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The Selection of Oat Extract with Content of Suitable Avenanthramides for Preparation of "Green Synthesis" Products, Poses Increased Biological Activity †

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Abstract: Avenanthramides (AVNs) are secondary metabolites category, produced exclusively by genus *Avena sp*. AVNs are amides of anthranilic acid with various polyphenolic acids and express antioxidant, anti-inflammatory and antineoplastic effect. The limited largest of the AVNs allows the increase of biological activity by dimerization or by preparation of nanoparticles via green synthesis. For green synthesis are suitable oat extracts with maximal AVNs content. The maximal content of AVNs was observed on the 8-th day of germination for oat variety TATRAN—610.13 μg/g of dried material. This water-methanol extracts were subjected to preparation of green synthesis products by reaction with $AgNO_3$.

Keywords: oat; avenanthramide; HPLC; green synthesis

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1. Introduction

The motivation for this work was aveanthramides, which were first isolated from oat grains in 1986 [1]. Oats contain a unique group of approximately 40 different types of AVNs that consist of an anthranilic acid derivatives and hydroxycinnamic acid derivatives [2]. They have been classified according to nature of the alkaloid: anthranilic acid (type 1), 5-hydroxyanthranilic acid (type 2) and 5-hydroxy- 4-methoxyanthranilic acid (type 3), and that of the linked cinnamic acids: *para*-coumaric acid (type p), caffeic acid (type c), ferulic acid (type f) [1]. Three avenantramides are most represented in the oat grains: N-[3′, 4′-dihydroxy-(E)-cinnamoyl]-5-hydroxyanthranilic acid (2c), N-[4′-hydroxy-(E)-cinnamoyl]-5-hydroxyanthranilic acid (2p) and N-[4′-hydroxy-3′-methoxy-(E)-cinnamoyl]-5-hydroxyanthranilic acid (2f). They act as antipathogens (phytoalexins) produced by the plant in response to exposure to pathogenic micro-organisms [3,4]. The avenanthramides exhibit significant biologically properties, such as antioxidant activity in and in vivo, antiproliferative, anti-atherogenic, anti-inflammatory and antipruritic effects [5,6]. These low-molecular-weight compounds have been found in various concentrations in oat grain [7,8]. Our work is focused on selection an oat variety with a high content of avenanthramides in the grain, then monitoring the formation of these substances during germination for green synthesis. It is well known, that nanoparticles, prepared from plant extracts express increases pro-oxidant activity, what is one of the co-mechanisms of antimicrobial and cytostatic effect.

2. Materials and Methods

2.1. Chemicals

Formic acid (HCOOH) was obtained from Mikrochem (Bratislava, Slovakia). Acetonitrile and methanol (both gradient grade) were obtained from Centralchem (Bratislava, Slovakia). Ultrapure water was prepared using Simplicity UV device. All commercially available standards of AVNs—A (2p), B (2f) and C (2c) were obtained from Merck/Sigma Aldrich/(Darmstadt, Germany) with declared purity of 98%.

2.2. Plant Materials

The oat variety TATRAN was selected from 96 oats varieties, cultivated in the experimental area of the Research and Breeding Station at Vígľaš-Pstruša of the National Agricultural and Food Centre, Research Institute of Plant Production Piešťany, Slovak Republic, harvest in year 2019, 2020 and 2021.

2.3. Germination and Sample Processing

Oat grains of the TATRAN variety were stored in a refrigerator. For germination 10.0 g of grains were weighed for each harvest and after washing, they were soaked in distilled water for 4 h at a laboratory temperature of 25 °C. The grains prepared in this way were placed in a single layer on filter paper in cultivation vessels. During the germination period, humidity was maintained by regular washing with distilled water. The germinated material was collected successively on the first, second, etc. up to the tenth day. After rinsing with distilled water and drying with paper cotton, it was weighed and stored in a deep-freezer box, at −80 °C. Subsequently, the plant material was dried by lyophilisation, at −50 °C to a constant weigh. After grinding, the samples were stored in a freezer at −20 °C until extraction. A methanol solution in ultrapure water (70% *v*/*v*) containing 1.1% formic acid was used for extraction. The ratio of lyophilized material and extraction agent was 1:5. The extraction was supported by ultrasound for 15 min at a temperature of 50 °C. After centrifugation for 15 min at 13,000 rpm, the supernatant was obtained, which was subsequently stored in a freezer (−20 °C) until HPLC analysis.

2.4. HPLC

HPLC analyzes of the extracts were performed on a Waters instrument equipment with Separations Module e2695, DAD detector 2998 and software Empower 3, on a reversed phase C-18 with hard-core particles (Cortecs 4.6 × 100 mm, 2.7 μm particle size) by step gradients. Component A of the mobile phase was 0.1% aqueous solution of formic acid, component B was acetonitrile with 0.1% formic acid, flow 1 mL/min., column temperature 35 °C, injection 10 μL, UV detection at 320 nm, UV spectra 210–400 nm. A shorter gradient was used to analyze grain extracts: 0–2 min. 12% B; 2–16 min. 12–28% B; 16–19 min. 100% B. A longer gradient was used for analysis of sprout extracts: 0–2 min. 12% B; 2–26 min. 12–40% B; 27–30 min. 100% B. Each experiment and each HPLC analysis was performed in triplicate.

2.5. The Preparation of Silver Nanoparticles

The selected water-methanol extracts with different AVNs content were subjected to preparation of green synthesis products—silver nanoparticles by reaction with $AgNO_3$ as follows: stirring a mixture of 1mM solution of $AgNO_3$ and prepared extract in ratio 9:1 during 30 min at 80 °C, followed by cooling and storing in the fridge at 4 °C.

2.6. Antioxidant and Pro-Oxidant Activity Determination

The modified DPPH/FRAP method on microplate was used to simultaneously measure the antioxidant and pro-oxidant properties of green synthesis products as described by Maliar et al. [9]. Green synthesis products solutions were diluted to achieve final

concentrations ranging from 1024 µg/mL to 2 µg/mL of dry matter. The microplate preparation involved tested samples dilution and applying conversion standards, assays started with adding 0.4 mM DPPH and FRAP reagents, achieving a final concentration of 0.3 mM. The microplate was incubated for 10 min for DPPH and 1 h for FRAP at room temperature, followed by measurements at 520 nm and 630 nm for DPPH and FRAP, respectively. $DPPH_{50}$ and $FRAP_{50}$ values were calculated. Each experiment was repeated three times with eight replicates, with results presented as mean ± SD. Statistical significance was determined using the Spearman method (* $p < 0.1$).

3. Results and Discussion

3.1. HPLC Analysis Plant Materials

In the first part of the experiments, grains of 96 different genotypes of *Avena* sp. were analyzed. Detailed results and their statistical processing, which includes the influence of genotype and meteorological influences on the content of avenanhramides in the grain, are the subject of a research work that is in preparation. The content of the major avenanthramides expressed as the sum of avenanthramides A (2p), B (2f) and C (2c) in µg/g of grain for 96 varieties harvested in 2020 is shown in appendix Figure A1. Based on the results, we can mark eight naked oat varieties that reached the content of the majority AVNs above 150 µg/g of grains. These varieties are marked with an asterisk in the picture: TAIDON, POMOR (Russian varieties), OLIVER, PATRIK, HYNEK (Czech varieties), BAY YAN (Chinese variety) and TATRAN, DUNAJEC (Slovak varieties). The TATRAN variety was selected for the study of the dynamics of avenanthramide formation during germination. On the Figure 1 is presented a comparison of the HPLC chromatogram of extracts from the dry grain (a) and after 8 days of germination (b).

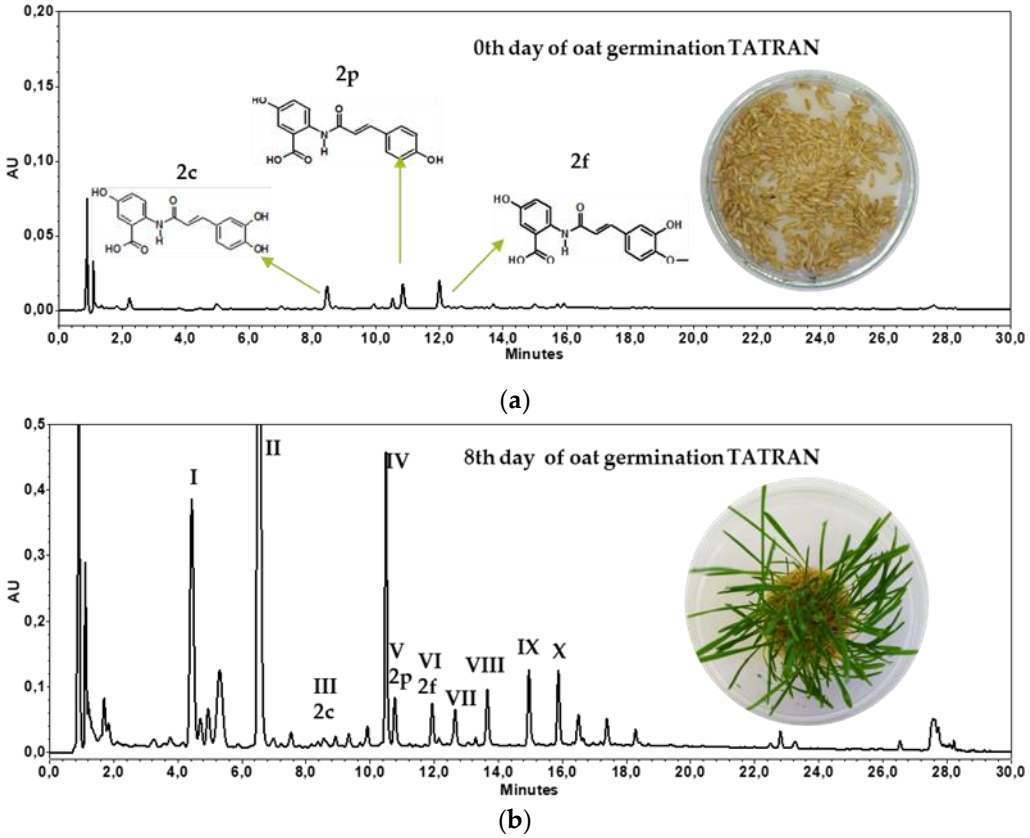

Figure 1. Chromatogram of HPLC analysis of dry grain extract—0th day of oat germination (**a**) and sprouted grain extract—8th day of germination (**b**).

The detected peaks were identified by comparing their retention times and ultraviolet (UV) spectra with the avenanthramide standards A (2p), B (2f and C (2c). The UV spectra of the detected peaks in the extract of germinated grains (peaks I–X) are shown in appendix Figure A2. The agreement to retention times and UV spectra was found for peak III, which was identified as avenantramide 2c, similarly peak V as avenantramide 2p, and finally peak VI as avenantramide 2f. Peak I and II they don't have a characteristic course of UV spectra, as they have up three maxima in the UV region and are probably flavonoids. Peak IV it has a similar UV spectrum to the major avenanthramides. UV spectra of peak VII–X have a second maximum shifted to a wavelength of 350–360 nm, which according to publication [10] belong avenanthramides with a longer chain. For major AVNs was realized quantification by external calibration by avenantramide standards A (2p), B (2f) and C (2c), other avenanthramides were analyzed semi-quantitative. The maximal content of total AVNs was found on the 8th day of germination for variety TATRAN, 610.13 μg/g of dried material.

3.2. Antioxidant and Pro-Oxidant Activity of Silver Nanoparticles

The oat variety TATRAN was only chosen for preparation of the water-methanol extracts in two different days of germination process (the 1-st and the 8-th day). Green synthesis was carried out on these two extracts samples only, to obtain only two, different samples of nanoparticles as green synthesis products by procedure mentioned therein before. The samples were marked as follows: **GS**_TATRAN_day1 and **GS**_TATRAN_day8, whereas "mother" extracts were marked as **_E**_TATRAN_day1 and **E**_TATRAN_day8. The Figure 2 presents the antioxidant and pro-oxidant activity of mentioned extract and green synthesis samples.

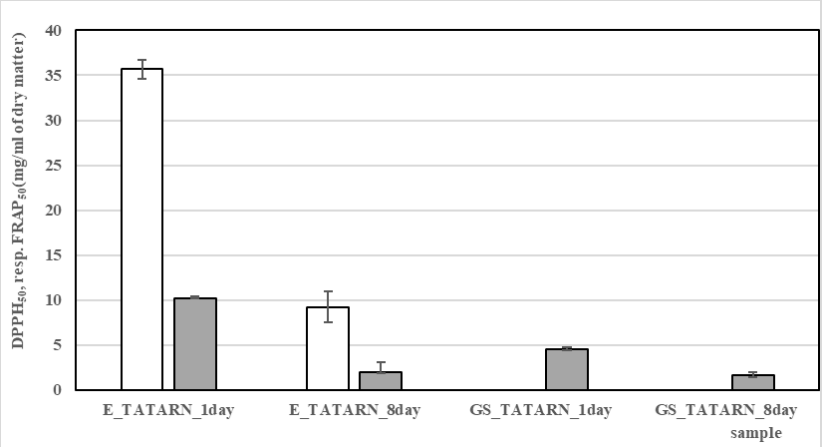

Figure 2. The antioxidant and pro-oxidant activity of following samples: E_TATRAN_day1, E_TATRAN_day8, GS_TATRAN_day1 and GS_TATRAN_day8, expressed as $DPPH_{50}$ and $FRAP_{50}$ parameters.

From the Figure 2 it is evident, that the samples from the 8-th day of germination is more potent (lower $DPPH_{50}$ and $FRAP_{50}$ value indicated more intensive effect) approximately three times from both antioxidant—prooxidant point of view. This more intensive effect does exist probably due to higher content of avenanthramides or minoritlly other polyphenols in the water-methanol extract of germinated oat, variety TATRAN, what were documented and supported by HPLC analysis. Interesting is the lack of antioxidant effect of both green synthesis samples due to lost of aromatic hydroxy groups, which are the subject of coordination bond after splitting hydrogen atom from hydroxy group by HAT mechanism. On the other hand, this coordination compounds—nanoparticles as products of green synthesis, expressed significant measure of pro-oxidant effect, what is currently associated with antibacterial, antimicrobial or cytostatic effect. The presumption

is, that avenanthramides, dominant secondary metabolite, presented in water-methanol extract of germinated oat, are linked in the complex with silver, which is reduced from plus one oxidation status to zero, as it was published for similar green synthesis with quercetin [11]. Moreover, it was determined a significant antibacterial activity with MIC value under 4 mg/L on broad spectrum of bacterial strains (yet not published data). The molecules of avenanthramides are suitable for coordination due to minimally 4 heteroatoms in the structure, and thus on avenanthramides rich extracts are suitable for green synthesis with significant perspective of biological activity, what is a subject of our future research.

4. Conclusions

This short research study clearly demonstrates the perspectives of AVN rich extracts, and green synthesis products, prepared from these, as a base for systematic research biologically and therapeutically effective entities.

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