Journal of Coastal Life Medicine

journal homepage: www.jclmm.com

Original article doi: 10.12980/jclm.4.2016j5-187

Screening potential genotoxic effect of aquatic plant extracts using the mussel micronucleus test

Bettina Eck-Varanka[1], Nora Kováts[1*], Katalin Hubai[1], Gábor Paulovits[2], Árpád Ferincz[3], Eszter Horváth[1]

[1]*Department of Limnology, University of Pannonia, Veszprém, Hungary*

[2]*Balaton Limnological Institute, Centre for Ecological Research, Hungarian Academy of Sciences, Budapest, Hungary*

[3]*Department of Aquaculture, Szent István University, Gödöllő, Hungary*

ARTICLE INFO

Article history:
Received 25 Sep 2015
Received in revised form 19 Oct 2015
Accepted 20 Nov 2015
Available online 22 Dec 2015

Keywords:
Genotoxicity
Mussel micronucleus test
Unio pictorum
Tannin

ABSTRACT

Objective: To assess the genotoxic potential of selected aquatic macrophytes: *Ceratophyllum demersum* L. (hornwort, family Ceratophyllaceae), *Typha angustifolia* L. (narrowleaf cattail, family Typhaceae), *Stratiotes aloides* L. (water soldier, family Butomaceae), and *Oenanthe aquatica* (L.) Poir. (water dropwort, family Umbelliferae).
Methods: For genotoxicity assessment, the mussel micronucleus test was applied. Micronucleus frequency was determined from the haemolymph of *Unio pictorum* L. (painter's mussel). In parallel, total and hydrolisable tannin contents were determined.
Results: All plant extracts elucidated significant mutagenic effect. Significant correlation was determined between tannin content and mutagenic capacity.
Conclusions: The significant correlation between genotoxicity as expressed by micronucleus frequency and tannin content (both total and hydrolisable tannins) indicate that tannin is amongst the main compounds being responsible for the genotoxic potential. It might be suggested that genotoxic capacity of these plants elucidate a real ecological effect in the ecosystem.

1. Introduction

In general, allelopathy is defined as the release of organic compounds (allelochemicals) by plants or bacterial species that affect other plants or bacterial species, facilitating competition. In aquatic habitats, allelopathy is considered an adaptive strategy of macrophytes in competing with phytoplankton for light and nutrients. It is hypothesised that allelopathy can drive the change in algal communities[1,2].

In most of the studies, plant extracts are used and tested against algae for allelopathic capacity, without isolating active ingredients[3,4]. However, in some plants, it is already known which bioactive compounds are responsible for the allelopathic activity. Phenolics and tannin are considered rather widely distributed[5]. *Myriophyllum spicatum*, which is considered to have high allelopathic potential, contains a wide variety of these compounds, such as ellagic acid, gallic acid, pyrogallic acid or hydrolysable tannins[6,7].

While these studies have been targeted at determining the allelopathic effect of higher plants on algal communities, some bioactive compounds such as phenolics and tannins play a role not only in competitive interactions but have been shown to exert geno- and/or cytotoxicological effect on other elements of the aquatic ecosystem. It was demonstrated that the naturally occurring phenolic acids (tannic, ellagic and gallic acids) caused DNA strand breakage in Chinese hamster cell line B14 as shown by the comet assay and cytotoxic effect was also found[8]. The same compounds were reported to induce DNA damage in the digestive gland cells of freshwater mussel *Unio tumidus* L. (*U. tumidus*) at the concentrations of 15, 30 and 60 μmol/L[9].

The aim of the study was to assess the genotoxic potential of different aquatic plants using the micronucleus test and to find possible correlation with the total polyphenol and tannin content. This test is a widely established, relatively easy-to-perform assay. Micronuclei formation indicates chromosomal DNA damage occurring as a result of either chromosome breakage or chromosome mis-segregation during mitosis[10].

As test organism, the freshwater bivalve *Unio pictorum* L. (*U. pictorum*) (painter's mussel) was selected. Bivalves are sentinel and filter-feeding organisms, which make them ideal for toxicity testing as they are able to accumulate and bioconcentrate most pollutants. *Unio* species, *U. tumidus* and *U. pictorum* have already proven sensitive test organisms in genotoxicological studies[11-14].

*Corresponding author: Nora Kováts, Department of Limnology, University of Pannonia, Veszprém H-8200, Hungary.
Tel/Fax: +36 88 624747
E-mail: kovats@almos.uni-pannon.hu
Foundation Project: Supported by Hungarian State and European Union (Grant No. TÁMOP-4.2.2.B-15/1/KONV-2015-0004).

2. Materials and methods

Plants were collected in the Kis-Balaton Water Protection System (west of Lake Balaton, Hungary). The following species were used for further analysis: *Ceratophyllum demersum* L. (*C. demersum*) (hornwort, family Ceratophyllaceae), *Typha angustifolia* L. (*T. angustifolia*) (narrowleaf cattail, family Typhaceae), *Stratiotes aloides* L. (*S. aloides*) (water soldier, family Butomaceae), and *Oenanthe aquatica* (L.) Poir. (*O. aquatica*) (water dropwort, family Umbelliferae).

Aqueous extracts were prepared as follows: 30 g of ground tissue of dried aerial parts was diluted in 500 mL of deionised water and then treated by shaking for 24 h at room temperature[15].

U. pictorum specimens were collected from Lake Balaton and were kept in a flow-through aquarium. Water source was Lake Balaton water, therefore not only proper oxygenation was ensured but a constant food supply as well. Animals were acclimatized for 4 weeks prior to testing.

For the test, specimens with length of 5–8 cm were used. Treatments were performed in three replicates. Aquaria of 3 L capacity were used. They were aerated during the experiment, and the temperature was set at 22 ˚C. Exposure time was 4 days. The assay was performed based on the protocol described by Wozniczki *et al.* with some modifications[16]. First, a range finding test was performed, using 6 and 60 g/L concentrations. As even the 6 g/L concentration proved to be lethal, for further test series 1 g/L concentration was chosen. Also, based on the experience of the range finding test which was a static test (test solution was not renewed), a semi-static test was conducted, that is, test solution was changed in the middle of the test, after 2 days. Lake Balaton water was also used for the control.

After 4 days of exposure, for the assessment of the number of micronuclei, haemolymph was taken from the posterior adductor using the non-lethal technique described by Gustafson *et al.*[17]. About 1 mL of haemolymph was mixed with 0.3 mL 10% acetic acid in methanol as a fixative and centrifuged at 1 000 r/min for 5 min. The supernatant was discarded and the rest was fixed in 1 mL 80 % ethanol. For processing the samples, refrigerated samples were centrifuged again at 1 000 r/min for 5 min. The supernatant was discarded, the pellet which contained the cells in a more concentrated form, was smeared onto a microscope slide and allowed to dry. After that the slides were fixed in 80% methanol, air dried and stained with 5% Giemsa in distilled water for 20 min.

Photos were taken by a Zeiss AxioScope A1 microscope with an AxioCam ICC1 camera and Zen 2011 program at 400× magnification. Micronuclei were identified according to Bolognesi and Fenech[10]. For each animal 250 cells were counted. Robust ANOVA of Welch with Tamahane T2 *post-hoc* test was used to compare the mean micronucleus (MN) numbers between the treatments. Spearman rank correlation was used to assess the correlation between the MN numbers and tannin forms.

Polyphenol detection technics were based on Folin-Phenol method[18]. Total polyphenol was detected from dry leaf samples[19]. For hydrolysed polyphenol detection, water samples and extracts were stored in refrigerator and samples were analysed within two weeks of extraction according to American Public Health Association[20]. Shortly, samples were thawed at 4 ˚C, and 1 mL Folin reagent and carbonate-tartrate reagent (Na_2CO_3 1.88 mol/L and Na-tartrate dihydrate 0.052 mol/L) were added to 50 mL volume of each sample. Absorbance was measured on 700 nm after 30 min. Tannic acid (99%) was used for calibration as standard.

3. Results

Table 1 summarises total and hyrolisable tannin content of dried aerial parts/plant extracts as well as number of micronuclei. In MN numbers, significant difference was observed between the control and all treatments (Welch-ANOVA: $F = 7.766$, $P < 0.00001$, Tamahane T2 *post-hoc*: $P < 0.05$ in all cases). It was clear that all plant extract proved to be genotoxic. Three plants exerted very similar and high allelopathic activity [*S. aloides* (MN 10.83), *O. aquatica* (MN 10.47), *T. angustifolia* (MN 9.70)] while *C. demersum* showed lower genotoxic potential (MN 7.65).

Table 1

Total and hydrolysable tannin content of dried aerial parts/plant extracts and number of micronuclei/250 cells.

	Total tannin content (mg/g)[a]	Hydrolysable tannin content (μg/mL)[b]	MN
Lake Balaton	-	-	3.26 ± 0.18
C. demersum	0.07 ± 0.01	1.52 ± 0.03	7.65 ± 0.29
S. aloides	0.20 ± 0.02	4.54 ± 1.40	10.83 ± 1.38
T. angustifolia	0.08 ± 0.00	3.42 ± 0.14	9.70 ± 1.05
O. aquatica	0.20 ± 0.02	3.99 ± 1.89	10.47 ± 1.24

[a]: mg/g dry aerial part; [b]: μg/mL extract.

In a comparative work of Hilt and Gross, aquatic plants were grouped in three categories according to their allelopathic potential[6]. *C. demersum* belonged to the group of the highest activity, while *S. aloides* into the group of medium one. In our study, however, the trend was just the opposite.

4. Discussion

Exposure pathway to allelochemicals such as phenolics and tannins is still relatively less clear. Laboratory experiments discussed above do not deal with the problem how these compounds are emitted during the vegetation period by fresh, healthy and active plants. Most possibly, most of the tannins and phenolics get to the water via leaching in the phase of decomposition. The study of Chen *et al.* suggests that decomposition enhances toxicological effects[5].

On the other hand, efficient allelopathy requires that these compounds are released during the vegetation period. As such, mesocosm studies indicate that aquatic plants may directly release allelopathically active compounds into the surrounding medium[21-24].

There are some studies indicating that the biologic actions of tannins are not so uniform in the aquatic ecosystem. The results of Labieniec *et al.* demonstrate that tannins exert antioxidative effect at low concentrations, as concentrations of 1 and 5 μmol/L significantly decreased the amount of lesions induced by H_2O_2[9]. Higher concentrations, however, trigger single strand breaks in the DNA of the digestive of *U. tumidus*. Similar concentration-dependent pattern was experienced by Labieniec and Gabryelak[25].

Although some studies demonstrated that apart from polyphenols and tannins, other compounds might be responsible for the allelopathic effect, our results showed significant correlation between total tannin content and MN number ($R = 0.900$, $P = 0.05$) as well as between hydrolisable tannin content and MN number ($R = 0.975$, P

= 0.01)[26-28].

Our results clearly demonstrate that all examined plans had significant bioactive and genotoxic potential. The significant correlation between genotoxicity as expressed by MN number and tannin content (both total and hydrolisable tannins) indicate that tannin is amongst the main compounds being responsible for the genotoxic potential. As in our study aqueous extract was used, mimicking a quasi-natural exposure pathway, it should be supposed that genotoxic capacity of this plants might pose a real ecological effect in the ecosystem, especially in the phases of senescence and decomposition. However, further studies are needed to clarify real-world exposure conditions.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

We acknowledge the financial support of the Hungarian State and European Union (Grant No. TÁMOP-4.2.2.B-15/1/KONV-2015-0004).

References

[1] Rojo C, Segura M, Rodrigo MA. The allelopathic capacity of submerged macrophytes shapes the microalgal assemblages from a recently restored coastal wetland. *Ecol Eng* 2013; **58**: 149-55.

[2] Zuo S, Fang Z, Yang S, Wan K, Han Y. Effect of allelopathic potential from selected aquatic macrophytes on algal interaction in the polluted water. *Biochem Syst Ecol* 2015; **61**: 133-8.

[3] Zhang T, Liu L, Yang X, Zhang S, Xia W, Li C. Allelopathic control of freshwater phytoplankton by the submerged macrophyte *Najas minor* All. *Acta Ecol Sin* 2014; **34**: 351-5.

[4] Ghobrial MG, Nassr HS, Kamil AW. Bioactivity effect of two macrophyte extracts on growth performance of two bloom-forming cyanophytes. *Egypt J Aquat Res* 2015; **41**: 69-81.

[5] Chen J, Zhang H, Han Z, Ye J, Liu Z. The influence of aquatic macrophytes on *Microcystis aeruginosa* growth. *Ecol Eng* 2012; **42**: 130-3.

[6] Hilt S, Gross EM. Can allelopathically active submerged macrophytes stabilise clear-water states in shallow lakes? *Basic Appl Ecol* 2008; **9**: 422-32.

[7] Wang H, Liang F, Zhang L. Composition and anti-cyanobacterial activity of essential oils from six different submerged macrophytes. *Pol J Environ Stud* 2015; **24**(1): 333-8.

[8] Labieniec M, Gabryelak T. Effects of tannins on Chinese hamster cell line B14. *Mutat Res* 2003; **539**: 127-35.

[9] Labieniec M, Gabryelak T, Falcioni G. Antioxidant and pro-oxidant effects of tannins in digestive cells of the freshwater mussel *Unio tumidus*. *Mutat Res* 2003; **539**: 19-28.

[10] Bolognesi C, Fenech M. Mussel micronucleus cytome assay. *Nat Protoc* 2012; **7**(6): 1125-37.

[11] Eck-Varanka B, Horváth E, Paulovits G, Kováts N. Comparative assessment of the mussel micronucleus test *vs*. bacterial bioassays for genotoxicity testing of benzo[a]pyrene. *Hung J Ind Chem* 2014; **42**(1): 1-5.

[12] Gačić Z, Kolarević S, Sunjog K, Kračun-Kolarević M, Paunović M, Knežević-Vukčević J, et al. The impact of *in vivo* and *in vitro* exposure to base analogue 5-FU on the level of DNA damage in haemocytes of freshwater mussels *Unio pictorum* and *Unio tumidus*. *Environ Pollut* 2014; **191**: 145-50.

[13] Vuković-Gačić B, Kolarević S, Sunjog K, Tomovi J, Knežević-Vukčević J, Paunović M, et al. Comparative study of the genotoxic response of freshwater mussels *Unio tumidus* and *Unio pictorum* to environmental stress. *Hydrobiologia* 2014; **735**: 221-31.

[14] Kolarević S, Kračun-Kolarević M, Kostíć J, Slobodnik J, Liška I, Gačić Z, et al. Assessment of the genotoxic potential along the Danube River by application of the comet assay on haemocytes of freshwater mussels: The Joint Danube Survey 3. *Sci Total Environ* 2016; **540**: 377-85.

[15] Economou G, Travlos IS, Folinas A, Karamanos AJ. Greek oregano (*Origanum vulgare* ssp. hirtum) as allelopathic plant. *J Food Agric Environ* 2007; **5**(1): 348-51.

[16] Woźnicki P, Lewandowska R, Brzuzan P, Ziomek E, Bardega R. The level of DNA damage and the frequency of micronuclei in haemolymph of freshwater mussels *Anodonta woodiana* exposed to benzo[a]pyrene. *Acta Toxicol* 2004; **12**(1): 41-5.

[17] Gustafson LL, Stoskopf MK, Bogan AE, Showers W, Kwak TJ, Hanlon S, et al. Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a freshwater bivalve (Mollusca: Unionidae). *Dis Aquat Organ* 2005; **65**: 159-65.

[18] Kalaiselvi V, Binu TV, Radha SR, Vijayakumari B. Antioxidant activity of various leaf extracts of *Mimusops elengi* L. *Human J* 2015; **3**: 231-7.

[19] Azrul M, Nurulaini R, Adzemi MA, Marina H, Effendy AWM. Tannins quantification in *Terminalia catappa* leaves extract and antihelmenthic potential evaluation. *J Nat Prod* 2014; **7**: 98-103.

[20] Rice EW, Bridgewater L. *Standard methods for the examination of water and wastewater.* 22 ed. Washington, D.C.: American Public Health Association, American Water Works Association; 2012.

[21] Juan M, Casas JJ, Elorrieta MA, Bonachela S, Gallego I, Fuentes-Rodríguez F, et al. Can submerged macrophytes be effective for controlling waterborne phytopathogens in irrigation ponds? An experimental approach using microcosms. *Hydrobiologia* 2014; **732**: 183-96.

[22] Švanys A, Paškauskas R, Hilt S. Effects of the allelopathically active macrophyte *Myriophyllum spicatum* on a natural phytoplankton community: a mesocosm study. *Hydrobiologia* 2014; **737**: 57-66.

[23] Vanderstukken M, Declerck SAJ, Decaestecker E, Muylaert K. Long-term allelopathic control of phytoplankton by the submerged macrophyte *Elodea nuttallii*. *Freshwater Biol* 2014; **59**(5): 930-41.

[24] Gette-Bouvarot M, Mermillod-Blondin F, Lemoine D, Delolme C, Danjean M, Etienne L, et al. The potential control of benthic biofilm growth by macrophytes - a mesocosm approach. *Ecol Eng* 2015; **75**: 178-86.

[25] Labieniec M, Gabryelak T. Antioxidative and oxidative changes in the digestive gland cells of freshwater mussels *Unio tumidus* caused by selected phenolic compounds in the presence of H_2O_2 or Cu^{2+} ions. *Toxicol In Vitro* 2007; **21**: 146-56.

[26] Kato-Noguchi H, Moriyasu M, Ohno O, Suenaga K. Growth limiting effects on various terrestrial plant species by an allelopathic substance, loliolide, from water hyacinth. *Aqua Bot* 2014; **117**: 56-61.

[27] Xian Q, Chen H, Zou H, Yin D. Allelopathic activity of volatile substance from submerged macrophytes on *Microcystin aeruginosa*. *Acta Ecol Sin* 2006; **26**: 3549-54.

[28] Le Thi H, Lin CH, Smeda RJ, Leigh ND, Wycoff WG, Fritschi FB. Isolation and identification of an allelopathic phenylethylamine in rice. *Phytochemistry* 2014; **108**: 109-21.