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# SUPPLEMENTARY MATERIAL

## Antioxidant volatiles of the freshwater bryozoan *Hyalinella punctata*

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Abstract

Two volatile samples of the bryozoan *Hyalinella punctata* (seasonally collected from the same locality) were isolated (hydrodistillation, Clevenger apparatus), identified (GC and GC-MS) and *in vitro* screened (EPR) for anti-hydroxyl radical activity. The main components of sample **1** (2-ethyl-1-hexanol 37.00%, dodecanol 21.40% and hexanal 8.40%) and sample **2** (2-ethyl-1-hexanol 30.50%, 7-tridecanol 24.60% and 1-hexadecanol 11.80%) were relatively similar. However, more components were present in the sample **2** (17 and 25, respectively). EPR measurements showed significant anti-hydroxyl radical activity of the both samples (75.00±6.00% and 87.00±8.00%, respectively) whereas the generation of other types of free radicals in reaction with hydroxyl radicals was not observed. According to the best of our knowledge, this is the first report of 31 organic compounds for the phylum Bryozoa. Alcohols, aldehydes, ketones, esters and ethers of lower molecular mass seem to be characteristic for the volatiles of these organisms commonly known as moss animals.

**Keywords:** Invertebrata; volatile organic compounds; GC & GC-MS; EPR; anti-hydroxyl radical activity

## Experimental

### *Biological material*

The samples of *Hyalinella punctata* (Hancock, 1850) were collected in Belgrade (the river Danube, Serbia) in November 2011 and May 2012, respectively. Voucher specimen has been deposited in the Zoology Collection of the Department of Biology and Ecology of the University of Novi Sad, Serbia (BRY 003).

### *Isolation of volatile components*

After carefully cleaning from contaminants, the bryozoan samples were lyophilised and used for the hydrodistillation (60 g). They were ground and steam distilled in a Clevenger apparatus for about two hours to obtain colourless volatiles dispersed in water. That is indeed the reason why the yield could not be determined.

### *Identification of volatile components*

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) analyses were performed using an Agilent 7890A GC equipped with inert 5975C XL EI/CI MSD and FID detector connected by capillary flow technology 2-way splitter with make-up. A HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm) was used. The GC oven temperature was programmed from 60°C to 300°C at a rate of 3°C/min and hold for 10 min. Helium was used as the carrier gas at 16.255 psi (constant pressure mode). The samples were analysed in the splitless mode. The injection volume was 1 μL. GC detector temperature was 300°C. MS data was acquired in EI mode with scan range 30-550 *m/z*, source temperature 230°C, and quadrupole temperature 150°C; solvent delay was 3 min. The components were identified based of their retention index and comparison with reference spectra (Wiley and NIST databases) as well as by retention time lock (RTL) method and RTL Adams data base. The retention indices were experimentally determined using the standard method (Van Den Dool & Kratz 1963) involving retention times of *n*-alkanes, injected after the each sample separately under the same chromatographic

conditions. The percentage (relative) of the identified compounds was computed from their GC peak area. The quantitative composition of the samples was GC (FID) analysed by internal normalisation assuming an identical mass response factor for all compounds. In this study, only those components present in the samples in amounts higher than 0.1% were taken into consideration.

***Determination of anti-hydroxyl radical activity of volatile components***

Hydroxyl radicals were generated using the standard Fenton reaction (0.2 mM $FeSO_4$ and 0.2 mM $H_2O_2$) in the presence of 0.1 M DEPMPO (5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide) (Enzo Life Sciences, Lausen, CH) (Savić & Mojović 2012). Solutions were prepared using deionised water of resistivity not less than 18.2 MΩ cm. The spin-trap DEPMPO was purified according to the procedure proposed by Jackson et al. (2002). The applied sample volume was 1 µL, while the incubation time was 10 min. Conditions for spectral recording of DEPMPO spin-trap adducts were: scan range 200 G, microwave frequency 100 kHz, modulation amplitude 2 G, microwave power 10 mW and time constant 0.032 s. EPR spectra were recorded using a Varian E104-A X-band EPR spectrometer (Varian, Palo Alto, CA, USA) at room temperature (22 °C). The spectra were processed by EW software (Scientific Software, Bloomington, IL, USA), while the additional analyses were performed in self developed scripts for MatWorks Matlab 2007. The operations done by script involve the subtraction of the baseline (approximated with the smoothing spline) and measuring of the peaks height.

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