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Antigenotoxic, anti-photogenotoxic and antioxidant activities of natural naphthoquinone shikonin and acetylshikonin and *Arnebia euchroma* callus extracts evaluated by the *umu*-test and EPR method

Agata Skrzypczak [a,*], Natalia Przystupa [a], Anna Zgadzaj [a], Andrzej Parzonko [b], Katarzyna Sykłowska-Baranek [c], Katarzyna Paradowska [d], Grzegorz Nałęcz-Jawecki [a]

[a] Department of Environmental Health Sciences, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland
[b] Department of Pharmacognosy and Molecular Basis of Phytotherapy, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland
[c] Department of Biology and Pharmaceutical Botany, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland
[d] Department of Physical Chemistry, Faculty of Pharmacy, Medical University of Warsaw, Banacha 1, 02-097 Warsaw, Poland

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ABSTRACT

The aim of this study was to evaluate the antigenotoxic and antioxidant potential of shikonin (SH), acetylshikonin (ACS) and *Arnebia euchroma* callus extract (EXT). The antigenotoxic activity was investigated by the *umu*-test as the inhibition of the SOS system induction caused by genotoxic chemical agents — 4-nitroquinoline oxide and 2-aminoanthracene. Moreover the ability of SH, ACS and EXT to prevent photogenotoxicity triggered by chlorpromazine under UVA irradiation was measured. The cytotoxicity of EXT toward V79 Chinese hamster cell line was additionally assessed. Shikonin and acetylshikonin had no effect on 4-NQO induced genotoxicity whereas EXT demonstrated an unclear effect. The protection against 2AA induced genotoxicity was observed for all tested substances. The highest protection was demonstrated for EXT with inhibition of 66%. SH and ACS reduced 2AA genotoxicity with inhibition of about 60%. Under UVA the strongest and dose-dependent activity was observed for EXT. Acetylshikonin was a weak anti-photogenotoxin whereas shikonin had no clear effect. EXT was highly cytotoxic toward the V79 cell line — the cells' morphology was affected seriously and apoptosis was impacted. The antioxidant activity of SH, ACS and EXT was studied by means of electron paramagnetic resonance spectroscopy using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. All three samples exhibited radical scavenging properties.

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1. Introduction

Genotoxic factors are ubiquitous in the human environment due to increasing pollution. Since somatic cell mutations are considered to be involved in the early, initiation stage of carcinogenesis, there is considerable interest in identifying agents which display anti-cancer activity. Much attention is drawn to the natural compounds and plants' extracts especially those used in traditional medicine (Bhatia et al., 2011; Verschaeve and Van Staden, 2008). Naturally occurring antigenotoxins appear to be beneficial and at the same time safe bioactive constituents of foods or individual diet supplements which may inhibit or delay the formation of cancer (Thèriault et al., 2006). The research on the structure–activity relationship indicates that some chemical groups may demonstrate particular activity against mutagen agents. Such a group seems to be for example polyphenols or naphthoquinone derivatives (Thèriault et al., 2006; Kumar et al., 2013). The mechanism of action of such compounds can vary: free radical scavenging, p450 enzymes inhibiting which does not allow for the transformation of the precarcinogen in the active form, inactivating or blocking the mutagen molecule or protecting the DNA chain. There are also many compounds with several simultaneous modes of action (Słoczyńska et al., 2014).

Shikonin (SH) and its derivatives are the naphthoquinone red pigments widely occurring in species of the *Boraginaceae* family. The extracts from the plants included in this family have long been used in traditional medicine as wound healing and anti-inflammatory agents (Papageorgiou et al., 1999). Shikonin and its enantiomer alkanin are potent pharmaceutical substances with many well-established biological properties like antimicrobial, anti-inflammatory, antioxidant, anticancer, antithrombotic and wound healing (Papageorgiou et al., 1999). Shikonin is commercially obtained from *Lithospermum* cultures (Seo et al., 1992) and used as a natural pigment for food coloring and cosmetics (Papageorgiou et al., 1999). The cytotoxicity of SH and its derivatives against cancer cells is currently of a special interest and the results obtained to date are very encouraging (Andújar et al., 2013; Damianakos et al., 2012). On the other hand the antigenotoxic properties of these compounds have not been widely investigated so far. Several reports published in the 1980s revealed that alkanin acted

* Corresponding author.
E-mail address: agata.skrzypczak@wum.edu.pl (A. Skrzypczak).

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antimutagenic against benzo[a]pyrene and 4-nitroquinoline N-oxide toward *Salmonella typhimurium* TA98 in Ames test (Papageorgiou et al., 1999; Bhatia et al., 2011). However the potency of naphthoquinone to diminish the photogenotoxic effect of chemicals has not been discussed as yet.

The aim of this study was to evaluate the antigenotoxic and antiphotogenotoxic potency of naturally occurring naphthoquinone compounds: shikonin and acetylshikonin as well as *Arnebia euchroma* extract. *A. euchroma* is from the *Boraginaceae* family and contains high concentrations of naphthoquinone derivatives. The evaluation was made by the bacterial *umu*-test toward known direct and indirect genotoxic agents — 4-nitroquinoline N-oxide and 2-aminoanthracene respectively and the photogenotoxic agent chlorpromazine. The cytotoxicity assays with Chinese hamster lung fibroblast cell line V79 were also carried out. The morphology observation was made as well as calcein and propidium iodide staining. In addition the free radical scavenging capacity of the tested compounds and the extract was assessed by the EPR method using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The DPPH scavenging test performed by UV–vis spectrophotometer is generally the most popular and as such the chosen method. However in the case of color antioxidant compounds such as shikonin derivatives or flavonoids having spectra that overlap the absorbance of DPPH the use of electron paramagnetic resonance (EPR) spectroscopy is more applicable and preferred.

2. Materials and methods

2.1 . Chemicals

4-nitroquinoline N-oxide (4-NQO) (CAS no. 56-57-5), 2-aminoanthracene (2-AA) (CAS no. 613-13-8), and chlorpromazine (CPZ) (CAS no. 200-701-3) were purchased from Sigma-Aldrich (Poznań, Poland). 4NQO and 2-AA were dissolved in DMSO and CPZ was dissolved in PBS. Shikonin (SH) (CAS no. 54952-43-1) was purchased from Sigma-Aldrich (Poznań, Poland) and dissolved in methanol. Nicotinamide adenine dinucleotide phosphate (NADP) (CAS no. 24292-60-2) was purchased from MP Biomedicals (Warsaw, Poland). D-glucose 6-phosphate (G-6-P) disodium salt hydrate (CAS no. 3671-99-6), and 2-nitrophenyl β-D-galactopyranoside (ONPG) (CAS no. 369-07-3), which was the β-galactosidase enzyme substrate were purchased from Sigma-Aldrich (Poznań, Poland). Methanol (CAS no. 67-56-1) was purchased from Merck (Warsaw, Poland). DMSO (CAS no. 67-68-5) was purchased from POCH (Gliwice, Poland). Sodium lauryl sulfate (CAS no. 151-21-3) was purchased from BDH chemicals (Warsaw, Poland). Propidium iodide solution (1.0 mg/mL in water) (CAS no. 25535-16-4) was from Sigma-Aldrich (Poznań, Poland) and calcein AM Fluorescent Dye (CAS no. 354216) was from BD Biosciences (Warsaw, Poland). The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (CAS no. 1898-66-4) was purchased from Sigma-Aldrich (Poznań, Poland).

2.2 . Extraction of acetylshikonin (ACS)

Acetylshikonin was extracted from roots of *Lithospermum canescens* (*Boraginaceae*) collected in Canada. A voucher specimen of *L. canescens* (Michx.) Lehm. was deposited at the W. P. Fraser Herbarium (SASK), University of Saskatchewan. Saskatoon, Saskatchewan, Canada (accession no. 94815). Dried crumbled roots were extracted for 72 h with n-hexane using Soxhlet apparatus. From the resulted evaporated to dryness red residue, among others, acetylshikonin were isolated and its structure was determined by NMR methods (Pietrosiuk and Wiedenfeld, 2005).

2.3. A. euchroma callus extract preparation

A. euchroma callus extract was prepared from lyophilized callus tissue cultivated *in vitro* on solid MSA medium as described earlier (Sykłowska-Baranek et al., 2012). To obtain red dye fraction the powdered callus tissue was sonicated with n-hexane. The extraction was done for 15 min in 40 °C till the red color extinction. The extract was evaporated under reduced pressure. Dry residue was dissolved in methanol and analyzed using HPLC-UV–VIS method as described earlier by Pietrosiuk et al., 2006. The DIONEX HPLC system equipped with an automated sample injector (ASI-100) and UVD 340S detector was used. The gradient elution was applied: acetonitrile (0–40%) + 0.04 M orthophosphoric acid (100–60%) with the flow rate 1.5 mL/min, column EC 250/4.6 Nucleosil 120–127 mm C18 (Macherey-Nagel, Düren, Germany) and monitoring eluent at 215, 278, 514, and 320 nm. SH (Wako, Tokyo, Japan) and its two derivatives ACS and isobutyrylshikonin (IBS) isolated previously from natural roots of *Lithospermum canescens* (Pietrosiuk and Wiedenfeld, 2005) were used as standards and analyzed under the same conditions. Peaks were assigned by spiking the samples with the standards and comparison of the retention times and UV spectra. The results of HPLC analysis are mean ± SD of five subsequent analyses. The amount of active ingredients in the extract was evaluated: acetylshikonin 137.40 μg/mg ± 31.27; isobutyrylshikonin: 70.20 μg/mg ± 18.61. All chemicals except the standards were purchased in Sigma-Aldrich (Poznań, Poland).

2.4. Determination of the antioxidant properties by EPR

72 mg/L of *A. euchroma* callus extract (EXT), and 10 mg/L of SH or ACS were mixed with methanol solution (100–500 μl) of DPPH (1.3 mM). The mixture was shaken and left at room temperature at darkness for 45 min and then EPR spectra were recorded. The DPPH samples with added appropriate amounts of water were used as intensity standards. Results were expressed as Trolox equivalents (TE micromoles per ml) with the use of a previously prepared standard curve. The intensity was taken as the double integral of the spectra. All experiments were performed in triplicate.

EPR spectra were recorded on a Miniscope MS200 spectrometer (Magnettech GmbH). The decay of DPPH signal was monitored in time. The spectra were recorded with the following spectrometer settings: center field 334 mT, sweep range 8 mT, sweep time 30 s, microwave power 10 mW, modulation amplitude 0.1 mT.

2.5 . Antigenotoxic properties (umu-test)

2.5.1. Umu-test

Umu-test detects the induction of the SOS system in the strain *S. typhimurium* TA1535/pSK1002. SOS system is the bacterial response to the DNA-damaging agents. One of the genes involved in the SOS system is *umuC*. The bacteria used in the test carries the fusion of *umuC* and *lacZ* gene which is placed at the plasmid pSK1002. As the normal *lacZ* region is deleted β-galactosidase activity strictly depends on *umuC* expression. Therefore activity of β-galactosidase measured by the colorimetric assay with the enzyme substrate ONPG indicates the level of the SOS system induction level and the genotoxic potency of the tested compound (Oda et al., 1985; Reifferscheid et al., 1991). In this study the *umu*-test was carried out in the micro-plate variant according to the ISO guideline [ISO/DIS 13829, 2000]. β-galactosidase activity of the tested compound was presented in units according to the formula:

$$\beta\text{-gal units} = OD420_{SAMPLE} / OD600_{SAMPLE}$$

OD420 was the absorption at 420 nm and indicated the intensity of enzymatic reaction and OD600 indicated the bacteria growth as being the optical density at 600 nm. The measurements were performed with Asys UVM340 Hightech microplate spectrophotometer. In the analogous way the basic β-galactosidase activity for negative control (which was the solvent or water) was calculated. The induction ratio

IR was calculated as the β-galactosidase activity of the tested compound relatively to the negative control:

$$IR = \beta\text{-gal units}_{SAMPLE} / \beta\text{-gal units}_{CONTROL}$$

The 1.5 fold and greater increase of the β-galactosidase activity resulting in IR value of 1.5 and greater indicated the genotoxicity of the sample. The assay was quantitative and linear relationship between dose and effect can be observed due to the genotoxicity of the sample in particular range of concentration (Oda et al., 1985). The minimal genotoxic concentration can be thus established at IR = 1.5.

2.5.2. Bacterial strain

S. typhimurium TA1535/pSK1002 was purchased from Deutsche Sammlung von Mikroorganismen Und Zellkulturen GmbH (Germany).

2.5.3. Metabolic activation

Metabolic activation was obtained with s9 fraction prepared from livers of male Sprague–Dawley rats. Rats were pre-treated five days before the isolation with a single dose of Aroclor 1254 in soya oil (500 mg/kg body weight). After isolation the evaluation of cytochrome p450 level was performed and s9 aliquots were stored at −80 °C. For the *umu*-test s9 fraction was diluted in the bacteria culture and relevant cofactors (NADP and G-6-P) were dissolved in the bacteria medium according to the ISO guideline [ISO 13829].

2.5.4. Determination of antigenotoxicity by umu-test

The antigenotoxic potential was evaluated against the genotoxic action of two known genotoxic agents 4NQO and 2-AA in the absence and presence of metabolic activation with rat's liver s9 fraction respectively. Additionally the anti-photogenotoxicity was measured using chlorpromazine as the agent which causes the genotoxic response under UVA irradiation. CPZ is known to be photo-activated to form the unstable promazyl radical which is able to bind to DNA (Oppenländer, 1988). The protocol of the *umu*-test was modified to perform it under UVA irradiation as previously described (Skrzypczak et al., 2010). The lamp emitting UVA 365 nm (0.231 mW/cm^2) was used.

Firstly the genotoxic potential of 4NQO and 2-AA *per se* was measured by the *umu*-test in the range of concentration 0.0078–0.25 mg/L and 0.078–10 mg/L respectively. The photogenotoxicity of CPZ was evaluated in the range of concentration 1.25–10 mg/L by the modified *umu*-test method under UVA irradiation. At the same time the (photo)genotoxicity of SH (1–25 mg/L), ACS (1–10 mg/L) and EXT (36–144 mg/L) *per se* was investigated. Next the inhibition of 4-NQO and 2-AA genotoxicity or CPZ photogenotoxicity by SH, ACS and *Arnebia* extract was investigated. SH or ACS was added to the (photo)genotoxins at concentrations of 1 and 10 mg/L and SH additionally in one experiment at a concentration of 25 mg/L. The *Arnebia* extract was added to the (photo)mutagens at concentrations of 36, 72 and 144 mg/L which corresponded to the ACS concentration of 5, 10 and 20 mg/L. While the concentration of the tested compounds (SH, ACS and EXT) was fixed at the abovementioned levels, the reference (photo)genotoxin was tested at the whole range of concentrations for each level of the tested compound. The final concentration of the solvents namely methanol and DMSO did not exceed 3% during the test performance which had no additional harmful effect on bacteria. The solvents were neither toxic nor genotoxic for the tested strain.

The rate of anti-(photo)genotoxicity (%) of the tested sample (SH, ACS or EXT) was calculated as the inhibition of β-galactosidase activity induced by the (photo)genotoxic agent (4NQO or 2-AA or CPZ) at the particular concentration according to the quotation:

$$\text{Antigenotoxicity } \% = [1\text{-}(\beta\text{-galactosidase unit}_{GENOTOXIN\ +\ SAMPLE} / \beta\text{-galactosidase unit}_{GENOTOXIN})] * 100\%.$$

2.6. Qualitative determination of EXT cytotoxicity toward V79 cell line

2.6.1 . The cell line and the treatment conditions

The Chinese hamster lung fibroblast cell line V79 (ATCC® CCL-93™) was purchased from the American Type Culture Collection. V79 cells were cultured under standard conditions in DMEM (Lonza) supplemented with 10% FBS heat-activated and HEPES (0.1 M), 100 IU/mL penicillin and 0.1 mg/mL streptomycin. Cells were kept in tissue-culture flasks at 37 °C in a humidified atmosphere with 5% CO_2. Then the cells were harvested by treatment with 0.05% trypsin–0.02% EDTA in PBS and the cell suspension of 10^5 cells/mL was prepared in the culture medium and dispensed into the 12-well tissue culture plate (1 mL/well). Cells were incubated for 24 h (5% CO_2, 37 °C, >90% humidity). At the end of incubation each well was examined under a phase contrast microscope to ensure cells form a half-confluent monolayer. In the meantime the dilution series of the EXT in the treatment medium with a reduced concentration of serum (5%) was prepared. The range of examined two fold dilutions included eight concentrations from 720 mg/L to 5.6 mg/L. After incubation the culture medium was discarded from the wells. 1 ml of each variant of treatment medium with EXT was added to wells as follows: Two wells were treated only with the treatment medium and two were treated with treatment medium containing 1% of methanol (as a solvent control). As a positive control sodium lauryl sulfate was used. Cells were incubated once more for 24 h under the same conditions and examined microscopically to assess changes in general morphology, detachment, cell lysis and membrane integrity. Neutral red staining was not performed because of the intense color of the EXT solutions. A second plate with cells was prepared and exposed to EXT in the same way. After 24 h incubation the calcein (2 μM) and propidium iodide (1.5 μM) staining were performed. The wells were examined under a fluorescent microscope.

2.7. Statistical analysis

The statistical analyses were made on the results of at least 3 independent biological replicates performed with completely fresh bacteria cultures (all measurements were performed in triplicate or more being true independent experiments). All data were analyzed using STATISTICA software package. As there was no compliance with the normal distribution (tested with Shapiro–Wilk test) all data were subjected to analysis with non–parametric U Mann–Whitney method (the comparison of the median value of a range of values). The results were considered to be statistically significantly different at a probability level of $p < 0.05$.

3. Results

3.1 . Antigenotoxic activity (umu-test)

The tested compounds did not reveal any genotoxic or photogenotoxic potential in the wide range of concentrations: SH (1–25 mg/L), ACS (1–10 mg/L) and EXT (36–144 mg/L). The assays were carried out in the absence or presence of metabolic activation with s9 fraction as well as under UVA irradiation (Table 1). On the basis of these results it can be concluded that the tested naphtoquinones have no effect toward *S. typhimurium* TA1535.

Results on the IR values obtained for the genotoxin 4-NQO and the mixture of the genotoxin and the tested naphthoquinones are presented in Table 2. Two fixed levels of SH and ACS (1 and 10 mg/L) as well as three fixed levels of EXT (36, 72 and 144 mg/L) were used on the range of 4-NQO concentrations (0.078–0.25 mg/L). The control of the tested compounds in each concentration in the absence of the genotoxin (4-NQO concentration 0) was also performed. The IR value represents the induction of the β-galactosidase activity over the baseline due to the induction of the SOS system by the mutagen agent. The IR values

Table 1
IR values for the tested compound: ACS, SH and EXT respectively under different conditions.

IR (mean ± SD)	Tested material concentration [mg/L]								
	0.125	0.25	0.5	1	1.25	2.5	5	10	25
ACS − s9	1.00 ± 0.06	0.99 ± 0.13	0.96 ± 0.14	0.84 ± 0.07	0.95 ± 0.10	0.98 ± 0.09	1.10 ± 0.07	0.89 ± 0.11	
ACS + s9	0.81 ± 0.09	0.78 ± 0.09	0.87 ± 0.09	0.85 ± 0.07	1.11 ± 0.06	1.13 ± 0.12	1.20 ± 0.10	1.04 ± 0.08	
ACS UVA	0.82 ± 0.08	0.94 ± 0.18	0.99 ± 0.22	0.92 ± 0.11	0.87 ± 0.08	1.00 ± 0.17	0.96 ± 0.13	1.10 ± 0.13	
SH − s9	0.94 ± 0.06	1.00 ± 0.12	0.86 ± 0.07	0.84 ± 0.08	0.86 ± 0.06	0.95 ± 0.03	0.97 ± 0.06	0.91 ± 0.06	
SH + s9	0.83 ± 0.12	0.80 ± 0.09	0.89 ± 0.14	0.98 ± 0.17	1.03 ± 0.07	1.07 ± 0.10	1.05 ± 0.08	1.07 ± 0.14	0.94 ± 0.04
SH UVA					1.03 ± 0.07	1.04 ± 0.11	0.97 ± 0.12	1.19 ± 0.16	

IR (mean ± SD)	Tested material concentration [mg/L]		
	36	72	144
EXT − s9	0.92 ± 0.05	0.70 ± 0.13	0.71 ± 0.16
EXT + s9	0.94 ± 0.03	0.90 ± 0.08	0.75 ± 0.10
EXT UVA	0.99 ± 0.09	1.00 ± 0.11	0.98 ± 0.16

Each value is expressed as mean ± standard deviation from at least 3 independent biological evaluations.

Table 2
IR values for the control — genotoxin 4-NQO *per se* and the mixture of the genotoxin with the tested compound: ACS, SH and EXT respectively.

IR (mean ± SD)	4-NQO concentration [mg/L]						
	0	0.0078	0.0156	0.0312	0.0625	0.125	0.25
4-NQO	1.00 ± 0.09	1.33 ± 0.13	1.76 ± 0.21	2.27 ± 0.35	3.23 ± 0.39	5.22 ± 0.66	7.81 ± 0.90
ACS 1.00 mg/L	0.84 ± 0.09	1.48 ± 0.18	1.88 ± 0.24	2.37 ± 0.29	3.47 ± 0.49	4.72 ± 0.48	6.94 ± 0.77
ACS 10.0 mg/L	0.89 ± 0.11	1.40 ± 0.14	1.74 ± 0.06	2.28 ± 0.19	3.28 ± 0.13	4.54 ± 0.26	6.39 ± 0.13
SH 1.00 mg/L	0.84 ± 0.08	1.52 ± 0.14	1.76 ± 0.24	2.24 ± 0.31	3.53 ± 0.44	4.82 ± 0.48	6.79 ± 0.44
SH 10.0 mg/L	0.91 ± 0.06	1.57 ± 0.18	2.04 ± 0.28	3.06 ± 0.31	4.13 ± 0.45	5.50 ± 0.29	7.09 ± 0.66
EXT 36 mg/L	1.07 ± 0.14	1.60 ± 0.20	2.06 ± 0.29	2.56 ± 0.50	3.48 ± 0.29	5.53 ± 1.20	8.11 ± 1.36
EXT 72 mg/L	0.97 ± 0.11	2.09 ± 0.27	2.92 ± 0.37	3.83 ± 0.69	**6.10 ± 0.96**	**8.47 ± 1.94**	**13.64 ± 2.35**
EXT 144 mg/L	0.91 ± 0.16	1.11 ± 0.21	1.48 ± 0.40	2.56 ± 0.23	3.09 ± 0.51	4.50 ± 0.73	**5.55 ± 0.52**

Each value is expressed as mean ± standard deviation from at least 3 independent biological evaluations.
IR values revealing genotoxicity are underlined.
Means that are significantly different are bolded ($p < 0.05$).

obtained for the genotoxin *per se* indicate a clear relationship between the concentration and the genotoxic effect at the tested range of 4-NQO concentrations. The IR values obtained for acetylshikonin, shikonin and *Arnebia* extract *per se* did not reveal any genotoxic potential of the tested compounds for the *Salmonella* strain (IR < 1.5 at 0 mg/L of 4-NQO). There was no significant antigenotoxicity against 4-NQO observed for the tested compounds — ACS, SH or EXT regardless of the concentration applied. The IR values obtained for the mixtures of 4NQO and the naphthoquinones or the extract were comparable with those for the genotoxin itself. Moreover for the mixture of 4-NQO and the *Arnebia* extract at a concentration of 72 mg/L the significant increase of the IR values could be observed especially at higher concentrations of the genotoxin (0.0625–0.25 mg/L). This indicated the additional induction of the 4-NQO genotoxicity caused by the extract. Only the *Arnebia* extract at the concentration of 144 mg/L in the mixture with 4-NQO at a concentration of 0.25 mg/L exhibited a clear and significant inhibition of the SOS system induction by 4-NQO. The SOS system induction was decreased by 43%. This indicated strong antigenotoxic activity of the extract. However at the concentration of the genotoxic agent lower than 0.25 mg/L such an effect was not observed and thus the minimal genotoxic concentration of 4-NQO was not affected. This does not confirm antigenotoxic activity of the extract.

Results on the effect of the tested compounds against 2-AA genotoxicity are presented in Table 3. Three fixed levels of SH (1, 10

Table 3
IR values for the control — genotoxin 2-AA *per se* and the mixture of the genotoxin with the tested compound: ACS, SH and EXT respectively.

IR (mean ± SD)	2-AA concentration [mg/L]								
	0	0.0781	0.156	0.313	0.625	1.25	2.50	5.00	10.0
2-AA	1.00 ± 0.06	1.36 ± 0.14	1.59 ± 0.21	1.96 ± 0.24	2.35 ± 0.19	2.56 ± 0.47	2.93 ± 0.39	3.35 ± 0.42	3.46 ± 0.42
ACS 1.00 mg/L	0.85 ± 0.07	1.10 ± 0.05	**1.25 ± 0.11**	**1.31 ± 0.12**	**1.69 ± 0.12**	**1.73 ± 0.29**	**1.90 ± 0.21**	**2.00 ± 0.33**	**2.13 ± 0.36**
ACS 10.0 mg/L	1.04 ± 0.08	0.80 ± 0.09	**0.87 ± 0.08**	**0.92 ± 0.03**	**0.96 ± 0.03**	**0.94 ± 0.15**	**1.08 ± 0.18**	**1.08 ± 0.15**	**1.21 ± 0.15**
SH 1.00 mg/L	0.98 ± 0.17	0.84 ± 0.07	**0.96 ± 0.05**	**1.07 ± 0.15**	**1.16 ± 0.06**	**1.16 ± 0.20**	**1.25 ± 0.22**	**1.41 ± 0.24**	**1.55 ± 0.25**
SH 10.0 mg/L	1.07 ± 0.14	0.79 ± 0.05	**0.85 ± 0.08**	**0.88 ± 0.09**	**0.97 ± 0.03**	**0.98 ± 0.14**	**1.14 ± 0.17**	**1.16 ± 0.18**	**1.40 ± 0.19**
SH 25.0 mg/L	0.94 ± 0.04	–	–	–	–	**0.85 ± 0.04**	**0.94 ± 0.07**	**0.92 ± 0.08**	**1.27 ± 0.01**
EXT 36 mg/L	1.00 ± 0.10	0.95 ± 0.09	**0.98 ± 0.10**	**0.99 ± 0.09**	**1.06 ± 0.13**	**0.99 ± 0.11**	**1.01 ± 0.04**	**1.05 ± 0.07**	**1.22 ± 0.14**
EXT 72 mg/L	1.00 ± 0.07	0.93 ± 0.08	**0.99 ± 0.09**	**1.01 ± 0.10**	**0.91 ± 0.05**	**0.95 ± 0.19**	**1.04 ± 0.09**	**1.14 ± 0.08**	**1.12 ± 0.21**
EXT 144 mg/L	1.00 ± 0.17	0.88 ± 0.09	**0.96 ± 0.08**	**0.84 ± 0.08**	**0.95 ± 0.07**	**0.82 ± 0.11**	**0.90 ± 0.05**	**0.85 ± 0.02**	**1.36 ± 0.23**

Each value is expressed as mean ± standard deviation from at least 3 independent biological evaluations.
IR values revealing genotoxicity are underlined.
Means that are significantly different are bolded ($p < 0.05$).

Table 4
β-galactosidase (β-gal) activity and antigenotoxicity of ACS, SH and EXT against 2-AA (10 mg/L) and anti-photogenotoxicity of ACS, SH and EXT against CPZ (2.5 and 5 mg/L) in *umu*-test.

	2-AA 10 mg/L		CPZ 2.5 mg/L		CPZ 5 mg/L	
	β-gal	Antigenotox.	β-gal	Antigenotox.	β-gal	Antigenotox.
(photo)genotoxin per se	2.27 ± 0.27		2.42 ± 0.34		3.22 ± 0.37	
+ ACS 1.00 mg/L	1.46 ± 0.16	**35.7%**	2.05 ± 0.18	**15.3%**	3.13 ± 0.18	**2.8%**
+ ACS 10.0 mg/L	0.82 ± 0.10	**63.9%**	1.92 ± 0.15	**20.7%**	2.87 ± 0.33	**10.9%**
+ SH 1.00 mg/L	1.03 ± 0.09	**54.6%**	2.3 ± 0.47	**5.0%**	3.34 ± 0.57	**0**
+ SH 10.0 mg/L	0.91 ± 0.15	**59.9%**	2.72 ± 0.45	**0**	3.73 ± 0.23	**0**
+ SH 25 mg/L	0.91 ± 0.01	**59.9%**	–	not tested	–	not tested
+ EXT 36 mg/L	0.84 ± 0.13	**63.0%**	1.33 ± 0.19	**45.0%**	2.90 ± 0.34	**10%**
+ EXT 72 mg/L	0.77 ± 0.23	**66.1%**	1.14 ± 0.18	**52.9%**	1.55 ± 0.32	**51.9%**
+ EXT 144 mg/L	1.09 ± 0.19	**52.0%**	0.89 ± 0.15	**63.2%**	–	–

and 25 mg/L) and EXT (36, 72 and 144 mg/L) as well as two fixed levels of ACS (1 and 10 mg/L) were used on the range of 2-AA concentrations (0.078–10 mg/L). The control of the tested compounds with the absence of the genotoxin (2-AA concentration 0) was also performed. The IR values were determined under the metabolic activation with s9 rats' liver fraction. 2-AA was relatively a weaker genotoxin than 4-NQO. The linear dose dependent relationship could be observed in the range of concentrations 0.078–0.625 mg/L. SH, ACS and EXT with the absence of the genotoxin did not reveal genotoxicity under metabolic activation for any of the concentrations tested. They acted however antigenotoxic when tested in the mixture with 2-AA and decreased the IR values in the entire range of the genotoxin concentration (Table 3). The antigenotoxicity was calculated for SH, ACS and the *Arnebia* extract as the inhibition of the SOS system induced by 2-AA at the concentration of 10 mg/L (Table 4). Acetylshikonin at 1 mg/L clearly acted weaker than at 10 mg/L whereas shikonin efficacy at 1 mg/L was comparable to that at 10 mg/L. Shikonin at the concentration of 25 mg/L was additionally tested. Its antigenotoxic strength was however the same as at the concentration of 10 mg/L. The *Arnebia* extract acted as a strong antigenotoxin and suppressed completely the 2-AA genotoxicity irrespective of the concentration used — IR values never exceeded 1.5 (Table 3).

The anti-photogenotoxic potential of SH, ACS and EXT was assessed as the inhibition of chlorpromazine induced genotoxicity under UVA irradiation (Table 5). Two fixed levels of SH and ACS (1 and 10 mg/L) as well as three fixed levels of EXT (36, 72 and 144 mg/L) were used on the range of CPZ concentrations (1.25–10 mg/L). The control of the tested compounds in each concentration with the absence of the photogenotoxin (CPZ concentration 0) was also performed. The tested naphthoquinones and the extract did not demonstrate photogenotoxic activity under UVA irradiation at 0 concentration of CPZ. CPZ was not phototoxic up to 10 mg/L while it demonstrated photogenotoxicity within the range of concentrations 2.5–10 mg/L. The mixture of CPZ 10 mg/L with SH (10 mg/L) or the *Arnebia* extract irrespective of the concentration demonstrated high toxicity against the bacteria and inhibited *S. typhimurium* TA1535 growth. For the lower concentration of CPZ such an effect did not occur for SH but the extract at 144 mg/L in combination with CPZ 5 mg/L was still highly toxic for bacteria (Table 5). Due to the high toxicity the *umu*-test results were not valid for these mixtures. Conversely the protection of the *Arnebia* extract against CPZ induced photogenotoxicity could be noticed if CPZ was applied at the concentration of 2.5 mg/L. The antigenotoxic effect of the EXT was strong irrespective of the concentration tested and exceeded 60% at 144 mg/L (Table 4). If CPZ was applied at the concentration of 5 mg/L such a strong effect was revealed for the extract only at the concentration of 72 mg/L. On the other hand ACS acted only as a weak anti-photogenotoxin toward CPZ 2.5 mg/L (21% of inhibition) and had no activity against CPZ at the concentration of 5 mg/L whereas shikonin had entirely no anti-photogenotoxic potential toward chlorpromazine (Table 4).

3.2. EXT cytotoxicity toward V79 cell line

V79 cells incubated with the EXT solutions exhibited a tendency to absorb the purple dye of the treatment medium. Based on the observations it was assumed that the pigment stained not only the cytoplasm but also the nuclei. Staining intensity decreased in accordance with the reduction of the EXT concentration in the medium. In the range of concentration between 90 and 720 mg/L the majority of the cells were well attached and spindly shaped (Fig. 1), although some of them exhibited morphological abnormalities such as enlarged nucleus, cytoplasm reduction and changes of shape — these observations may indicate the beginning of apoptosis. However in the subsequent four dilution series the cells morphology was seriously affected. The majority of cells were round shaped, shrunken and detached (Fig. 2). The calcein and

Table 5
IR values for the control — photogenotoxin CPZ *per se* and the mixture of the photogenotoxin with the tested compound: ACS, SH and EXT respectively.

IR (mean ± SD)	CPZ concentration [mg/L]				
	0	1.25	2.5	5.00	10.0
CPZ	1.00 ± 0.07	1.37 ± 0.22	1.86 ± 0.31	2.46 ± 0.34	4.30 ± 0.61
ACS 1.00 mg/L	0.92 ± 0.11	1.03 ± 0.14	**1.44 ± 0.20**	2.20 ± 0.30	3.50 ± 0.59
ACS 10.0 mg/L	1.10 ± 0.13	1.02 ± 0.15	**1.42 ± 0.22**	2.03 ± 0.43	3.36 ± 0.55
SH 1.00 mg/L	1.03 ± 0.07	1.14 ± 0.19	1.74 ± 0.55	2.20 ± 0.52	3.97 ± 1.01
SH 10.0 mg/L	1.19 ± 0.16	1.46 ± 0.16	1.93 ± 0.51	2.62 ± 0.31	tox
EXT 36 mg/L	0.99 ± 0.09	1.05 ± 0.17	**1.37 ± 0.16**	2.25 ± 0.27	tox
EXT 72 mg/L	1.00 ± 0.11	1.01 ± 0.14	**1.07 ± 0.16**	**1.41 ± 0.18**	tox
EXT 144 mg/L	0.90 ± 0.16	1.23 ± 0.14	**1.18 ± 0.20**	tox	tox

Each value is expressed as mean ± standard deviation from at least 3 independent biological evaluations.
IR values revealing genotoxicity are underlined.
Means that are significantly different are bolded ($p < 0.05$).
'tox' — toxicity toward *S. typhimurium* TA1535.

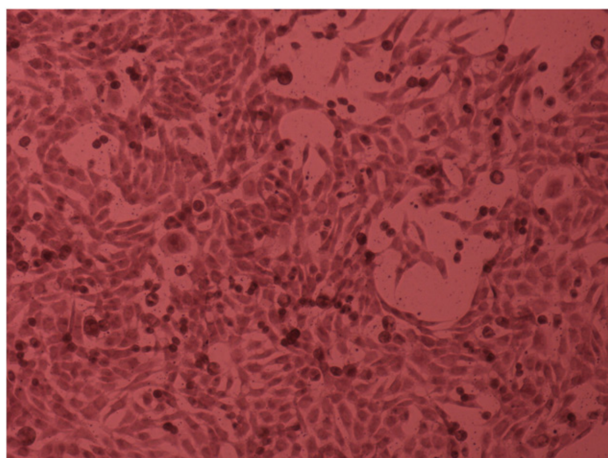

Fig. 1. Morphology of V79 cells after treatment with EXT 720 mg/L.

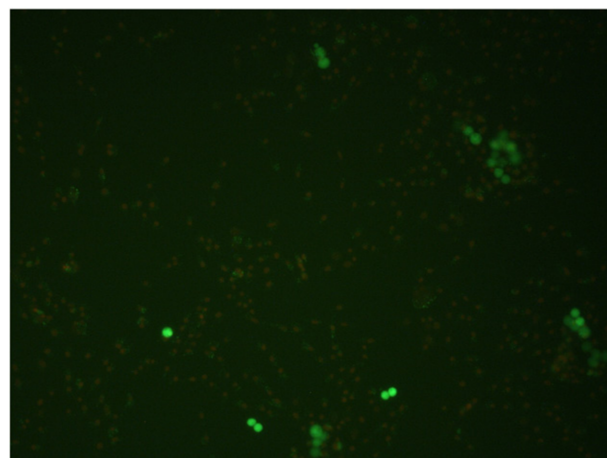

Fig. 3. The calcein staining of EXT 5,6 mg/L.

propidium iodide staining confirmed that all examined cells were dead for all EXT concentrations between 5.6 mg/L and 360 mg/L. The examination of the highest EXT concentration was impossible due to the UV-absorbing ability of the EXT. Several living fibroblasts were found only in the lowest examined concentration (Fig. 3). The differences in the morphology of cells in the range of the examined concentrations may indicate that the treatment of the cells with EXT concentrations from 90 mg/L to 720 mg/L impacted the fibroblasts death before they detached and became apoptosis.

3.3 . Antioxidant activity

Antioxidant assays (DPPH-EPR) were performed for 3 samples, namely ACS, SH and *A. euchroma* callus extract (EXT). Ascorbic acid (AA) was used as a reference antioxidant compound. Even though the antioxidant activities of the studied samples were lower than those of ascorbic acid all three samples exhibited radical scavenging properties and can be considered as antioxidants (Fig. 4).

Moreover the antioxidant activity of the EXT was not a sum of ACS and SH antioxidant activities. This result suggests that there could be other antioxidant compounds in the *A. euchroma* callus extract which are responsible for its properties. For example compounds such as isobutyrylshikonin and a number of shikonin derivatives which are also present in the extract could be responsible for a higher antioxidant activity of the extract as compared to pure ACS and SH. That effect is more pronounced when the decay of the DPPH-EPR signal is analyzed with time (Fig. 5).

In the EPR assay the values of antioxidant activity usually increase with the higher content of total polyphenols (Jamróz et al., 2014). Initially the DPPH scavenging reaction is extremely fast and after several minutes radical reactions slow down. Less active scavengers slowly decrease the amount of DPPH radical. The compounds like procyanidins and larger polyphenolic polymers seemed to be responsible for the long reaction time with DPPH, as was demonstrated in the studies of apple juice and apple puree (Oszmiański et al., 2008). Additionally, these compounds may exhibit a variety of radical-scavenging mechanisms. This supports the assumption that ACS and SH are not the main compounds responsible for the antioxidant and radical scavenging activities of *A. euchroma* callus extract.

4. Discussion and conclusion

In the *umu*-test performed without metabolic activation with 4-NQO as the direct genotoxic agent no antigenotoxic effect of shikonin and its derivative acetylshikonin was observed. The *Arnebia* extract had an unclear effect: it induced the 4-NQO genotoxicity if applied at the lower concentration and inhibited genotoxic activity if applied at the higher concentration. Such an

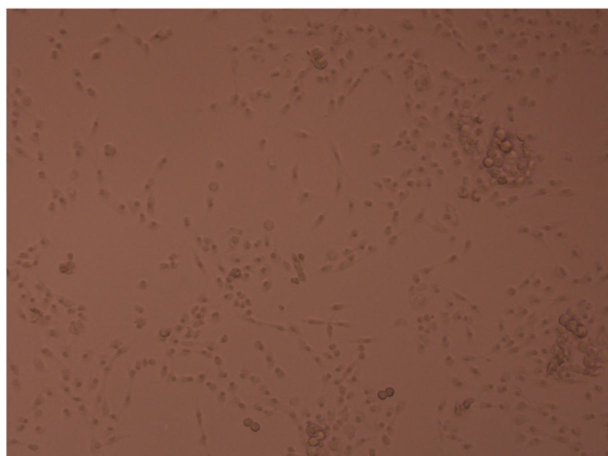

Fig. 2. Morphology of V79 cells after treatment with EXT 5,6 mg/L.

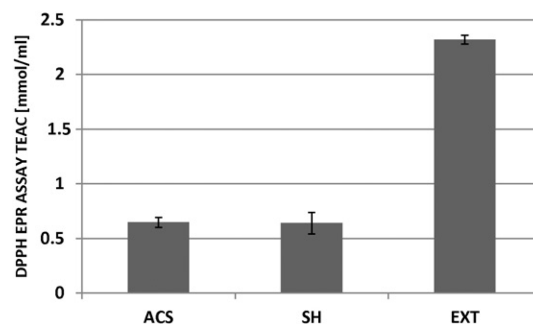

Fig. 4. Antioxidant properties of acetylshikonin (ACS), shikonin (SH) and *Arnebia euchroma* callus extract (EXT) infusions DPPH-EPR. Results are presented as mean of three independent experiments with standard deviation.

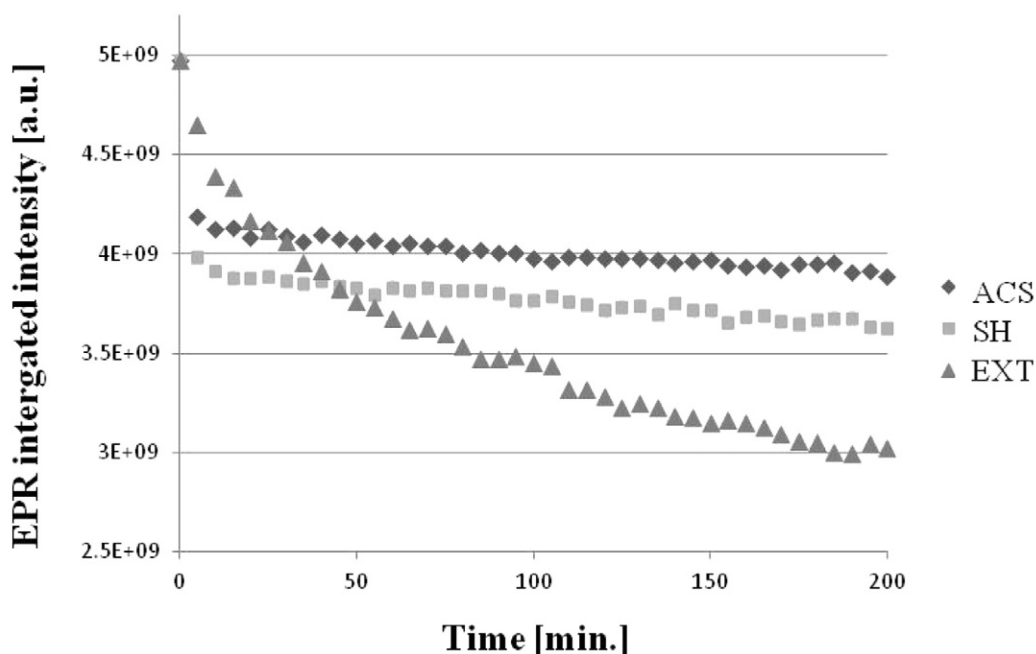

Fig. 5. The decay of DPPH· signal in time after addition of acetylshikonin (ACS), shikonin (SH) and *Arnebia euchroma* callus extract (EXT).

action however was only observable if the genotoxin was applied in higher concentrations. On the basis of the results obtained it cannot be concluded that the extract exhibits antigenotoxic potential against 4-NQO. On the other hand the tested naphthoquinones and the extract exhibited antigenotoxic properties under metabolic activation against 2-AA. The antigenotoxicity of ACS toward a fixed concentration of 2-AA depended on the ACS level: moderate effect at lower concentration and strong effect at the higher concentration. Shikonin and *Arnebia* extracts demonstrated strong antigenotoxic properties toward a fixed concentration of 2-AA regardless of the dose used (inhibition by 50–60%). The obtained results suggest that the tested naphthoquinones need to be activated to reveal an antigenotoxic effect. Alternatively they may not be involved in the mechanism of 4-NQO genotoxic action whereas they may block or modify the metabolic activation of 2-aminoanthracene thus not allowing its transformation to the active form.

In the paper by Li et al. (1999) the *in vitro* metabolism of shikonin through rat liver microsomes was investigated. The main metabolites were dihydroxylated or monohydroxylated SH in the different positions of the naphthoquinone ring. The biotransformation of shikonin by microorganism proceeds differently. Sun et al. (2000) have reported that shikonin was transformed using a human intestinal bacterial strain into two monomeric metabolites and into four dimeric metabolites. Monomeric ones were yielded through dehydration and reduction of the side chain and the dimeric through tautomerization and condensation of shikonin and the monomer or two monomers. These data suggest that the hydroxylation of the naphthoquinone ring causes the protective action of the tested naphthoquinones at least against 2-AA mutagenicity. Otherwise the biotransformation of the side chain or the arising of the bigger molecule resulting from polymerization taking place in the cytosol does not affect the genotoxic action of 4-NQO.

The mechanism of mutagenesis by 4-NQO consists of base-pair substitution (principally G to A transitions but also G to T transversion) (Bailleut et al., 1989). The compound needs to be transformed by the cellular enzymes to an active compound which can covalently bind to guanine or adenine of DNA (Bailleut et al., 1989). Such a basic conversion involving the reduction of the nitro-group was observed in both prokaryotic and eukaryotic cells and led to the formation of two products: 4-hydroxyaminoquinoline 1-oxide (4HAQO) and 4-aminoquinoline 1-oxide (4AQO). Only the first, 4HAQO, acted as a carcinogen and was more active than the parent compound 4-NQO (Bailleut et al., 1989; Paul et al., 1971). The results obtained in our study suggest that the tested naphthoquinones are not involved in this mechanism and thus do not modify the genotoxicity of 4-NQO. Moreover it seems that the naphthoquinones' transformation by bacteria as mentioned above apply to other cytosol enzymes as the transformation of 4-NQO and therefore no competitive effect could be noticed.

The second known mechanism of the carcinogenic action of 4-NQO is intracellular oxidative stress (Nunoshiba and Demple, 1993). It can undergo redox cycling and generates reactive oxygen species as superoxide radical or hydrogen peroxide. The oxidative stress contributes to tumor promotion (Kanojia and Vaidya, 2006). Although the tested naphthoquinones in this study revealed an antioxidative capacity no antigenotoxic effect against 4-NQO was observed. This could be explained by the fact that the oxidative stress is one of the possible mechanisms of action of this mutagen which contributes to others.

Genotoxic agent 2-aminoanthracene is a pre-carcinogen. It is a reactive frameshift mutagen after liver activation (McCann et al., 1975). The biotransformation involves the initial oxidation consisting of N-hydroxylation. The N-hydroxyl derivatives are then converted to the unstable acetyl and sulfate esters forming the highly reactive nitrenium cation which is responsible for the binding with DNA (Veres et al., 2005; Marczyło and Ioannides, 1999). On the basis of these reports and data on liver transformation of SH (Li et al., 1999) it could be concluded that the naphthoquinone compounds compete with 2-AA molecules for the oxidative enzymes from the liver's s9 fraction. This results in the decreased activation of 2-AA and its weaker genotoxicity.

The anti-photogenotoxic activity of naphthoquinones has not been examined as yet. In the present study such an attempt was made using a modified *umu*-test. Chlorpromazine was employed as a model photogenotoxic agent since it has been known to be photo-activated to form the unstable promazyl radical which is able to damage the DNA molecule (Ciulla et al., 1986). The modified procedure of the *umu*-test to perform it under UVA irradiation and examine the

genotoxicity of photoproducts (even if only by short-living radicals) was established in our previous study (Skrzypczak et al., 2010). The usefulness of this novel tool to test photogenotoxicity was confirmed. Chlorpromazine is often used as a positive control assessing the photogenotoxic potential of chemicals with various testing models both prokaryotic and eukaryotic (Struwe et al., 2007; Struwe et al., 2011; Chetelat et al., 1996).

Shikonin had no anti-photogenotoxic properties toward CPZ. Acetylshikonin exhibited weakened antigenotoxicity by up to 15–20% if chlorpromazine was applied in the lower concentration and had no particular effect with higher CPZ doses. *Arnebia* extract demonstrated the best inhibitory effect which was over 45% toward lower doses of chlorpromazine regardless of the EXT dose. If CPZ was applied in the higher concentrations the *Arnebia* extract was as effective only if the higher concentration (72 mg/L) was used. The tested compounds under UVA irradiation may act as UV filters and therefore reduce the CPZ transformation to the genotoxic derivative. Indeed the absorption spectra made for SH, ACS and EXT solutions revealed the best absorption properties for the *Arnebia* extract for those which stay in accordance with the results of the *umu*-test. Cheng et al. (1995) reported that shikonin was decomposed under solar light while Feng et al. (2007) demonstrated that the extract containing naphthoquinone derivatives behaved like a UV filter. Moreover shikonin and the *Arnebia* extract increased the toxicity of CPZ high doses under UVA irradiation — the growth of *Salmonella* TA1535 was inhibited. In our previous study chlorpromazine under UVA irradiation demonstrated high toxicity toward *S. typhimurium* TA1535 but in concentrations higher than 10 mg/L. It was concluded that the genotoxic action of UVA irradiation itself in addition to the genotoxicity of promazyl radical formed under the test conditions made the growth of bacteria insufficient to perform the *umu*-test. Chlorpromazine at the concentration of 10 mg/L was however not toxic to bacteria under UVA irradiation. In this study SH and EXT induced the toxicity of irradiated CPZ in high concentrations in an unknown mechanism but protected the bacteria from the photogenotoxic action of CPZ in lower concentrations. Shikonin and the *Arnebia* extract may operate as free radical scavengers and in such a way protect the bacterial cells. Accepting such an assumption shikonin activity should also be observed, however it was not. The antioxidative properties of the extracts containing naphthoquinone derivatives as well as isolated shikonin and its derivatives were investigated by several groups. Generally speaking the obtained results suggest efficient antioxidant and radical scavenging activity of SH and derivatives. Weng et al. (2000) reported that SH and ACS in a concentration of 0.06% were as strong radical scavengers as α-tocopherol. Another research group (Gao et al., 2000) demonstrated highly efficient antioxidative activity of SH against several types of reactive oxygen species such as singlet oxygen, superoxide anion radical, hydroxyl radical and *tert*-butyl peroxyl radical. These results were obtained with the EPR method. Ordoudi et al. (2011) tried to assess the structure–radical scavenging activity relationship among a wide range of SH derivatives. The results indicated that the hydroxyl group at the C-1′ position of the side chain has a positive effect to the radical scavenging properties. Thus SH was more potent than deoxyshikonin. Moreover esterification larger than with acetyl group for the hydroxyl group was critical for the activity. The activity of SH and ACS was estimated at 28% and 32% respectively and isobutyrylshikonin was almost inactive probably due to the steric effect. Our findings are not in accordance with the abovementioned results. Antioxidant activity of SH and ACS toward DPPH was 4–6 fold weaker than those by Ordoudi et al. (2011). On the other hand the *Arnebia* extract had the best radical scavenging capacity but it contained only acetylshikonin and isobutyrylshikonin, supposedly almost inactive, whereas no shikonin. On the other hand EXT activity could be due to other active compounds which were not determined by the HPLC method applied in this study.

On the basis of the obtained results of cytotoxicity assays it can be concluded that the *Arnebia* extract is virulent and highly cytotoxic toward the V79 cell line. In the present study the addition of the EXT in all concentrations (5.6 mg/L to 720 mg/L) resulted in significant cytotoxicity. It suggests a much higher sensitivity of mammalian cell line than the bacteria strain toward *A. euchroma* callus extract. The cytotoxicity of shikonin and its derivatives has been widely reported however mostly against tumors not having normal cell lines. Inhibition of proliferation, blocking the cell cycle or apoptosis induction is only a few of the possible mechanisms of action observed for shikonin derivatives in cell culture studies (Andújar et al., 2013). Nevertheless none of them have entered into clinical trials because of their serious toxicity, even though they show great tumor inhibitory effects (Wang et al., 2014).

Generally speaking it can be concluded that all of the tested compounds and the extract have comparable antigenotoxic potential against 2-aminoanthracene under s9 metabolic activation toward the bacteria strain *S. typhimurium* TA1535. *A. euchroma* callus extract demonstrates however better antioxidant and anti-photogenotoxic potential than isolated naphthoquinone compounds. Nevertheless it needs to be applied in particular and suitable concentrations to be deemed safe and beneficial. Furthermore *in vitro* and *in vivo* studies are needed to evaluate the mechanism of antigenotoxic action of shikonin and its derivatives. Moreover more detailed studies on *Arnebia* extract should be conducted. While high cytotoxicity of the *Arnebia* extract toward V79 cells was observed further investigation with different ranges of concentrations and different cell lines to exclude the particular sensitivity of the V79 cells is essential. Moreover isolated the naphthoquinone compound should be examined *in vitro* next to the *Arnebia* extract. Naphthoquinone derivatives seem destined to become bioactive foods' components with health benefits and not only the coloring agents.

Acknowledgments

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