Post-print of: Hudecova A., Kusznierewicz B., Hasplova K., Huk A., Magdolenova Z., Miadokova E., Galova E., Dusinska M.: *Gentiana asclepiadea* exerts antioxidant activity and enhances DNA repair of hydrogen peroxide- and silver nanoparticles-induced DNA damage. FOOD AND CHEMICAL TOXICOLOGY. Vol. 50, iss. 9 (2012). p. 3352 - 3359. DOI: 10.1016/j.fct.2012.06.017

Gentiana asclepiadea exerts antioxidant activity and enhances DNA repair of hydrogen peroxide- and silver nanoparticles-induced DNA damage

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a b s t r a c t

Exposure to high levels of different environmental pollutants is known to be associated with induction of DNA damage in humans. Thus DNA repair is of great importance in preventing mutations and contributes crucially to the prevention of cancer. In our study we have focused on quantitative analysis of *Gentiana asclepiadea* aqueous or methanolic extracts obtained from flower and haulm, their antioxidant potency in ABTS post-column derivatisation, and their potential ability to enhance DNA repair in human lympho-cytes after hydrogen peroxide (H_2O_2) treatment (250 μM, 5 min). We also studied DNA repair in human kidney HEK 293 cells after exposure to 20 nm silver nanoparticles (AgNPs) (100 μg/ml, 30 min) in the presence and absence of the plant extract. We have found that mangiferin along with unidentified polar compounds are the most pronounced antioxidants in the studied extracts. Extract from haulm exhibited slightly stronger antioxidant properties compared to flower extracts. However, all four extracts showed significant ability to enhance DNA repair in both cell types after H_2O_2 and AgNP treatments.

Keywords: DNA repair, DNA damage, *Gentiana asclepiadea,* Hydrogen peroxide, Silver nanoparticles, Human cells

1. Introduction

The balance between antioxidation and oxidation is believed to be a critical factor in maintaining a healthy biological system (Dudonné et al., 2009). In recent years, many studies have indicated the important role of free radicals and reactive oxygen species (ROS) in the aetiology and progress of many human diseases (Heo et al., 2010; Skandrani et al., 2010a,b). ROS such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), and singlet oxygen ($^1O_2$), are of the greatest biological significance. ROS are produced continuously in all cells, as metabolic byproducts of a number of intracellular systems (Martínez-Cayuela, 1995).

In addition to endogenous sources of free radicals, there are many exogenous factors that cause ROS production in cells. For example some nanomaterials are considered to cause oxidative damage (Halamoda Kenzaoui et al., 2012). Among the 580 nanotechnology-based consumer products, silver nanoparticles (AgNPs) are the most common material (Woodrow Wilson International Center, 2007). Apart from applications in medicine, AgNPs are being widely used for water purification, food service and personal care products, and also in indoor air quality management (Cheng et al., 2004; Jain and Pradeep, 2005; Park et al., 2010; Zhang and Sun, 2007). There is evidence that AgNP exposure induces the generation of free oxygen radicals. This oxidative stress subsequently results in DNA damage and ultimately apoptosis (Ahamed et al., 2010; Hudecova et al., 2012d).

DNA is prone to oxidation by endogenous ROS as well as exogenous agents (including radiation and chemicals) and this can lead to different types of DNA damage (Sevcovicova and Hercegova, 2010). This damage can be either repaired or tolerated. Misrepaired or unrepaired DNA lesions are likely to be mutagenic and thus can lead to carcinogenesis. The ability to repair DNA damage is associated with a decreased risk of cancer and possibly other human diseases. Protection of DNA from damage or modulation of DNA repair thus contribute to preventing mutations and maintaining genomic stability (Kovacs, 2002; Ramos et al., 2010). Mechanisms of oxidative DNA damage repair can differ significantly between organisms and cell types (Aherne et al., 2007; Baute and Depicker, 2008; Dianov and Parsons, 2007). The repair pathways include base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) (Friedberg, 1985).

The comet assay is a simple and reliable method to detect DNA damage and can also be used for assessment of DNA repair (Azqueta et al., 2011). Both the in vitro DNA repair assay (Collins et al., 2012) as well as cellular DNA repair phenotype assays, called challenge assays (Decordier et al., 2010) have been successfully used to assess activity of different DNA repair pathways. Collins et al.

http://dx.doi.org/10.1016/j.fct.2012.06.017

(2012) reviewed evidence that DNA repair can be affected by phytochemicals and various components of diet.

There has been an increasing demand for antioxidants of plant origin in the food, beverage and cosmetic industries, and efforts have been made to identify new natural sources for active antioxidant phytochemicals (Dudonné et al., 2009; Skandrani et al., 2010a,b). Plants synthesize antioxidant compounds as secondary products, mainly phenolics serving in plant defence mechanisms that counteract ROS and thus avoid oxidative damage. The antioxidant activities of phenolics are due to a number of different mechanisms, such as free-radical scavenging, hydrogen donation, singlet oxygen quenching, metal ion chelation, antioxidant enzyme activation, and acting as substrate for radicals such as superoxide or hydroxyl (Niciforovic et al., 2010).

Gentiana asclepiadea is a plant belonging to the family Gentianaceae. The dried roots and rhizomes have been traditionally used in folk medicine as remedies for poor appetite and digestive problems or even for hepatitis A virus infections (Mihailovic et al., 2011; Saric, 1989). It is also used in small amounts as food and beverage flavouring, in antismoking products and even as a substitute for hops in making beer. It contains a variety of phytochemicals (mangiferin, swertiamarine, gentiopicroside, homoorientin/isoorientin, amaroswerine, sweroside and others) with some positive bioactivities. In our previous studies we have shown antioxidant, antigenotoxic and biomodulatory effects of *G. asclepiadea* extracts on various cells (including lymphocytes and HEK 293 cells) exposed to different agents (H_2O_2, zeocin, AgNPs) (Hudecova et al., 2010, 2012a,b,c,d). Here we have studied the antioxidant activities of separated compounds by using the on-line method with 2,2́-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cations (ABTS), post-column derivatisation. We have also used the cellular DNA repair 'challenge' assay with the alkaline comet assay to examine the effect of *G. asclepiadea* extracts on DNA repair in human peripheral blood mononuclear cells treated with H_2O_2 and HEK 293 cells treated with AgNPs. Both agents were previously shown to cause DNA oxidation resulting in strand breaks and oxidised DNA lesions (Ahamed et al., 2010; Piao et al. 2011; Hudecova et al., 2010, 2012a,c,d).

2. Material and methods

2.1. Preparation of extracts from G. asclepiadea

G. asclepiadea extracts were prepared as follows: plants were harvested from the Garden of Medicinal Plants, Faculty of Pharmacy, Comenius University, Bratislava (Slovakia) in August 2008. The separated plant material, weighing about 60 g, was air-dried to dryness at room temperature, cut into small pieces and then extracted with 150 ml of methanol or water at 65 °C. This procedure was repeated 5-times. The extract was then filtered and concentrated in vacuum and the rest of the water was removed by azeotropic distillation with benzene. The final extracts were kept in the dark at +4 °C until tested. Four different extracts were prepared – haulm methanolic (HM), haulm aqueous (HA), flower methanolic (FM) and flower aqueous (FA).

2.2. Reagents

HPLC grade methanol and formic acid (98–100%) were from Merck (Germany) and 2,2′-azinobis(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) from Sigma–Aldrich (USA). Water was purified using a QPLUS185 system from Millipore (USA). The following standard compounds were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and mangiferin from Sigma–Aldrich (USA) gentiopicroside, sweroside, swertiamarin, homoorientin and isovitexin from LGC Standards (Germany).

2.3. Chromatographic determination of bioactive compounds

Phenolic compounds and iridoids were analysed by the Agilent Technologies 1200 Series HPLC-DAD-MS system (Agilent Technologies, USA) equipped with a Zorbax XDB C8 (150 × 4.60 mm, 3.5 μm) column. The phytochemical resolution was carried out using a mobile phase composed of 4.8% formic acid in water (solvent A) and methanol (solvent B) at a flow rate of 1 ml/min; the injection volume of all samples was 5 μl. Elution was conducted with a linear gradient program to ensure the following ratio of solvent B to A: from 0 to 30 min (15:85–70:30 v/v). MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 120 V; drying gas temperature, 350 °C; gas flow (N_2), 12 l/min; nebulizer pressure, 35 psig. The instrument was operated in positive and negative ion mode, scanning from *m/z* 100 to 1000. Individual compounds were identified by comparing their retention times and spectra with those for standards or on the basis of available literature data and UV and mass spectra. For quantitative determination of analytes, the calibration curves were generated by integration of the areas of absorption peaks determined during chromatographic analysis of serial dilutions of available standards.

2.4. On-line profiling of antioxidants

For on-line profiling of antioxidants, the HPLC-DAD system (Agilent Technologies, USA) was connected with a Pinnacle PCX Derivatisation Instrument (Pickering Laboratories, Inc., USA) and a UV–VIS detector (MWD Agilent Technologies, USA). The conditions of chromatographic separation of bioactive compounds present in *G. asclepiadea* extracts were identical with those described above. The post-column derivatisation procedure was done according to Kusznierewicz et al. (2011). In all experiments, a 0.5 ml (PTFE, 0.25 mm, 10 m) reaction coil heated to 130 °C was used. The derivatisation reagent was prepared as follows: ABTS was dissolved in aqueous sodium persulphate (2.45 mM) to obtain a concentration of 7 mM. The mixture was stored in the dark at room temperature for 12 h and then diluted with methanol to the concentration of 30% (v/v). ABTS solution was fed into the system at a flow rate of 0.1 ml/min. Chromatograms after derivatisation with ABTS were registered at 734 nm. The equation of the standard line – Trolox concentration = f(peak area) – determined for ABTS derivatisation reagent was used to calculate TE values from the peak areas of analytes obtained for plant extracts following derivatisation.

2.5. Cells

Human peripheral blood mononuclear cells (lymphocytes) were isolated from a finger prick sample, mixed with 1 ml of PBS (Invitrogen, Oslo, Norway), left on ice for 30 min, underlayed with 100 μl of Lymphoprep (Axis-Shield, Oslo, Norway), centrifuged (200 × *g*, 3 min, 4 °C), and 100 μl of lymphocytes was retrieved from just above the boundary and used for the experiments.

HEK 293 cells (derived from human embryonic kidney cells) were cultured in Dulbeccós Modified Essential Medium (DMEM – Sigma–Aldrich, Oslo, Norway) supplemented with 10% fetal calf serum (FCS – Sigma–Aldrich, Oslo, Norway), 100 U/ml of penicillin (Invitrogen, Oslo, Norway) and 100 μg/ml of streptomycin (Invitrogen, Oslo, Norway).

2.6. Dispersion of AgNPs

AgNPs of nominal size 20 nm and purity 99.5% were purchased from Plasmachem GmbH, Germany. A stock solution at 2 mg/ml was prepared just before use in filtered distilled water, vortexed and sonicated (Labsonic®P, Sartorius Stedim Biotech, Goettingen, Germany) for 3 min (100% cycle, 100 watts) on ice. Immediately after sonification bovine serum albumin (BSA) and PBS were added to a final BSA concentration of 1.5%. Three concentrations of AgNPs (1, 25 and 100 μg/ml) were prepared in DMEM medium with 10% fetal calf serum and added to the HEK 293 cells for 30 min at 37 °C in a 5% CO_2 atmosphere.

2.7. Cellular DNA repair – challenge assay

2.7.1. Lymphocyte treatment with H_2O_2 and post-treatment with plant extract

Isolated lymphocytes were exposed to 250 μM H_2O_2 (Sigma–Aldrich, Oslo, Norway) for 5 min. After the treatment cells were washed with PBS and incubated in RPMI medium either in presence or absence of *G. asclepiadea* extracts (0.25 mg/ml) at 37 °C. Residual DNA damage was measured at 4, 8, 16, 24 or 32 min post-treatment, with the alkaline comet assay. Untreated cells and cells treated with *G. asclepiadea* extracts (0.25 mg/ml) but without H_2O_2 were used as negative controls.

2.7.2. Treatment of HEK 293 cells with AgNP and post-treatment with plant extract

HEK 293 cells were inoculated on 12-well plates (1×10^5/well) and incubated in DMEM medium. Next day, cells were treated with 100 μg/ml of AgNPs for 30 min in DMEM at 37 °C, then washed with PBS and incubated in DMEM media in the presence or absence of *G. asclepiadea* extracts (0.25 mg/ml). Residual DNA damage was measured at 5, 15, 30, 60, 90 or 120 min post-treatment, with the alkaline comet assay. Untreated cells and cells treated with *G. asclepiadea* extracts (0.25 mg/ml) but without H_2O_2 were used as negative controls.

2.7.3. Single-cell gel electrophoresis (SCGE; comet assay)

Microscope slides were pre-coated with a base layer of 100 μl of 1% normal melting point agarose in water. The cells were re-suspended in 1% low melting point agarose in PBS buffer (Ca^{2+} and Mg^{2+} free). A volume of 60 μl of the cell

suspension (approximately 2×10^4 cells) was dropped on a pre-coated slide, and a glass coverslip placed on top to spread the gel, which was left to set at 4 °C. All slides were then placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris–HCl, pH 10 and 1% Triton X-100) at 4 °C for 1 h to remove cellular membranes, cytoplasm, and histones, leaving DNA as nucleoids. After lysis, the slides were transferred to an electrophoresis tank containing alkaline solution (300 mM NaOH, 1 mM Na_2EDTA, pH > 13) for 20 min at 4 °C to allow the DNA to unwind. The electrophoresis was run under the following conditions: 25 V, 300 mA, 30 min. The slides were removed and neutralised in PBS (10 min) and ddH2O (10 min) at 4 °C. Each sample was stained with 20 μl of SYBRgold (0.1 μl/ml in TE buffer – 10 mM Tris–HCl, 1 mM Na_2EDTA, pH 7.5–8) and 100 nucleoid 'comets' per sample were scored by computerised image analysis (Comet Assay IV 4.2, Perceptive Instruments Ltd) for determination of% DNA in the tail, which is linearly related to the frequency of DNA strand breaks.

2.8. Statistical analysis

The results represent the mean of 3 experiments ± standard deviation (SD). The significance of differences between means was evaluated by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3. Results and discussion

3.1. Composition of bioactive compounds in G. asclepiadea extracts

The most abundant bioactive components of family *Gentianaceae* are iridoids, xanthones, mangiferin, and C-glucoflavones (Jensen and Schripsema, 2002). On the basis of comparison of the retention times, peak orders, and spectral data with those of available standards three secoiridoids (swertiamarin, gentiopicroside, sweroside), two C-glucoflavones (isovitexin, homoorientin) and one xanthonoid (mangiferin) were identified in *G. asclepiadea* extracts. In the absence of other reference compounds, the presence of loganic acid, luteolin-diglucoside, isovitexin-glucoside, amaroswerine, depressin and gentioside was confirmed by the comparison of UV and mass spectra with literature data. In the MS spectra, quasimolecular ions of these compounds in positive and negative modes respectively were as follows: $m/z = 399$ ($[M + Na]^+$) and $m/z = 375$ ($[M - H]^-$) for loganic acid; $m/z = 611$ ($[M + H]^+$) and $m/z = 609$ ($[M - H]^-$) for luteolin-diglucoside; $m/z = 595$ ($[M + H]^+$) and $m/z = 593$ ($[M - H]^-$) for isovitexin-glucoside; $m/z = 603$ ($[M + H]^+$) and $m/z = 601$ ($[M - H]^-$) for amaroswerine; $m/z = 711$ ($[M + Na]^+$) and $m/z = 687$ ($[M - H]^-$) for depressin, and $m/z = 575$ ($[M + Na]^+$) and $m/z = 551$ ($[M - H]^-$) for gentioside. The quantitative determination was accomplished only for the compounds for which standards were available. Additionally, in the case of luteolin-diglucoside and isovitexin-glucoside, the contents of these analytes were expressed respectively as homoorientin and isovitexin equivalents (Table 1). Gentiopicroside was found to be the most abundant component in all samples analysed, which is in agreement with previous reports estimating the abundance of this compound in different *Gentiana* species (Szucs et al., 2002; Zheng et al., 2011). The content and composition of the analytes did not differ significantly between the samples under study. The most visible differences were the occurrence of depressin and gentioside only in the flower extracts, as well as about four times higher content of mangiferin in the methanolic extract from haulm.

3.2. On-line antioxidant profiling with ABTS as derivatisation reagent

Over the past two decades, a number of analytical methods measuring antioxidative activity have been developed, most of which are based on the ability of antioxidants to quench free radicals by hydrogen donation. The same chemical reactions have been exploited here for the on-line HPLC-coupled profiling of antioxidants in *G. asclepiadea* extracts. This approach enabled the characterisation of antioxidant phytochemicals in *G. asclepiadea* extracts, as well as determination of their individual antioxidant activities. The examples of chromatograms for HM, HA, FM, and FA extracts of *G. asclepiadea* obtained during on-line antioxidant profiling with ABTS as derivatisation reagent are presented in Fig. 1.

The post-column detection of the ABTS radical reduction in relation to antioxidant content is reflected in the negative chromatogram at 734 nm. As can be seen, in all presented chromatograms obtained as a result of post-column derivatisation, mangiferin (5) and some unidentified polar compound (1) are the major analytes responsible for antioxidant activity of *Gentiana* samples studied. The share attributable to loganic acid (2), swertamarin (6), homoorientin (8), sweroside (9) and amaroswerie (11) is markedly smaller. Mangiferin exhibits a wide spectrum of pharmacological effects, including immunomodulatory, anti-inflammatory, antitumor, antidiabetic, lipolytic, antimicrobial, and antiallergic activities (Wauthoz et al., 2007). At least some of these effects could be attributed to its antioxidant property. In Table 1 the antioxidant activity of individual analytes based on peak areas obtained by post-column derivatisation and then recalculated with the use of the Trolox calibration curve are presented. As can be seen,

Table 1
The composition and content [mM] of bioactive compounds detected in methanolic and aqueous extracts prepared from flowers and haulms of *G. asclepiadea* combined with their antioxidant activity [TE mM] determined by HPLC-coupled postcolumn derivatisation with ABTS reagent.

Peak	Compound	Flower				Haulm			
		Methanolic		Aqueous		Methanolic		Aqueous	
		Content mM	TE mM	Content mM	TE mM	Content mM	TE mM	Content mM	TE mM
1	Unknown	–	0.687	–	1.097	–	0.560	–	1.105
2	Loganic acid	–	0.160	–	0.139	–	0.107	–	0.096
3	Luteolin-diglucoside	0.212	nd	0.293	nd	0.529	nd	0.410	nd
4	Isovitexin-glucoside	0.403	nd	0.361	nd	0.502	nd	0.389	nd
5	Mangiferin	0.264	0.537	0.295	0.538	1.153	1.489	0.554	0.881
6	Swertiamarin	0.684	0.107	0.686	0.106	0.802	0.118	0.620	0.099
7	Gentiopicroside	4.878	nd	3.111	nd	4.998	nd	1.987	0
8	Homoorientin	0.420	0.177	0.353	0.156	0.571	0.219	0.277	0.129
9	Sweroside	0.164	0.278	0.052	0.143	0.066	0.156	0.076	0.178
10	Isovitexin	0.226	nd	0.170	nd	0.264	nd	0.114	nd
11	Amaroswerine	–	0.113	–	0.094	–	0.134	–	0.142
12	Depressin	–	0.091	–	0.084	nd	nd	nd	nd
13	Gentioside	–	0.131	–	0.136	nd	nd	nd	nd
14	Unknown	–	0.348	–	0.252	–	0.115	–	0.098
	Others	–	1.988	–	2.735	–	3.051	–	2.958
	Total		4.617		5.480		5.949		5.686

The SD values do not exceed 1% in the case of analytes content determination and 5% in the case of antioxidant activity determination; nd, not detected.

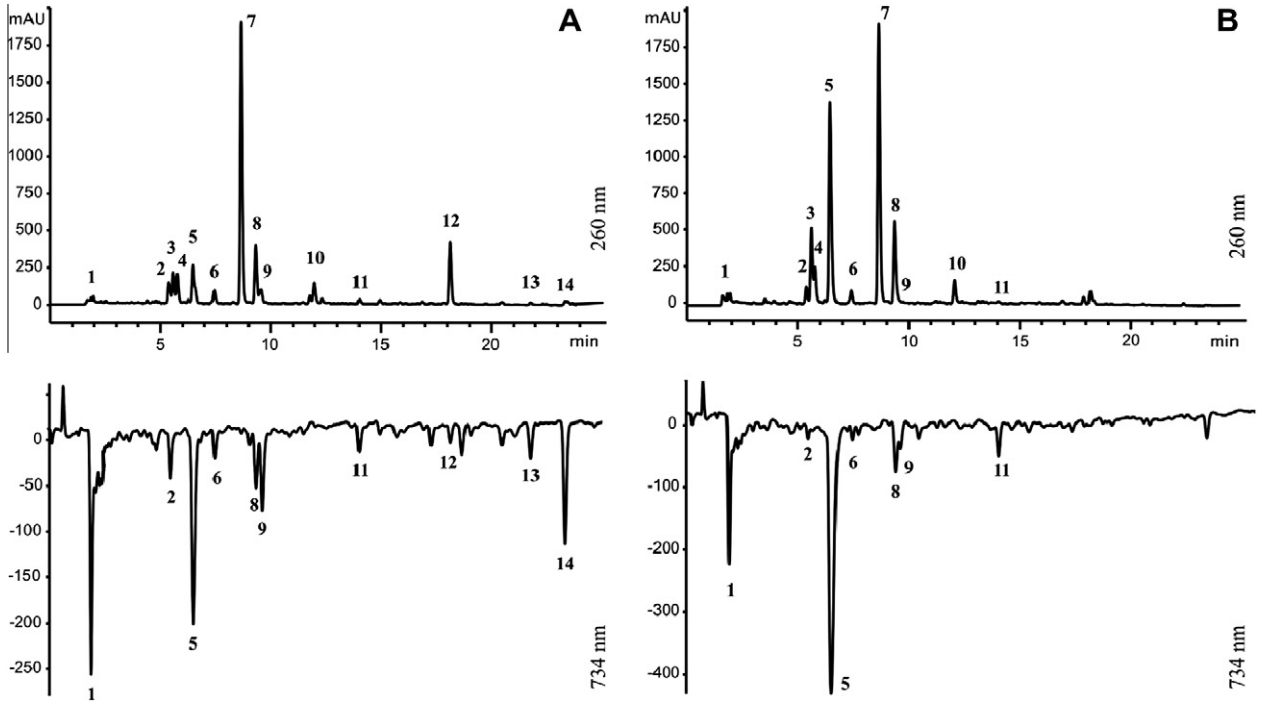

Fig. 1. Sample HPLC chromatograms of bioactive compounds (260 nm) obtained for methanolic extracts prepared from flowers (A) and haulms (B) of *G. asclepiadea* along with the profiles of antioxidants detected on-line with ABTS (743 nm). For identity of peaks see Table 1.

mangiferin displayed the strongest antioxidant potential among identified phytochemicals. In comparison with earlier data concerning antioxidant activities of phenolic compounds, the activity of mangiferin is comparable with that of rutin commonly used as an antioxidant for medical purposes (Kusznierewicz et al., 2011). A similar observation using a spectrophotomeric test with the DPPH radical was made by Dar et al. (2005). The total antioxidant activity of *G. asclepiadea* extracts studied ranged from 4.62 to 5.95 TE mM. Slightly stronger antioxidant properties were observed for extracts prepared from haulm. We have also confirmed scavenging activity of *G. asclepiadea* extracts using the DPPH assay (Hudecova et al., 2012a).

3.3. Kinetics of DNA repair in human peripheral blood mononuclear cells after H_2O_2 treatment and in HEK 293 cells after AgNPs exposure

Previously we found that pre-treatment of cells with *Gentiana* plant extract protects against H_2O_2– and AgNP-induced DNA damage (Hudecova et al., 2012a,b,c,d). Both H_2O_2 and AgNPs induce DNA damage in human cells. However, their mechanisms, promptness and rate of ROS production could be different, as could the underlying mechanisms leading to potential genotoxicity. The cells were challenged with H_2O_2 or AgNPs and then incubated for DNA recovery in the absence or presence of *Gentiana* extracts. Residual DNA damage was measured with the comet assay.

The ability of human peripheral blood mononuclear cells to rejoin strand breaks (SBs) induced by H_2O_2, was assessed by measuring DNA damage remaining at different recovery times (4, 8, 16, 24 and 32 min). DNA SBs were determined by the comet assay. Immediately after the treatment (at 4 min time point) the level of SBs was 70% DNA in tail. SBs decreased with the time of recovery and at 32 min the level of SBs was reduced to 12%. Fig. 2 and 3A and B show the background level of DNA SBs (control cells), DNA SBs induced with H_2O_2 (250 μM, 5 min) and the level of DNA SBs in cells incubated in the presence of *G. asclepiadea* extracts. The level of DNA SBs was measured also in untreated cells during the post-treatment period at the same time points – 4, 8, 16, 24 and 32 min. No significant change in the background level of SBs was found in untreated cells. To validate that none of the four plant extracts is genotoxic, cells were treated with the extracts at 0.25 mg/ml and none of them induced DNA damage; the levels of SBs did not significantly differ from those of the negative controls. These findings were in concordance with our previous experiments on human lymphocytes, where no plant extracts exhibited DNA damaging effects (Hudecova et al., 2012a,b). To assess effects of the plant extracts on the ability of human lymphocytes to rejoin DNA SBs, cells were treated with H_2O_2 for 5 min and then incubated with or without the *G. asclepiadea* extracts in fresh medium. The results showed significantly less DNA damage compared with post-treatment in medium alone, at different times of recovery (4, 8, 16, 24 and 32 min). Human lymphocytes treated with H_2O_2 and incubated in the absence of the plant extract after 8 min of recovery had rejoined ~40% of SBs (calculated as a decrease in% tail DNA compared to control), while in medium with 0.25 mg/ml of plant extracts, cells had rejoined ~90% (HA; Fig 2B), ~86% (FM; Fig 3A), ~81% (HM; Fig. 2A) and ~72% of SBs (FA; Fig 3B). After 8 min of DNA recovery in the presence of plant extracts we observed a plateau, and further decreases in% tail DNA were not significant. The only exception was when 0.25 mg/ml of flower/aqueous extract was used (Fig 3B), where the % tail DNA decreased between 8 and 16 min from ~19% to ~3% ($p < 0.05$). Thus the recovery of cells was greatly accelerated in medium supplemented with plant extract.

In the same way as with H_2O_2, we assessed the ability of HEK 293 cells to rejoin DNA SBs induced by AgNPs by measuring damage remaining at different times of recovery (5, 10, 15, 30, 60 and 120 min) in the DMEM medium either in presence or absence of plant extracts. After AgNP exposure the most pronounced decrease in SBs (from ~19% to ~7% tail DNA) was detected between 30 and 60 min of recovery. After 60 min, no further rejoining was observed but the level of SBs had not returned to the background values. The results in Figs 4 and 5A and B show that the untreated cells had

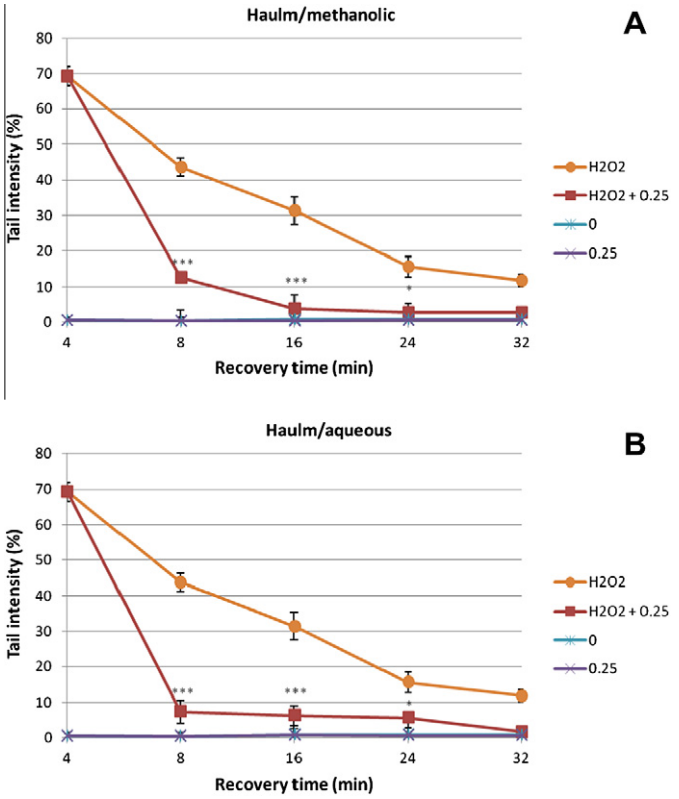

Fig. 2. Effects of *G. asclepiadea* haulm methanolic (A) and aqueous (B) extracts (0.25 mg/ml) on kinetics of DNA repair after treatment of human peripheral blood mononuclear cells (lymphocytes) with H_2O_2 (250 μM, 5 min) . H_2O_2 – cells treated with H_2O_2 (250 μM, 5 min) and incubated for 32 min in medium for DNA recovery; H_2O_2 +0.25 mg/ml – cells treated with H_2O_2 and incubated in medium in the presence of *G. asclepiadea* haulm methanolic (A) and aqueous (B) extracts for up to 32 min; 0 – untreated cells incubated in medium for 32 min; 0.25 mg/ml of *G. asclepiadea* extracts – cells incubated in medium in the presence of cell extract. The significance of differences between means was evaluated by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

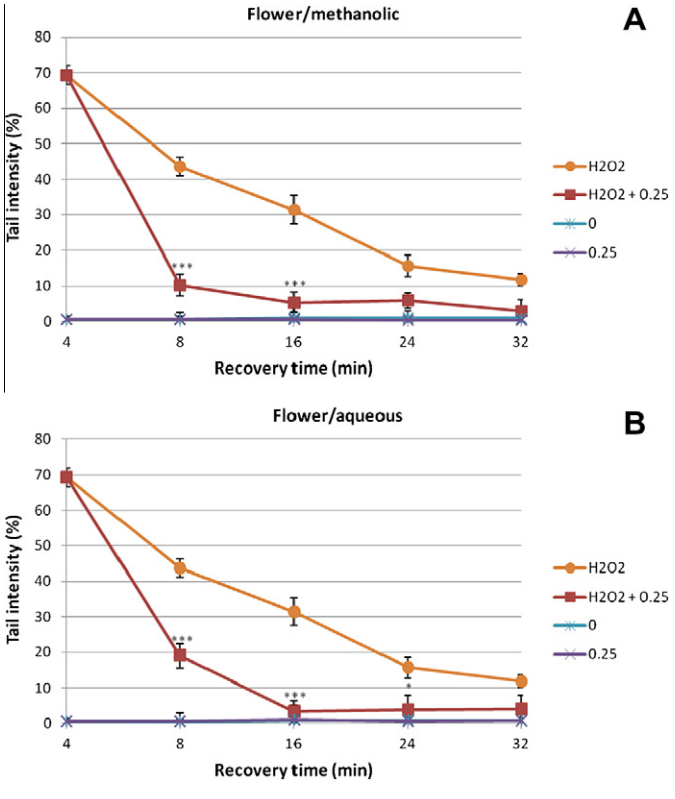

Fig. 3. Effects of *G. asclepiadea* flower methanolic (A) and aqueous (B) extracts (0.25 mg/ml) on kinetics of DNA repair after treatment of human peripheral blood mononuclear cells (lymphocytes) with H_2O_2 (250 μM, 5 min). H_2O_2 – cells treated with H_2O_2 (250 μM, 5 min) and incubated for 32 min in medium for DNA recovery; H_2O_2 +0.25 mg/ml – cells treated with H_2O_2 and incubated in medium in the presence of *G. asclepiadea* haulm methanolic (A) and aqueous (B) extracts for up to 32 min; 0 – untreated cells used incubated in medium for 32 min; 0.25 mg/ml of *G. asclepiadea* extracts – cells incubated in medium in the presence of cell extract. The significance of differences between means was evaluated by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

virtually no DNA damage. We also measured DNA damage in cells incubated only with plant extracts to assess whether extracts induce DNA damage. No significant difference compared to the negative control was found. These results confirm our previous finding that plant extracts in tested concentrations are non-genotoxic to kidney cells (Hudecova et al., 2010).

Cells incubated with plant extracts after AgNP (20 nm) exposure clearly recovered faster than without extracts. Results show the ability of HM (Fig 4A), HA (Fig 4B), FM (Fig 5A) and FA (Fig 5B) extracts to enhance the DNA rejoining after AgNP treatment compared with the recovery period only in medium. The most pronounced increase in SB rejoining compared to the initial DNA damage after AgNP exposure ($p < 0.001$) was between 5 and 10 min of recovery in all four extracts, where DNA damage decreased from 26.6% tail DNA (AgNPs – positive control) to ~13% tail DNA (HM, HA and FM) (Fig 4A and B, Fig 5A) and ~11% (FA) (Fig 5B). In cells recovered in medium supplemented with haulm extracts we have observed significant ($p < 0.05$) DNA repair between 30 and 60 min of recovery (Fig 4A and B) which was not observed after treatment with flower extracts. DNA damage decreased during these 30 min from 13.7% to 5.9% tail DNA in the cells incubated in DMEM with the 0.25 mg/ml concentration of HM extract (Fig 4A) and from 12.4% to 6.7% tail DNA in HA extract (Fig 4B). After 60 min of recovery, a plateau was observed with no significant decrease in the DNA damage.

Oxidative DNA damage induced by ROS and free radicals is important in the pathogenesis of many human diseases, including cancer, muscle degeneration, coronary heart disease, and is also thought to be a factor in ageing. ROS induce both cytotoxic and mutagenic damage. H_2O_2 is one of the main sources of ROS and is known to cause a spectrum of DNA lesions, including single and double strand breaks, in various cell types (Finnegan et al., 2010). We confirmed that AgNPs cause ROS formation and induce DNA damage including oxidative DNA lesions (Hudecova et al., 2012d; Ahamed et al., 2010; Piao et al., 2011).

As mentioned above, mangiferin was the most pronounced antioxidant compound in *G. asclepiadea* extracts. Rao et al. (2009) revealed that mangiferin had potent cytoprotective and antigenotoxic effects against $CdCl_2$-induced toxicity in the HepG2 cell line which could be attributed to a decrease in $CdCl_2$-induced ROS levels and resultant oxidative stress (Rao et al, 2009). Furthermore the findings of Das et al. (2011) indicated a protective effect of mangiferin against methylmercury-induced toxicity in human neuroblastoma cells. This may be attributed to its antigenotoxic, antiapoptic and anti-lipid peroxidative potential plausibly because of its free radical scavenging ability, reducing oxidative stress and in turn facilitating the down-regulation of mitochondrial apoptotic signaling pathways (Das et al., 2011). Based on obtained results as well as literature data, we assume that mangiferin along with an unidentified polar compound were the constituents which clearly attributed to the enhancement of DNA repair after treatment with both types of studied agents. We hypothesise that this can be based on both antioxidant and antigenotoxic properties of mangiferin. Though lower than mangiferin, homoorientin antioxidant capacity was also

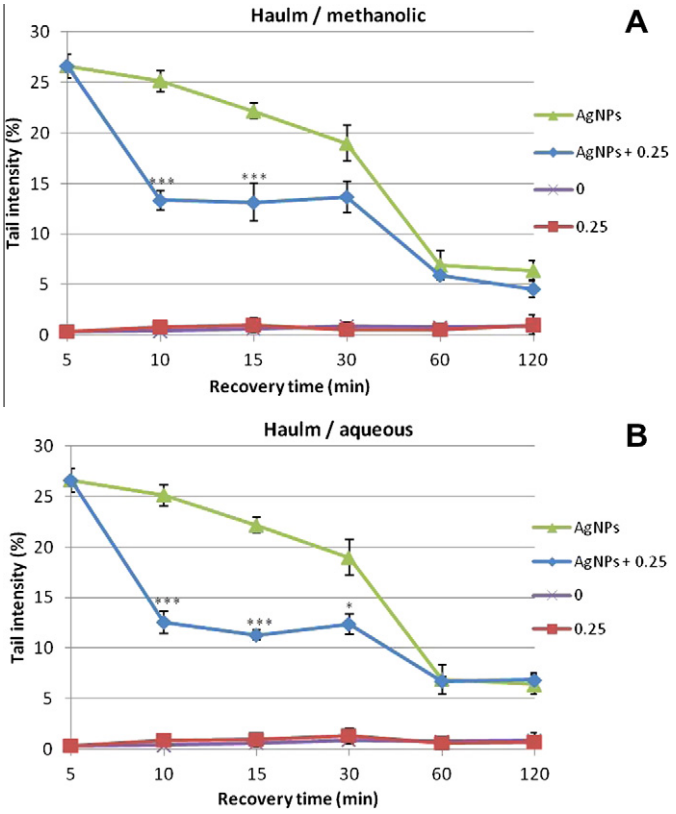

Fig. 4. Effect of *G. asclepiadea* haulm methanolic (A) and aqueous (B) extracts (0.25 mg/ml) on kinetics of DNA repair after AgNP treatment (20 nm, 100 μg/ml, 30 min) of HEK 293 cells during 120 min post-treatment. AgNPs – cells treated with AgNPs (100 μg/ml) and incubated in medium for 120 min; AgNPs +0.25 mg/ml – cells treated with AgNPs and left to recover in medium in the presence of *G. asclepiadea* haulm methanolic (A) or aqueous (B) extract at the 0.25 mg/ml concentration for 120 min; 0 – untreated cells incubated in meadia for 120 min; 0.25 mg/ml – untreated cells incubated in the presence of the 0.25 mg/ml of *G. asclepiadea* haulm methanolic or aqueous extracts. The significance of differences between means was evaluated by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

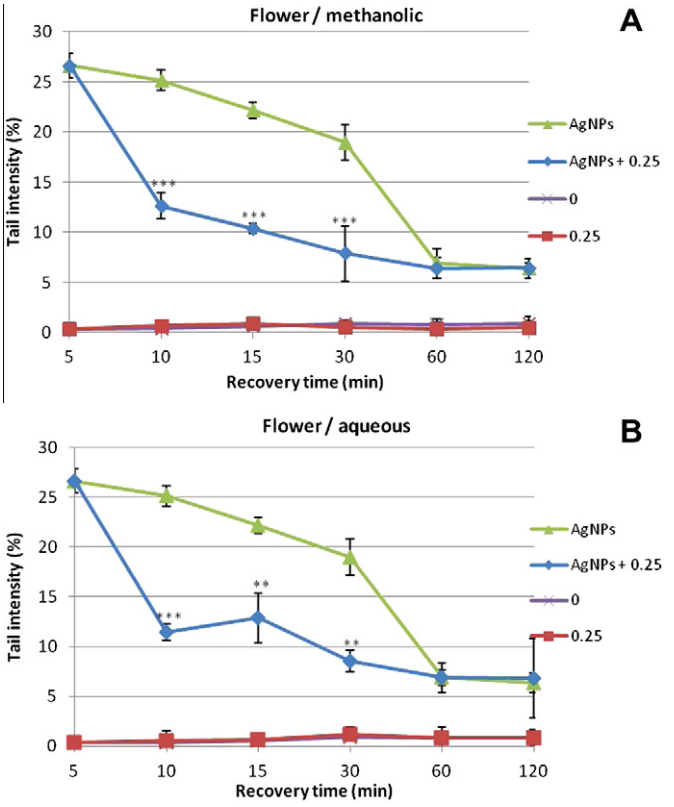

Fig. 5. Effect of *G. asclepiadea* flower methanolic (A) and aqueous (B) extracts (0.25 mg/ml) on kinetics of DNA repair after AgNP treatment (20 nm, 100 μg/ml, 30 min) of HEK 293 cells during 120 min post-treatment. AgNPs – cells treated with AgNPs (100 μg/ml) and incubated in medium for 120 min; AgNPs +0.25 mg/ml – cells treated with AgNPs and left to recover in medium in the presence of *G. asclepiadea* flower methanolic (A) or aqueous (B) extract at the 0.25 mg/ml of extract; 0 – untreated cells used incubated in medium for 120 min; 0.25 mg/ml – untreated cells incubated in the presence of 0.25 mg/ml of *G. asclepiadea* flower methanolic or aqueous extracts. The significance of differences between means was evaluated by Student's *t*-test: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

detected. Its activity was measured also against 2,2́-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cations (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), superoxide anion radical $O_2^{-\cdot}$ whilst the reducing activity was determined by the cyclic voltammetry method. Homoorientin showed pronounced antioxidant capacity in all tests used (Zielińska and Zieliński, 2011). These findings correlate with the research of Zeraik et al. (2011) who postulated that passion fruit (*Passiflora edulis*) extract is a potential source of molecules with strong antioxidant activities, and that homoorientin is particularly implicated in these antioxidant properties. Antioxidant properties of passion fruit were demonstrated by several methods such as ORAC, CAP, CAP-e and ROS PMN assays (Kang et al. 2010). Among other detected compounds, swertiamarine was found to possess significant antioxidant and hepatoprotective properties on rat liver damage induced by d-galactosamine (d-GalN). d-GalN causes significant hepatotoxicity by alteration of several hepatic parameters and also causes significant lipid peroxidation and reduces the level of antioxidant defence mechanisms (Jaishree and Badami, 2010).

All these biologically active compounds from *G. asclepiadea* that showed antioxidant potential are likely to include the phytochemicals that modulated the kinetics of DNA repair as was shown in our experiments. It is possible that these multiple constituents may work synergistically, antagonistically or additively in biological systems (cells) and the enhancing effect on repair may differ accordingly. Often, several components of the extract are responsible for the studied biological effect, whereas the isolated pure substances show little or no activity. The use of the mixture can however have many advantages, as the mixture can have a significantly higher clinical effectiveness than one active compound alone. Our findings about the ability of studied extracts (presenting a mixture of compounds) to accelerate the repair of H_2O_2 and AgNP induced lesions has an important implication for utilisation of plant extracts that have been used for centuries in traditional medicine.

DNA repair is an essential function in removing DNA damage before it can cause mutations and contribute to cancer. Several studies show that some micronutrients or phytochemicals can modulate DNA repair (Collins et al., 2012). However, the mechanisms involved in this process are not yet understood. The fact that DNA repair can be modulated by diet is striking. Different DNA repair pathways may be targets for specific phytochemicals. However, it is also possible that common underlying mechanisms are involved in this process. We hypothesise that phytochemicals may interfere with phase II metabolism and that modulation of repair may be via phase II enzymes which are involved in DNA damage signalling. Recently it was found that the activity of glutathione S transferases (GST) can influence DNA stability and repair of oxidative DNA damage. This suggests that GSTs may be involved in DNA damage signalling (Dusinska et al., 2012). GST enzymes are important in the control of oxidative stress and their roles as antioxidants and in phase II metabolic pathways are well known. However, GSTs can act also via non-enzymic mechanisms and are involved in cell

signalling through MAPK kinases (Laborde, 2010) and it was hypothesised that the cellular and nuclear defence systems may be connected (Dusinska et al., 2012). We previously found that *G asclepiadea* plant extracts protect against oxidative DNA damage (Hudecova et al., 2012a) which might support the hypothesis that stimulation of DNA repair goes through antioxidant defence pathways but the exact mechanisms how DNA repair is modulated should be further investigated.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

We would like to thank Fridrich Gregáň and Dagmar Vaculčíková (Department of Chemistry, Faculty of Natural Sciences, Matej Bel University, Tajovského 40, 974 01, Banská Bystrica, Slovakia) for providing us the *Gentiana asclepiadea* extracts and Andrew R. Collins for reviewing manuscript. This work was supported by Polish-Norwegian Research Fund 115 [PRNF/122-A I-1/07], VEGA: 1/0025/11; 2/0072/09; APVT-20-00-2604 and NILU internal grant 106170. Granted governmental scholarships of Ministry of Education, Science, Research and Sport of the Slovak Republic 1253/2009.

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