Cytotoxic and genotoxic effect of methanolic flower extract from *Gentiana asclepiadea* on COS 1 cells

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Abstract

OBJECTIVE: The purpose of this study was to assess whether a methanol extract isolated from the flower of *Gentiana asclepiadea* had potential cytotoxic or genotoxic effect on COS 1 (monkey kidney) cell line. Five various concentrations of the extract were investigated for cytotoxicity and genotoxicity and to determine non-cytotoxic and non-genotoxic concentrations suitable for utilization in pharmacology and medicine.

METHODS: Cytotoxicity was determined using the proliferation (growth activity) and the plating efficiency (colony forming ability) assays after 24 hour incubation of COS 1 cells with different concentrations of methanolic flower extract from *G. asclepiadea*. To assess potential genotoxicity, the comet assay or SCGE (Single-Cell Gel Electrophoresis) was used.

RESULTS: We found that only the highest (5 and 25 mg/ml) concentrations of the extract revealed cytotoxic and genotoxic effect. We have also determined concentrations that stimulated cell growth (0.25 mg/ml) and colony forming ability (0.25–2.5 mg/ml) and did not exhibit genotoxic effect (0.25–2.5 mg/ml).

CONCLUSIONS: We found out that extract of *G. asclepiadea* was neither cytotoxic nor genotoxic in a wide range of concentrations (0.25–2.5 mg/ml) and thus can be used to further investigate potential beneficial usage in pharmacology and medicine.

ORIGINAL ARTICLE

INTRODUCTION

Plants and plant extracts have been used for centuries as a valuable source of natural products for maintaining human health and their use can be of great significance in many aspects of human life. The medicinal values of plants lie in their component phytochemicals such as alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body (Hill 1952). These components exhibit a wide range of biological activities. Increasing public demand for the replacement of synthetic antioxidants by natural ones has initiated intense research activity that resulted in the screening of a wide variety of plant extracts and stimulated efforts to discover effective preservatives for a wide range of foodstuffs (Nadova *et al.* 2008). Plants, including herbs and spices, contain many phytochemicals which are potential sources of natural antioxidants, e.g. phenolic diterpenes, flavonoids, tannins and phenolic acids. These compounds possess antioxidant, anti-inflammatory and anticancer activities (Miadokova *et al.* 2006). In addition, plant food-derived antioxidants are increasingly proposed as important dietary antioxidant factors. Natural products are cheap and claimed to be safe (Horvathova *et al.* 2006; Slamenova *et al.* 2002). Additionally, many plant extracts are nowadays considered as a powerful alternative to some of the synthetic medications (Nadova *et al.* 2008) due to the high content of natural bioactive substances which may have an important function in protection against numerous diseases. A problematic aspect in understanding potential toxicological events relevant to the use of herbs is that the exact amounts of active chemical constituents are frequently unknown. These multiple constituents may work synergistically and could hardly be separated into active parts (Silva *et al.* 2008).

Plants belonging to *Gentianaceae* are widely used in traditional medicines in many countries for stimulation of appetite and gastric secretion, gastro-duodenal protection, liver protection, antifungal treatment, and in some cases for gynaecological diseases (Matsukawa *et al.* 2006). It is generally known, that *Gentiana* is used in small amounts as a food and beverage flavouring, in anti-smoking products and even was used as a substitute for hops in making beer. Experimental studies have showed, that *Gentiana* contains as main components compounds such as secoiridoid-glycosides (gentiopicroside, sweroside, swertiamarin, amarogentin), xanthone glycosides (gentioside and its isomer), terpenes, xanthones (gentisin, isogentisin), secoiridoids (gentiopicrine), alkaloids (gentianine), anthocyanins (delphinidine) and flavone C-glucosides (Georgieva *et al.* 2005; Jiang *et al.* 2005; Jensen & Schripsema 2002; Ozturk *et al.* 2002, 2006; Wu *et al.* 2007; Wu *et al.* 2009). Iridoids are the most common constituents of genus *Gentiana*, and they have been found to exhibit a wide range of bioactivities including antibacterial, anticancer, antifungal, antioxidant, anti-inflammatory, anticoagulant, choleretic, cardioprotective, hepatoprotective, neuroprotective, hypoglycaemic, antispasmodic, immunomodulatory and purgative properties (Jensen & Schripsema 2002; Tundis *et al.* 2008; Dinda *et al.* 2007).

The purpose of this study was to examine cytotoxicity and genotoxicity of the methanolic flower extract obtained from *Gentiana asclepiadea* on COS 1 cell line and to determine concentrations that do not exhibit cytotoxic or genotoxic effect.

MATERIALS AND METHODS

Preparation of methanolic extract from Gentiana asclepiadea

Gentiana asclepiadea extract was prepared as follows: plants were harvested from the Garden of Medicinal Plants, Faculty of Pharmacy, Comenius University, Bratislava in August 2008. The separated plant material (flowers), weighing about 60 g, was air-dried to dryness at room temperature, cut into small pieces and then extracted with 150 ml of methanol at 65 °C. This procedure was repeated 5-times. The extract was then filtered and concentrated in vacuo and the rest of the water was removed by azeotropic destillation with benzene. The final extract was kept in the dark at 4 °C until tested.

Cells

Experiments were carried out on COS 1 cell line which was obtained by immortalizing a CV-1 cell line derived from kidney cells of the African green monkey with a version of the SV40 genome that can produce large T antigen but has a defect in genomic replication.

COS 1 cells were cultured in Dulbecco´s Modified Essential Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 µg/ml streptomycin in humidified atmosphere of 5% CO_2 at 37 °C.

Proliferation (growth activity) assay

The cells (2.5×10^5) were plated on 6 well plates and incubated at 37 °C in a 5% CO_2 atmosphere. After 24 hours, the cells were exposed to 5 different concentrations (0.25, 0.5, 2.5, 5, 25 mg/ml) of the extract for 24 hours. After trypsinization, detached cells in culture medium were counted and plated on 6 well plates at concentration 1.2×10^5 per well. Cells were counted every 24 hours for each concentration. Mean from three values (three independent experiments performed in different time) was calculated.

Plating efficiency (colony forming ability) assay

The cells (2.5×10^5) were plated on 6 well plates and incubated at 37 °C in a 5% CO_2 atmosphere. After 24 hours, the cells were exposed to 5 different concentrations (0.25, 0.5, 2.5, 5, 25 mg/ml) of the extract for 24 hours. After the treatment cells were washed, trypsinized and plated on 6 well plates (200 cells per well).

The cells were incubated for 10 days to form colonies, then stained with methylene blue (1% solution) and the number of colonies was counted. The relative Plating efficiency (PE in percentage) was calculated.

Single-cell gel electrophoresis (SCGE; comet assay)

The comet assay was performed according to Collins *et al.* (1996). Briefly: microscope slides were pre-coated with base layer of 100 µl of 1% NMP (normal melting point) agarose in water. The cells were trypsinized, centrifuged and re-suspended in 1% LMP (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 spread on pre-coated slides. Hydrogen peroxide (H_2O_2) was used as a positive control (slides were submerged for 5 min in 250 µM H_2O_2, on ice). All slides were 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 hour to remove cellular membrane and cytoplasm leaving nuclei. After lysis, the slides were transferred to an electrophoretic tank containing alkaline solution (300 mM NaOH, 1mM Na_2EDTA, pH>13) for 20 min at 4 °C to allow the DNA to unwind. The electrophoresis run under following conditions: 25 V, 300 mA, 30 min. The slides were removed and neutralized in PBS (10 min) and ddH_2O (10 min) at 4 °C. Each sample was stained with 20 µl SYBRgold (0.1 µl/ml in TE buffer – 10 mM TrisHCl, 1 mM Na_2EDTA, pH 7.5–8) and per each sample 100 nucleoids per sample were scored by computerized 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.

Statistical analysis

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

RESULTS

The cytotoxicity (proliferation – growth activity, plating efficiency – colony forming ability) and genotoxicity (SCGE) of methanolic extract from flower of *Gentiana asclepiadea* on COS 1 cells were determined as shown in Figures 1–3.

The result of the proliferation (growth activity) assay (Figure 1) shows, that the highest concentration of the extract (25 mg/ml) was cytotoxic and inhibited the proliferation of the COS 1 cells. The highest differences in cell growth were seen after 96 and 120 hours after incubation with the extract. The most effective in stimulating of the proliferation comparing to the control was the lowest concentration (0.25 mg/ml). Differences in cell growth between control cells and cells treated with 0.5, 2.5 and 5 mg/ml were not statistically significant.

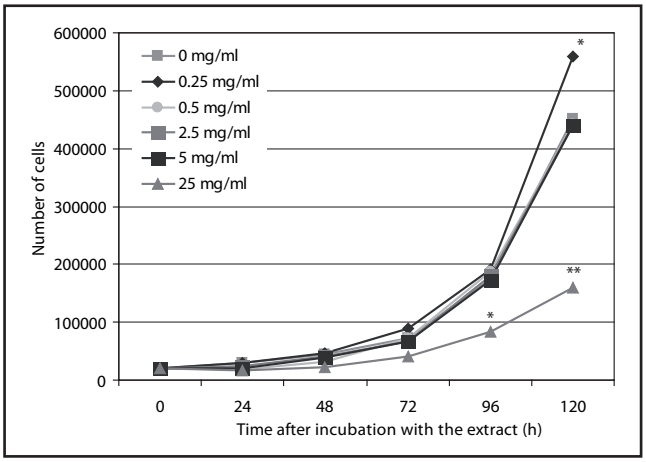

Fig. 1. Cell proliferation (growth activity) of COS 1 cells monitored after the 24 hour treatment in 0, 24, 48, 72, 96 and 120 hours incubation intervals with various concentrations (0.25–25 mg/ml) of methanolic flower extract from *G. asclepiadea*. The results show mean from three values (three independent experiments performed in different time). The significance of differences between means was evaluated by Student's *t*-test: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

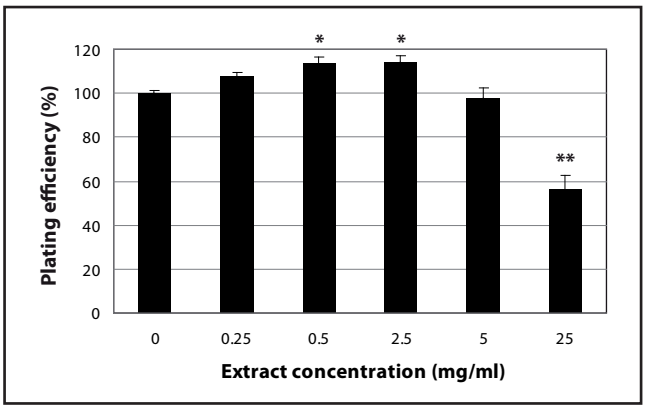

Fig. 2. Plating efficiency (Colony forming ability) of COS 1 cells after 24 hour incubation with various concentrations (0.25-25 mg/ml) of methanolic flower extract from *G. asclepiadea*. The significance of differences between means was evaluated by Student's *t*-test: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

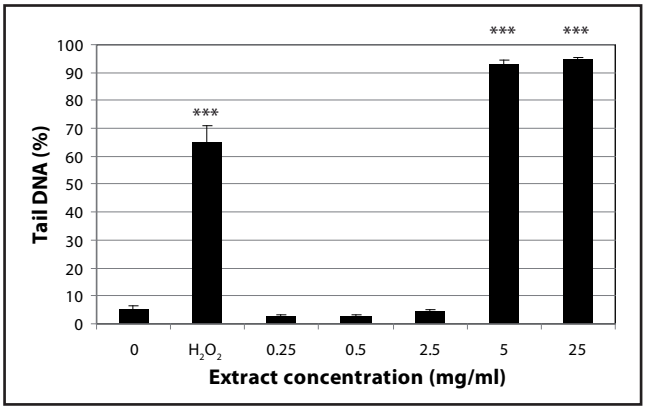

Fig. 3. DNA damage (strand breaks) measured by the Comet assay in COS 1 cells after the 24 hour treatment with various concentrations (0.25-25 mg/ml) of *G. asclepiadea* methanolic flower extract. H_2O_2 (250 µM) was used as a positive control. The significance of differences between means was evaluated by Student's *t*-test: $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Plating efficiency (colony forming ability) assay results (Figure 2) show that the highest concentration (25 mg/ml) of the extract inhibited the colony forming and was cytotoxic. Concentration 5 mg/ml slightly inhibited colony forming ability but the difference was not statistically significant comparing to the control. Concentrations 0.25, 0.5 and 2.5 mg/ml stimulated forming of colonies compared to the control.

After the treatment of COS1 cells for 24 hours with methanolic extract we investigated the level of strand breaks using the Comet assay (SCGE) (Figure 3). The results show that the low concentrations of the extract did not induce genotoxic effect. High induction of DNA damage was found after the treatment with 5 and 25 mg/ml of extract. Almost all the comets in samples treated with these concentrations had the highest tail intensity with almost 100% DNA damaged and exhibited even higher level of DNA damage than the positive control (H_2O_2).

DISCUSSION

Plant *Gentiana asclepiadea* L. is perennial herb and the root has been traditionally used as medicine for hepatitis A virus infections (Saric 1989). Many *Gentiana* species have been studied for their potential positive effect on human health due to content of many bioactive compounds with a wide range of biological activities. Root extract from *G. triflora* triggered cell death of human Daudi cells in culture. In addition, daily administration of the extract to mice inhibited growth of implanted solid tumors (Matsukawa et. al 2006). Methanolic extracts of *G. lutea* leaves and roots in two different systems using electron spin resonance (ESR) spectrometry were investigated for the free radical scavenging activity. This study showed that yellow gentian leaves and roots exhibit considerable antioxidant properties, expressed either by their capability to scavenge DPPH or superoxide radicals (Kussar *et al.* 2006) similarly as was revealed in our study on *G. asclepiadea* (Hudecová *et al.* submitted) and also G. decumbens which exhibited a strong scavenging activity for DPPH, superoxide and hydroxyl radicals (Myagmar & Aniya 2000). Various phenolic antioxidants, such as flavonoids, tannins, coumarins, xanthones and more recently procyanidins scavenge radicals dose-dependently, thus they are viewed as promising therapeutic drugs for free radical pathologies (Hollman & Katan 1998).

Interestingly, we found dose dependent effect of methanolic extract of *G. aslepiadea* on mammalian (monkey) cells. While low and mild concentrations up to 2.5 mg/ml show no cytotoxic and genotoxic effects, high concentration demonstrated clear cytotoxicity and potential genotoxicity. *G. aslepiadea* is an interesting plant due to the high contain of bioactive compounds (Georgieva *et al.* 2005; Jiang *et al.* 2005; Jensen Schripsema 2002; Ozturk *et al.* 2002, 2006; Wu *et al.* 2007; Wu *et al.* 2009) with potentially beneficial effect as it is also shown in our recent study (Hudecová *et al.* submitted) where we revealed that the extracts from *G. aslepiadea* may protect human cells against DNA oxidation. However, as we show here, careful consideration of dose range is needed. From the results obtained in this study by the proliferation (growth) activity, the plating efficiency and the comet assay we can conclude, that the most suitable concentrations of the *G. asclepiadea* methanolic flower extract that can be recommended in further research are concentrations between or below 0.25–0.5 mg/ml, since these concentrations neither induce cytotoxic nor genotoxic effects in COS1 cell line. However, the exact mechanisms of the potential protective effect of the extract in safe dose range remains to be further investigated.

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