STUDY OF THE EFFECT OF PARACETAMOL BINDED IN POLYMERIC NANOPARTICLES ON DAFNIA MAGNA

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Abstract

Drugs are important xenobiotics in the environment. Their use increases with the growth of the human population, but also in agricultural primary production. Paracetamol (PAR) is a widely used analgesic and antipyretic and its production is still growing. Commonly available drug production technologies are being developed very intensively with nanotechnological modifications for their gradual and targeted release. Nanoparticles (ST/PAR) from starch were prepared: PAR (0, 1, 2, 3, 5 and 10 mg/L) was mixed with citric acid ester in a 1:8 v/v ratio for 30 min at 25 ºC. By the centrifugation (16.000 g, 30 min) ST/PAR were obtained in the pellet. The effect of PAR was studied on *Daphnia magna* Straus (Cladocera, Crustacea). Adult females (70–400 mg) were used for self-evaluation. The EC50 was 3.749 mg/L after 48 h of PAR treatment. Total protein values determined by Lowry method were between 0.5–2.2 mg/mL and by Bradford method between 190–676 mg/L. Antioxidant activity values determined by CUPRAC method were between 4–15 μg/mL GAE and by ABTS method ranged between 40–103 μg/mL GAE. PAR values were between 9–40 μM. Subsequently, the biological activity of the prepared nanoparticles was tested.

Keywords: ecotoxicology; chemical and biochemical analysis; nanomedicine

1. INTRODUCTION

Drugs are important xenobiotics in the environment. Their use increases with the growth of the human population, but also in primary agricultural production. Paracetamol (PAR) is a non-steroidal anti-inflammatory drug widely used [1]. PAR enters the aquatic environment with wastewater and thus affects aquatic organisms

[2,3]. There is little information on the effect of PAR on detoxification processes. PAR induces oxidative stress, can manifest in endocrine disruption [3,4] and affect the expression of selected genes [1].

The increase in their environmental concentrations in individual components of the environment is obvious, and with the use of modern analytical techniques, residues are detected in soil, surface water, sediments, groundwater, and marine ecosystems [5]. It is highly probable that increasing concentrations of PAR in the aquatic environment and its transformation by the food chain may affect a number of biochemical and physiological processes determining the growth and development of aquatic biocenosis [6]. The importance of PAR monitoring stems from the results of monitoring studies that have shown its high concentrations in wastewater [7,8]. Wastewater treated plants cannot effectively eliminate most medicines, so they are a particularly significant environmental risk for aquatic ecosystems [9]. Little relevant information is available, in particular on the sublethal effects of PAR on aquatic organisms, in addition, the effects of nanoparticle-bound drugs (NPs) are completely unknown. Cladocera *Daphnia magna* Straus (Cladocera, Crustacea) is not only an important model species in ecotoxicology, but also a planktonic crustacean, which is a key consumer of algae and cyanobacteria, as well as food for many fish [10]. It thus serves as a link between primary phytoplankton production and higher trophic levels in the aquatic ecosystem. Commonly available drug manufacturing technologies are being developed very intensively by nanotechnological modifications for their gradual and targeted release [11,12]. Nanotechnological modification of PAR could find application in the treatment of cancer [13]. The aim of this project is to extend existing knowledge about the toxicity of PAR and its nanoform (NPs-PAR) to aquatic organisms. The determined values of acute and chronic toxicity of the monitored substances will allow the estimation of the risk to the aquatic environment.

2. MATERIAL AND METHODS

Biological experiments

The solution and elaboration of the topic presupposes the mastery of selected standard ecotoxicological tests on aquatic crustaceans – Cladocera *Daphnia magna*. Tests according to ČSN EN ISO 6341 (75 7751) was used to evaluate ecotoxicity. Immobilization test for *Daphnia magna* Straus (Cladocera, Crustacea) - Acute toxicity test and ČSN ISO 10706 (75 7752 - Determination of chronic toxicity of substances for *Daphnia magna* Straus (Cladocera, Crustacea). The acute test on Clacodera *D. magna* pearls consists in monitoring their immobilization in a selected range of test substance concentrations at exposure for 24 and 48 hours.

Chemical analysis

Amount of 150 µL LOWRY solution I (7 mM Na-K tartrate, 0.81 M sodium carbonate, 0.5 M NaOH) was pipetted into cuvettes and then in 9th period (81 s) 9 µL of barley extract was added. Afterwards, LOWRY solution II (70 mM Na-K tartrate, 40 mM $CuSO_4$ and 0.1 M NaOH) was added in 42nd period (378 s) in volume of 20 µL and finally 172 µL of LOWRY solution III (1:15 Folin-Ciocalteu reagent: H_2O) in 90th period (810 s) was added. Reaction was evaluated as a response in miliabsorbance units between period 93 to 96 (837 seconds to 864 seconds from pipetting of LOWRY I solution). Wavelength: 660 nm. Quality of reagents were controlled via regulation diagram for 188 mg/L bovine serum albumin standard with good reproducibility of reaction (8400 ± 258 mAU, RSD 3.07 %, 8 days).

PARACETAMOL methodology. Measurement in stems - Approximately 1 g of sample for each concentrations was crushed with sea sand and 2 mL of PBS, pH = 7 was added. Crushed stems and buffer were quantitatively transferred to 2 mL eppendorf microtubes and centrifuged for 30 minutes for 16.900 g. Supernatant was pipetted into clear microtubes and concentration of paracetamol was measured. Amount of 320 µL of Paracetamol solution I (17.9 mM sodium nitroprusside and 13.4 mM $K_3Fe(CN)_6$) was pipetted into cuvettes and then) 40 µL of barley extract was added. Afterwards, 40 µL of Paracetamol solution II (2 M NaOH) was

added in 43nd period (559 seconds). Reaction was evaluated as a response in absorbance units in period 146 (1898 s from pipetting of Paracetamol solution 1). Wavelength: 700 nm. 270 µL CUPRAC solution 1 (10 mM $CuSO_4$; 7.5 mM neocuproine in 99% ethanol and 1 M ammonium acetate in 1:1:1 ratio) was pipetted into cuvettes and then 9th period (81 s) 30 µL of sample was added. Reaction was evaluated as a response in miliabsorbance units between period 75 to 79 (675 s to 711 s from pipetting of PBM solution 1). Wavelength: 450 nm. Quality of reagents were controlled via regulation diagram for two concentrations of gallic acid; 25 µg/mL and 12.5 µg/mL. Based on 7 separate days of measurements, responses for concentration 25 µg/mL are in range of 58804 ± 1964 mAU, RSD 3.34 % and for concentrations 12.5 µg/mL are in range of 34461 ± 2360 mAU, RSD 6.85 %, which shows good reproducibility of reaction. 200 µl ABTS solution 1 (0.7 mM ABTS and 495 µM potassium persulfate) was pipetted into cuvettes and then in 9th period (81 s) 45 µL of sample was added. Reaction was evaluated as a response in miliabsorbance units between period 75 to 79 (675 seconds to 711 s from pipetting of PBM solution 1). Wavelength: 660 nm. Quality of reagents were controlled via regulation diagram for two concentrations of gallic acid; 11.5 µg/mL and 5.75 µg/mL. Based on 4 separate days of measurements, responses for concentration 11.5 µg/mL are in range of 11149 ± 2790 mAU, RSD 25.03 % and for concentrations 5.75 µg/mL are in range of 29636 ± 4209 mAU, RSD 14.20 %. Precision is lowered, because ABTS is based on decolourization of the reaction mixture, which is always connected with lower precision. 190 µl of Bradford solution I (0.1 g/L Coomassie Brilliant Blue; 8.5 % H_3PO_4; 3.4 % ethanol) was pipetted into cuvettes and then 10 µL of sample was added. Reaction was evaluated as a response in absorbance units in period 45 (598 s from pipetting of Bradford solution 1). Wavelength: 700 nm. 150 µL GST solution 1 (2 mM CDNB in 20 % methanol) was pipetted into cuvettes and then in 9th period (81 s) 45 µL of sample was added. Afterwards, GST solution II (12.5 mM GSH) was added in 42nd period (378 s) in volume of 20 µL. Reaction was evaluated as a response in miliabsorbance units in 80th (end of the reaction) and 42nd period (when GST solution II was added) and activity of GST was calculated via formula. PAR (0, 1, 2, 3, 5,10 mg/L) were used for acute toxicity testing. At the end of the test, *D.magna* was washed in distilled water and frozen at -20°C. Homogenized in 1 ml of water. PAR concentrations were determined photometrically at 700 nm. Total SH groups (Ellman's method at 405 nm), GSH, GSSG (at 450 nm), GST (at 480 nm) and metallothionein electrochemically by the Brdička method. Total protein levels were determined by pyrogallol, biuret and Bradford methods. In our experimental model, the acute toxicity of PAR was performed in three independent experiments (n = 3).

3. RESULTS AND DISUSSION

A photometric method based on a colour reaction was designed and tested for rapid analysis of PAR content in samples [14-17]. The method showed very good reproducibility and stability of the reaction, RSD ranged up to 5% (**Figure 1**). Subsequently, the method was applied to the determination of PAR in water and in cell homogenates of *D.magna*.

To date, very little attention has been paid to the evaluation of PAR toxicity to *D. magna*. Some selected summary information can be found in the work of Grung et al. [18]. Kim et al. found that after 21 days of NOEC exposure (5.72 mg/mL) no effects on reproduction were observed [10]. In a study by Daniel et al., no effect on reproduction was demonstrated in *D. magna* [19]. Even in the detailed assessment of wastewater no significant effects on biological assessment were found [20]. In a study, Ding et al observed chronic effects on reproduction at a concentration of 50 mg/mL [1]. In the case of acute exposure, an effect on the up-regulation of the HRP96, CYP360A8, CYP314, MRP4 and P-gp genes was observed. However, after 96 h, the expression was already reduced [1]. A detailed study of the effect of PAR was performed by Castro et al. In addition, he recommends assessing the reproduction of the offspring of the F-1 generation. There was an effect on maternal reproduction after PAR (F-1) exposure, although these effects were not very pronounced [21]. In our acute toxicity tests, we observed an EC50 of about 3.749 mg PAR/L (**Figure 2**).

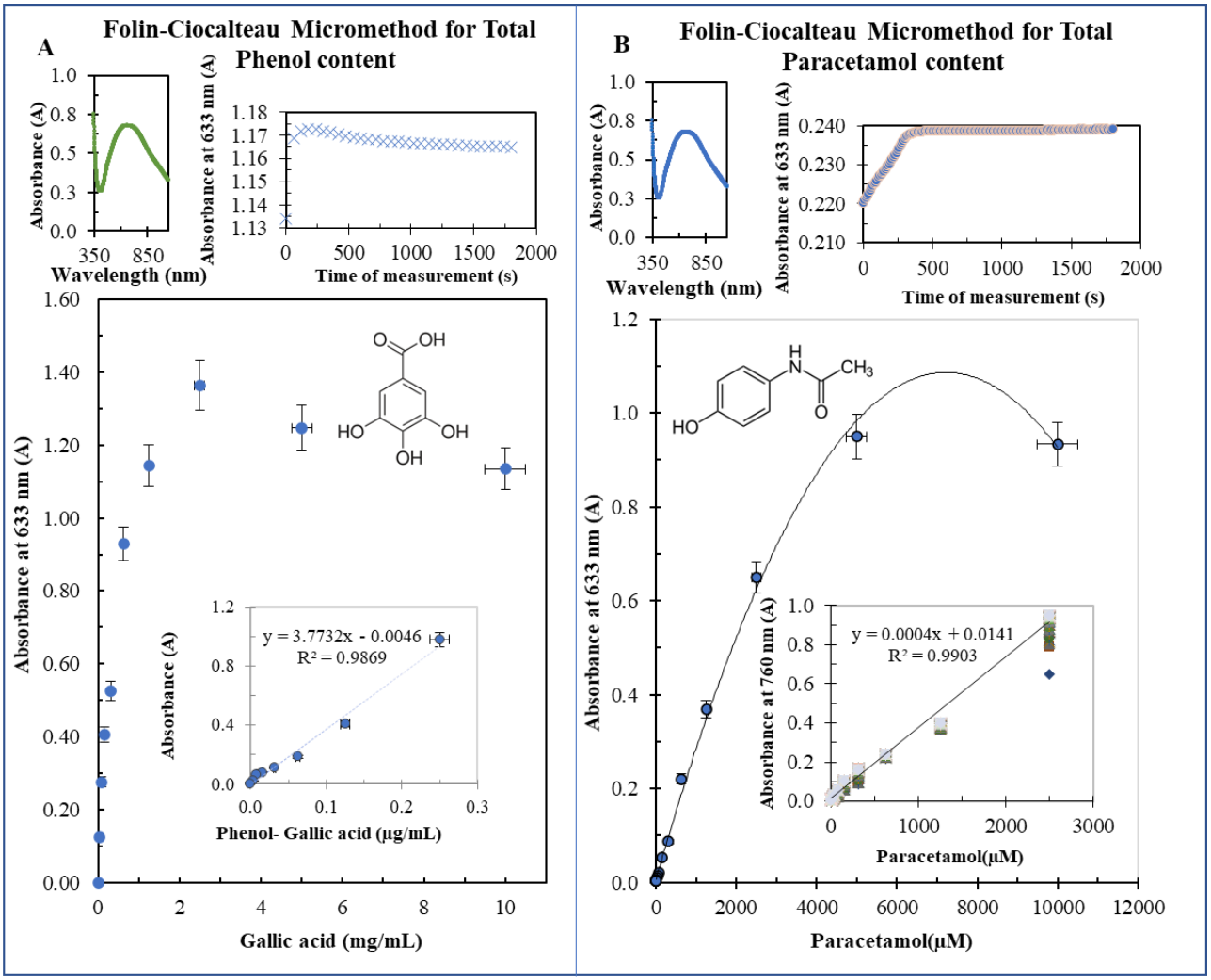

Figure 1 Typical dependence of absorbance on the concentration of gallic acid (GA) - A, and paracetamol (PAR) - B, determined by the Folin-Ciocalteau Micromethod. Typical VIS spectra of GA (5 mg/mL) and PAR (5 mM PAR). Stability of the reaction in time 0–2.000 s. Dependence of absorbance on the concentration 0–10 mg/mL GA and in the insert 0–0.3 µg/ml. Dependence of absorbance on the concentration of 0– 2.000 µM PAR in the insert 0–3.000 µM.

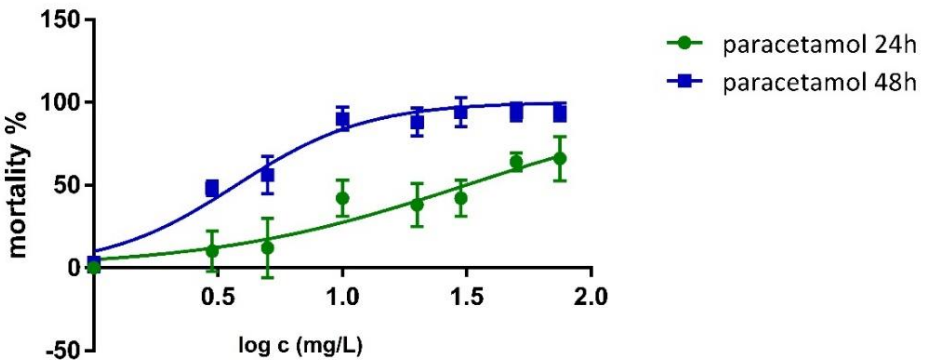

Figure 2 The EC50 for PAR in *D. magna* were determined at 24 and 48 h 21.48 (24.34 to 40.70) and 3.749 (3.283 to 4.282) mg/L, respectively

Table1 Chemical analysis of culture water after application of PAR. Each measurement was performed 5 times.

Sample name	Protein	sodium	potassium	chloride	pH	conductivity	density	redox potential	ammonia	electrochemical index	nitrates
PAR (1mg)	8.3	4.1	33.9	7.3	7.8	726.8	1.00028	19.7	415.4	16.2	1.4
PAR (2mg)	13.0	10.1	24.5	4.9	7.8	699.0	1.0003	21.9	314.6	14.2	1.3
PAR (5mg)	14.5	21.6	34.7	6.0	7.8	755.6	1.0003	22.1	480.7	14.5	4.9

Preparation of SPION starch nanoparticles

Starch (100 mg) was dissolved in 20 ml of distilled and deionized water at 80 °C under magnetic stirring. A 5 mL portion of solution containing 0.1 M Fe^{2+} and 0.2 M Fe^{3+} was poured into a prepared starch solution under vigorous stirring. The starch and iron ion mixture (25 mL) was then added dropwise into 250 mL of 0.1 M NaOH under vigorous mechanical stirring (2000 rpm/min) at 60 °C for 2 h. Around 50 wt % of water was evaporated, and the remaining solution was cooled to room temperature and allowed to stand 12 h. The gels formed were washed with deionized water until the pH became less than 8.5. Excess salt and ions were removed by using dialysis at 37 °C for 2-3 days against 5 L of distilled water. To cleave glycosidic bond and reduce the polymeric chain to an average molecular weight, the influence of oxidizing agent, used for cleavage of the polymeric starch chains, was investigated. H_2O_2 (5 mL of 0.46 M) was mixed with 20 mL (10 mg/mL) of starch-coated SPION. Starch citrate (0.8 mg/mL) was mixed with paracetamol. The concentration of paracetamol in the reaction mixture was 3205 mM. The reaction volume was 1.6 mL. The 2 mL tube was left on the rotator for 2 hours. Subsequently, it was centrifuged (5 min, 14.000 g) and the paracetamol concentration was measured. Subsequently, 1.6 mL of water were added, and the mixture was allowed to stir (40 rpm), after which the concentration of the supernatant was measured again. Samples were taken according to 0, 30, 60, 90, 120 and 150 min. The binding of 99% paracetamol to starch citrate was demonstrated, paracetamol was not released from the binding during the experiment [11,12].

4. CONCLUSION

The acute toxicity of PAR to *D. magna* was monitored and subsequently starch nanoparticles were prepared. These nanoparticles were modified with PAR. We found the binding of PAR to the surface of the prepared nanoparticles and PAR was gradually released from the nanoparticles.

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