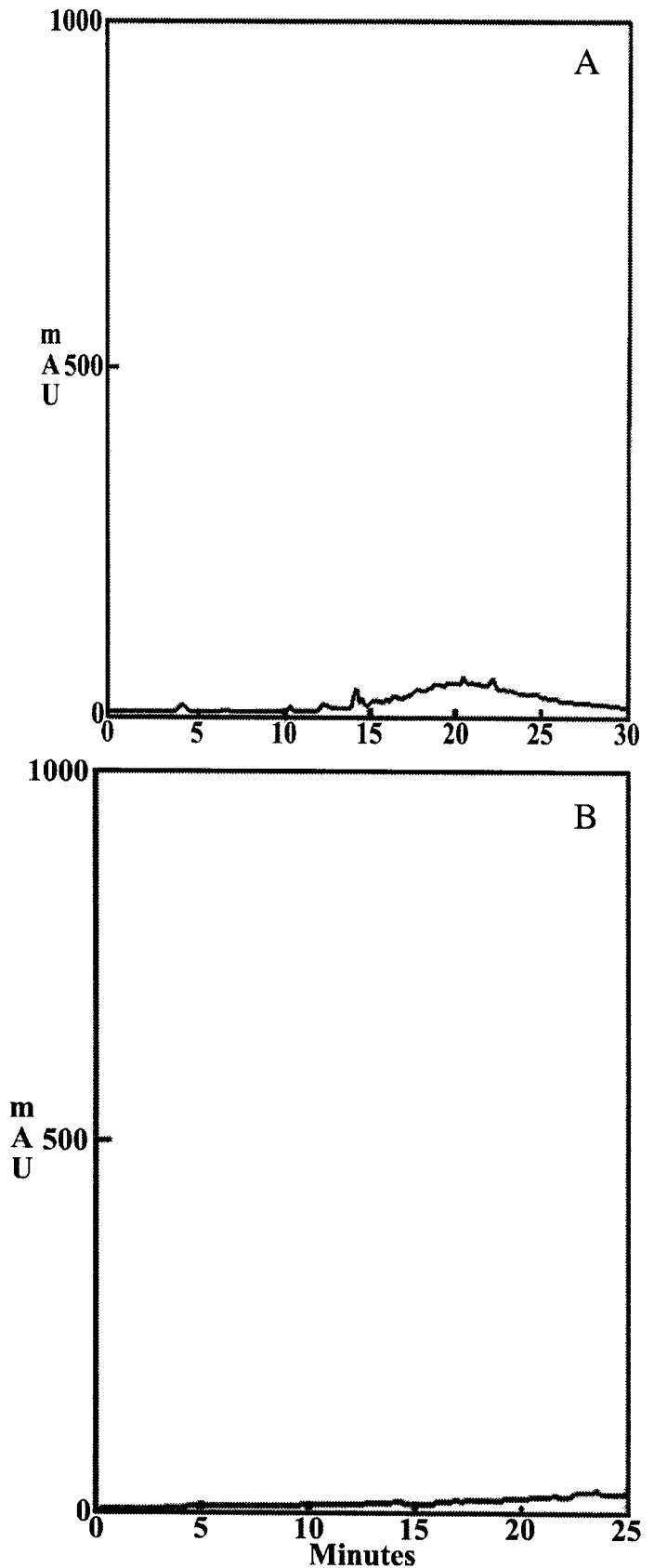


Figure 4. No compounds were detected with HPLC analysis at 280 nm of culture medium in which (A) Eurasian watermilfoil and (B) hydrilla were grown.

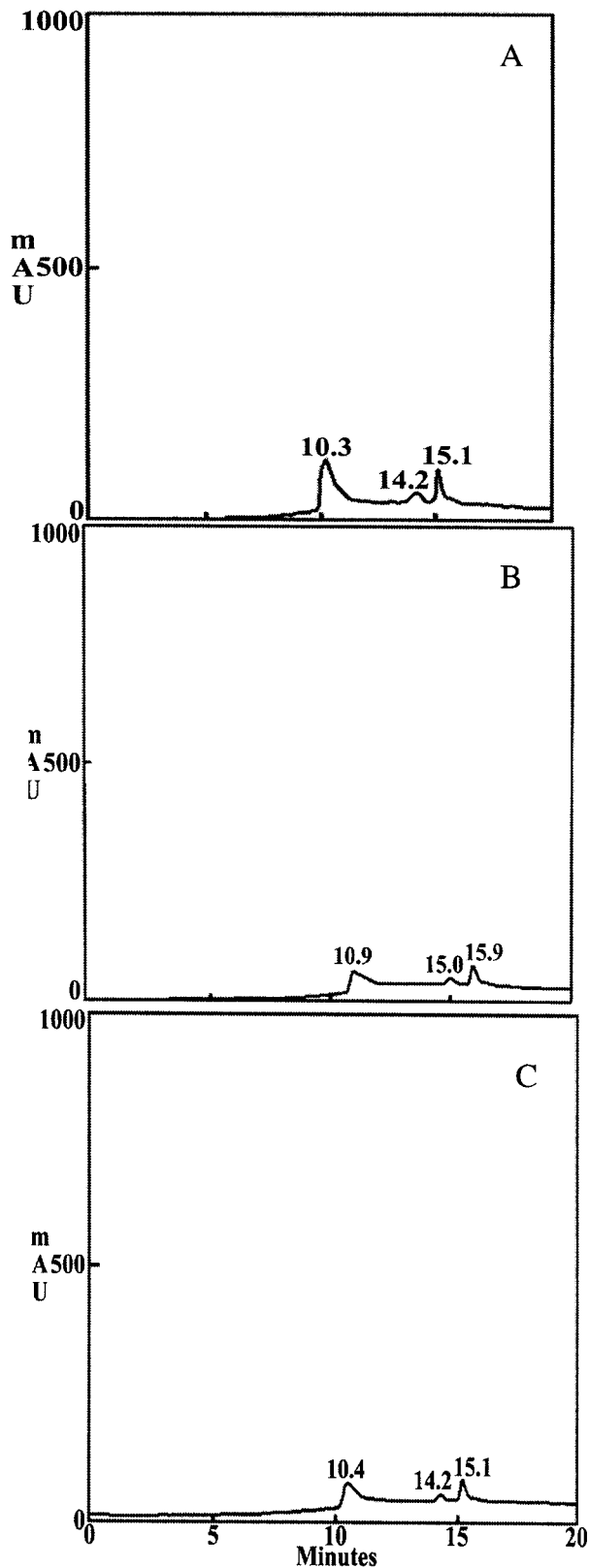


Figure 5. HPLC results at 280 nm from the analysis of pond water with (A) Eurasian watermilfoil, (B) hydrilla, and (C) no vegetation. Although peaks were detected, they were approximately the same at all three sites, indicating that plants were not releasing significant quantities of secondary compounds. Elution times vary slightly because of the impurity of compounds collected from nature.

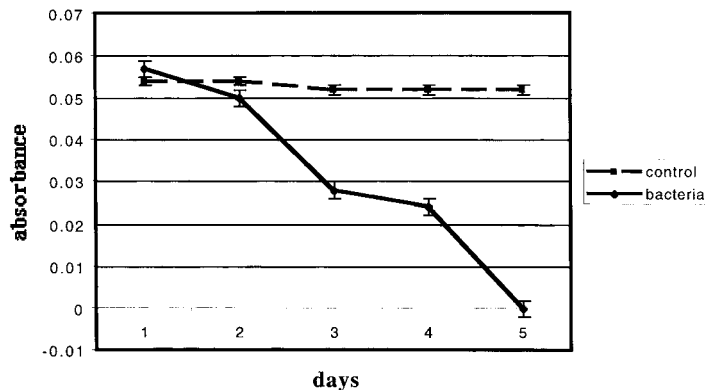


Figure 6. Bacterial degradation of gallic acid. Absorbance was measured at 258 nm.

ondary metabolites are synthesized by these plants and may be useful in preventing herbivory or disease, but either they are not being exuded into the water by healthy plants [or if they are exuded, it is at concentrations below our detection limits (pico-micromolar)] or they are rapidly being broken down by microbes. In the case of herbicide-treated plants that are undergoing significant deterioration, the compounds may become oxidized and conjugated to plant constituents such as cell wall materials, thus rendering them difficult to detect (Nicholson and Hammerschmidt 1992). Our study provides no evidence that Eurasian watermilfoil and hydrilla are successful competitors for reasons other than well-known non-allelopathic strategies (e.g., canopy formation, prolific propagule production).

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#### LITERATURE CITED

- Bhat, T. K., B. Singh and O. P. Sharma. 1998. Microbial degradation of tannins—A current perspective. *Biodegradation* 9: 343-357.
- Dhillon, M. S., M. S. Mulla and Y. Hwang. 1982. Allelochemicals produced by the hydrophyte *Myriophyllum spicatum* affecting mosquitoes and midges. *J. Chem. Ecol.* 8: 517-526.
- Dixon, R. A. and N. L. Paiva. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7: 1085-1097.
- Elakovich, S. D. and J. W. Wooten. 1989. Allelopathic potential of sixteen aquatic and wetland plants. *J. Aquat. Plant Manage.* 27: 78-84.
- Field, J. A. and G. Lettinga. 1992. Biodegradation of tannins, pp. 61-97. *In*: H. Sigel, ed. *Metal ions in biological systems*, V. 28. Degradation of environmental pollutants by microorganisms and their metalloenzymes. Marcel Dekker Inc., New York.
- Godmaire, H. and C. Nalewajko. 1990. Structure and development of secretory trichomes on *Myriophyllum spicatum* L. *Aquat. Bot.* 37: 99-121.
- Graham, T. L. 1991. A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. *Plant Physiol.* 95: 584.
- Gross, E. M., H. Meyer and G. Schilling. 1996. Release and ecological impact of algicidal hydrolysable polyphenols in *Myriophyllum spicatum*. *Phytochemistry* 41: 133-138.

- Gross, E. M. 1999. Allelopathy in benthic and littoral areas: Case studies on allelochemicals for benthic cyanobacteria and submersed macrophytes, pp. 179-200. *In*: K. M. M. Inderjit, Dakshini and C. L. Foy, eds. Principles and practices in plant ecology: allelochemical interactions. CRC Press, New York.
- Hipskind, J., K. V. Wood, B. Leite, T. Chand, C. A. Lembi and R. L. Nicholson. 1992. A fungitoxic phenolic compound in *Hydrilla verticillata*. *Biol. Control* 2: 51-58.
- Hipskind, J., K. Wood and R. L. Nicholson. 1996. Localized stimulation of anthocyanin accumulation and delineation of pathogen ingress in maize genetically resistant to *Bipolaris maydis* race O. *Physiol. and Mol. Plant Pathol.* 49: 247-256.
- Hoagland, D. R. and D. Arnon. 1950. The water-culture method for growing plants with soil. *Calif. Agric. Exp. Sta. Cir.* 347.
- Kulshreshtha, M. and B. Gopal. 1983. Allelopathic influence of *Hydrilla verticillata* (L.f.) Royle on the distribution of *Ceratophyllum* species. *Aquat. Bot.* 16: 207-209.
- Mann, C. J. and R. G. Wetzel. 1996. Loading and utilization of dissolved organic carbon from emergent macrophytes. *Aquat. Bot.* 53: 61-72.
- Nicholson, R. L. and R. Hammerschmidt. 1992. Phenolic compounds and their role in disease resistance. *Annu. Rev. Phytopathol.* 30: 369-389.
- Planas, D., F. Sarham, L. Dube, H. Godmaire and C. Cadieux. 1981. Ecological significance of phenolic compounds of *Myriophyllum spicatum*. *Verh. Int. Verein. Limnol.* 21: 1492-1496.
- Provasoli, L. and I. J. Pintner. 1959. Artificial media for freshwater algae; problems and suggestions, pp. 84-96. *In*: C. A. Tryon and R. T. Hartman, eds. *The ecology of algae*. Special Publication No. 2. Pymatuning Laboratory of Field Biol. University Pittsburg.
- Rice, E. L. 1984. *Allelopathy*. Academic Press, Orlando, FL. 422 pp.
- Rosenthal, G. A. 1986. The chemical defense of higher plants. *Sci. Am.* 254: 94-99.
- Scalbert, A. 1991. Antimicrobial properties of tannins. *Phytochemistry* 30: 3875-3883.
- Selim, S. A., S. W. O'Neal, C. A. Lembi and M. A. Ross. 1989. Bioassay of photosynthetic inhibitors in water and aqueous soil extracts with Eurasian watermilfoil (*Myriophyllum spicatum*). *Weed Sci.* 37: 810-814.
- Shabana, Y. M. and R. Charudattan. 1996. Microorganisms associated with hydrilla in ponds and lakes in North Florida. *J. Aquat. Plant Manage.* 34: 60-68.
- Stapleton, A. E. and V. Walbot. 1994. Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage. *Plant Physiol.* 105: 881-889.
- Sugui, J. A., C. Bonham, S-C. Lo, K. V. Wood and R. L. Nicholson. 1998. MALDI-TOF analysis of mixtures of 3-deoxyanthocyanidins and anthocyanins. *Phytochemistry*. 48: 1063-1066.
- Takeda, K., J. B. Harborne and R. Self. 1986. Identification and distribution of malonated anthocyanins in plants of the compositae. *Phytochemistry*. 25: 1337-1342.
- Wharton, P. S. and R. L. Nicholson. 2000. Temporal synthesis and radiolabeling of the sorghum 3-deoxyanthocyanidin phytoalexins and the anthocyanin, cyanidin 3-dimalonyl glucoside. *New Phytol.* 145: 457-469.