Toxicity assessment of *Gentiana lutea* L. root extract and its monoterpane compounds

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Received 20 December 2019; revised 05 August 2020

Root of *Gentiana lutea* commercially available as gentian root, a natural antidote for different types of poisons, possess antioxidative, immunomodulatory, cytoprotective and anti-inflammatory, and adverse, genotoxic and mutagenic effects. It has monoterpenes loganic acid, swertiamarin, gentiopicroside and sweroside as most abundant constituents. In this study, we assessed the toxicity of monoterpenes’ reactive molecular fragments using *in silico* prediction by VEGA-QSAR platform. Further, we compared the data obtained with *in vitro* geno- and cyto-toxicity testing of the above monoterpenes and the *G. lutea* root extract (GE), on human primary unstimulated and mitogen-stimulated peripheral blood mononuclear cells (PBMCs). Viability was assessed by TB and XTT tests after 48 h treatment. DNA damage was evaluated by alkaline comet assay on unstimulated cells, whereas cytokinesis-block micronucleus assay was employed on mitogen-stimulated PBMCs. Stability of compounds throughout treatment was monitored by UPLC. The observed *in vitro* results had highest compliance with *in silico* IRFMN/ISSCAN-CGX prediction model. Compounds showed high stability during experiment while treatment with single compounds reduced number of viable cells and increased DNA damage. GE treatment had toxic impact on unstimulated PBMCs but no significant genotoxic influence on mitogen-stimulated PBMCs. In summary, the mild GE effect suggests that the complexity of crude GE extract chemical composition may attenuate the toxicity of the tested monoterpenes loganic acid, swertiamarin, gentiopicroside and sweroside.

**Keywords:** Gentian root, Gentiopicroside, Loganic acid, Structural alerts, Sweroside, Swertiamarin, Yellow gentian

*Gentiana lutea*, also known as the yellow gentian, is a plant that belongs to the family of Gentianaceae widely distributed in the mountain regions of Europe, Southeast Asia, and North America. It is commonly used in traditional medicine, however root extract (GE), and its monoterpenes, including loganic acid (La), sweroside (Sw), gentiopicroside (Gp), and swertiamarin (Sm), beside beneficial1,2, antioxidative and immunomodulatory3,4 exert detrimental, genotoxic5, mutagenic and clastogenic effects6,7. For instance, depending of experimental conditions, gentiopicroside, a most dominant compound8, causes both cytoprotective9 and damaging outcomes6, while sweroside and swertiamarin, which are present in lower amounts than gentiopicroside, manifest non-toxic10,11 to noticeably cytotoxic effects12. Although listed monoterpenes share structural similarity, the slight differences in their structure may influence intensity of toxic potential13.

Toxicology assessment software offers rapid evaluation of chemical toxicity considering different toxicological endpoints and/or metabolic pathways. Two main approaches for the assessment of compounds’ toxicity are mostly used, Read-across and Quantitative Structure-Activity Relationship (QSAR)14,15. VEGA-QSAR platform combines both approaches, offering prediction for a number of outcomes through QSAR prediction models16. VEGA-QSAR provides five models for mutagenicity, four for carcinogenicity effect prediction and an independent algorithm for the evaluation of the result through the Applicability Domain Index (ADI)15,16. Genotoxicity and carcinogenicity correlates in aspects like chromosomal instability and unrepaired DNA damage17,18. Data of carcinogenic potency and cell viability studies originated from high-throughput screening (HTS) of environmental chemicals reveal that chemicals which affect cell viability are likely to be carcinogens19. Thus, VEGA-QSAR models’ prediction of mutagenic and carcinogenic compounds’ effects, could reflect both on geno- and cyto-toxicity outcomes20. Literature search suggests that the toxicity assessment obtained by *in silico* prediction should be further tested *in vitro*21.

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As exposure to chemicals in vivo leads to elevated concentrations of unchanged compounds and their metabolized products in peripheral blood, the peripheral blood mononuclear cells (PBMCs) are adequate and often used model system in toxicological studies. A part of PBMCs, T lymphocytes, can be transformed into blast forms with phytohaemagglutinin (PHA), and hence this model system enables testing on differentiated and dividing cells of the same origin. Since PHA-stimulated PBMCs exhibit higher level of repair capacity compared to unstimulated, their parallel testing could reveal toxicity potential grade of structurally similar compounds.

Here, we investigated monoterpene constituents of gentian root (Gentiana lutea) and their previously reported GE effects. We selected monoterpenes with possible toxic effects tested using the in silico toxicity analysis and monitored chemical stability during the treatment to confirm that the recorded effect due to the presence of these compounds, and further compared the in silico and in vitro results.

Materials and Methods

Chemicals/reagents

Commercially available Gentiana lutea L. radix (Gentian root) was purchased from the Institute of Medicinal Plant Research “Dr Josif Pantic”, Belgrade, Republic of Serbia (Product safety and quality management policy: SPRS ISO 9001:2015; codex alimentarius [CAC/RCP 1-1969, rev. 4-2003]). Chemicals used for cell cultures were acquired from Capricorn Scientific GmbH (Ebsdorf, Germany) and reference compounds were from Wuhan ChemFaces Biochemical Co (Wuhan, PRC). Trypan blue (TB), sodium 3,3′-[(phenylamino)carbonyl]-3,4-tetrazolium)-Bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT), phenazinemethosulfate (PMS), all used in viability assays were acquired from Serva (Heidelberg, Germany). Low melting point agarose (LMPA), normal melting point agarose (NMPA), cytochalasin B, Giemsa stain, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), analytical grade salts, solvents and buffer reagents were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). Vectashield solution was produced by Vector Laboratories Ltd (Peterborough, UK).

VEGA-QSAR analysis

For in silico analysis of the most common GE constituents (loganic acid, swertiamarin, gentiopicroside and sweroside) we used VEGA-QSAR software (http://www.vega-qsar.eu/). Namely, by inserting chemical structure of interest in the form of the simplified molecular-input line-entry system (SMILES) and selecting Tox models, VEGA-QSAR software provided us numerous information about compounds’ structure related effects. Among them were structural alerts (SAs) in chemical structure of tested compounds, based on analogy with known mutagenic and cancerogenic chemicals of similar structure. Additionally, software incorporated algorithm gave evaluation of reliability prediction as ADI value. We used positive results with ADI >0.5, as indicators of potential toxicity effect: low (0.5 <ADI <0.6), medium (0.6 <ADI <0.8) and high (0.8 <ADI <1).

Preparation of GE

Aqueous extract was prepared by heating ground G. lutea root (GE) in water, for 10 min, in ratio 1:5 (m/V). GE was filtered using Whatman Filter paper No.4 (GE Healthcare Life Sciences, MA, USA), supernatant was centrifuged at 10000 ×g for 20 min to separate non-soluble particles, lyophilized and stored in sample tubes at 4°C until use. Prior to analysis lyophilized powder was dissolved in 50% ethanol to obtain concentration 50 mg/mL, passed through 0.2 μm Minisart filter (Göttingen, Germany) and used as a stock solution in further experiments.

Chromatographic analysis

Waters ACQUITY Ultra Performance Liquid Chromatography (UPLC) system (Malvern Panalytical Ltd, Malvern, UK) with PDA detector and LUNA 3u, C18(2), 3 μm, 100 × 2 mm Phenomenex column (BO, Italy), as a stationary phase, was used for chromatographic separations. All analyses were done under gradient condition with mobile phase consisting of solvent A (0.1 wt.% HCOOH in water) and solvent B (0.1 wt.% HCOOH in methanol) at a constant flow rate of 0.3 mL/min. The solvent B content was changed from 5 to 55% up to 8 min, from 8-8.2 min the proportion of solvent B was decreased back to the starting and held constant up to 9 min. Autosampler and column compartment were maintained at 4 and 35°C, respectively. The 3D chromatograms were recorded in wavelength range from 210 to 500 nm and 2D chromatograms at 254 nm (for all reference compounds) and additionally at 271 nm for gentiopicroside. The run time was 9 min and injection volume 2-6 μL. Standard solutions of all investigated reference compounds (loganic acid, swertiamarin, gentiopicroside
and sweroside) were prepared for construction of calibration curve by dissolving 5 mM stock solutions in cell culture medium (concentration range from 25 to 50 µM). GE solution in concentration 1.0 mg/mL was used for quantification. Chromatograms of tested compounds in cell culture medium were recorded at the beginning (0 h) and end of treatment (48 h) to determine their stability at 37°C. Additionally, we analyzed media originated from both type of PBMCs cultures after 48 h of treatment. To provide clear samples, we precipitated serum proteins with HCl solution at final concentration 0.3% and centrifugation at 8000xg (Eppendorf AG, Hamburg, Germany). Furthermore, we tested the stability of compounds by titration to neutral pH with NaOH in a 10 mM final concentration.

**Cell culture**

Heparinized blood samples were obtained from healthy donors aged 20-40 years with informed consent. PBMCs were isolated using separation solution made with Ficoll™ density gradient media according to the manufacturer’s instructions. Cells were cultured in RPMI 1640, supplemented with 1% penicillin-streptomycin, 10% fetal bovine serum for all and 3% PHA–M solution for stimulated cultures. Reference compounds were dissolved in 50% ethanol to obtain 5 mM stock solutions.

PBMCs were resuspended in culture medium at a concentration of 1 x 10^6 viable cells/mL, treated with 50 µM reference compounds or 1.0 mg/mL GE, and placed in an incubator for 48 h at 37°C. At the end of the treatment, cultured cells were divided and used for viability and genotoxicity assays, while supernatant was aliquoted for chromatographic analysis.

**Viability tests**

Selection of optimal dose for *in vitro* treatments was performed by cell viability test (TB) employing different concentrations of plant extract (0.1-2 mg/mL) and single compounds (20-130 µM), on primary unstimulated PBMCs for 48 h. For TB assay, 100 µL of PBMC cell culture was mixed with the same volume of 0.4% TB suspension was counted using a haemocytometer (Cambridge Instruments Inc. NY, USA). Cell counting and calculation of number of viable cells in samples were performed according to Strober. Concentration of compound with lowest toxic effect, that significantly reduced number of viable cells after 48 h treatment, was chosen as further equimolar treatment concentration. Equimolar concentration of single compounds was used to discriminate some SAs or to validate cumulative effect of other.

Cell viability after 48 h treatment was assessed with TB and XTT viability assay. For XTT assay, 50 µL (1.0 mg/mL) of reagent activated with PMS was added to 100 µL aliquot of cultured cells per well and put in incubator at 37°C, for 2-4 h until development of colour. Colorimetric measurements were performed at 470 nm with cut off at 660 nm, using a Sunrise microplate reader apparatus (Tecan Group Ltd, Männedorf, Switzerland).

**Alkaline comet assay**

Alkaline comet assay was performed using an adaptation of the method of Singh et al. In brief, after 48 h of treatment, PBMCs were washed in 1×PBS, suspended in 1% LMPA in PBS, pH 7.4, at 37°C, and 100 µL were pipetted onto a glass microscope slide pre-coated with 100 µL layer of 1% NMPA. Slides were put at 4°C for 10 min for agarose to set, and then immersed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, NaOH to pH 10.0, and 1% Triton X-100) at 4°C for 1 h. Slides were then placed in an electrophoresis tank containing 0.3 M NaOH and 1 mM Na2EDTA, pH >13, for 20 min before electrophoresis. Electrophoresis was done at 25V (1 V/cm, 300 mA) for 20 min at 4°C. Slides were then washed 3 times for 5 min, each with 0.4 M Tris-HCl, pH 7.5, at 4°C, dried and counterstained with (DAPI)-containing Vectashield solution (5 µg/mL). For each slide at least 300 cells were evaluated for the percentage of DNA in the comet tail using Zeiss-Axioimager A2 microscope with automated Metafer, MetaSystem Metafer CometScan software (Altussheim, Germany).

**CBMN assay**

The CBMN assay was performed as described by Fenech. Slides were scored using an Axioimager 1 (Carl Zeiss, Jena, Germany), light optical microscope with magnification 400X, following the scoring criteria outlined by the International Human Micronucleus (HUMN) Project. Each sample was evaluated for the frequency of micronuclei (MNi-BN) and CBPI.

**Statistical analysis**

All experimental procedures were performed at least three times in duplicate and data were expressed as mean±SEM. Statistical significance was assessed by One-Way ANOVA accompanied by Tukeys’ post hoc test, using software SPSS for Windows 10.0. All results were considered significant at *P* <0.05.
Results

Positive results of VEGA-QSAR in silico prediction of tested compounds are presented in Table 1 and Fig. 1. VEGA-QSAR models, used for in silico mutagenic and carcinogenic prediction, marked structural alerts (SAs) number 29 and 76 in all analyzed molecules. Small differences in molecular structure of tested compounds were presented as additional SAs for loganic acid (La) (No 106) and gentiopicroside (Gp) (No 13, SM 55) (Fig. 1). From five mutagenicity models, only CAESAR 2.1.13 marked La as potential mutagen (ADI >0.8). Mutagenicity predictions for other tested compounds, in all models, were inconclusive since both positive and negative predictions were out of highly reliable AD. Carcinogenicity models CAESAR (2.1.9) and ISS (1.0.2) predicted non-carcinogenic effect of examined compounds with medium to high AD, while other two carcinogenicity models, IRFMN/Antares and IRFMN/ISSCAN-CGX, had opposite predictions (Table 1).

Quantitative UPLC analysis of Gentiana lutea root extract (GE) in medium is given in Table 2. Identification of peaks in GE chromatograms was performed by comparing their retention times and correspondent UV spectra with reference compounds, according to the calibration curves. Although retention times for Gp and sweroside (Sw) solutions in medium are similar, better resolution could be achieved through comparative analysis of chromatograms extracted at 254 and 271 nm (Fig. 2). Peak area corresponding to Sw is much lower for chromatogram recorded at 271 nm (Fig. 2B), while for Gp, peak area obtained at 271 nm (Fig. 2D) is higher than in the case of 254 nm (Fig. 2C).

As presented in Fig. 3, UPLC analysis of control GE treatment medium after 48 h, compared with initial condition, displayed stability of tested compounds. Analyses of complete cell culture medium, after 48 h of PBMCs cultivation with compounds, showed substantial change in chromatograms (Fig. 4). Numerous cell metabolites/

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<th>Table 1 — Positive prediction reliability of VEGA-QSAR models [Applicability Domain Index (ADI) values]</th>
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<td>Mutagenicity (CAESAR) 2.1.13</td>
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<tr>
<td>Mutagenicity (SarPy/IRFMN) 1.0.7</td>
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<tr>
<td>Carcinogenicity (IRFMN/Antares) 1.0.0</td>
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<tr>
<td>Carcinogenicity (IRFMN/ISSCAN-CGX) 1.0.0</td>
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[*low reliability prediction; **medium reliability prediction; and ***high reliability prediction]
constituents were eluted simultaneously with Gp and Sw with no change in retention times (Fig. 4B).

In primary unstimulated PBMCs, treatments with 50 µM of single compounds and treatment with GE at concentration of 1.0 mg/mL, significantly decreased number of viable cells ($P <0.05$, df=5, $F=21.5$) compared to control (Fig. 5A). Gp and Sw reduced the number of viable cells for 25%, standing out as more toxic than other tested compounds (Tukey's subset $1=1$, $a=0.05$). Moreover, all investigated single compounds significantly reduced the number of viable PHA-stimulated PBMCs compared to control, Fig. 4 — UPLC chromatograms of reference compounds and GE recorded at 254 nm, after 48 h lasting treatment: (A) control medium; and (B) PBMCs treatments. [0, medium; 1, Loganic acid (La); 2, Swertiamarin (Sm); 3, Gentiopicroside (Gp); 4, Sweroside (Sw); GE, Gentiana lutea root extract; injection volume 4.5 µL; * peak corresponds to component present in medium]
range from 13 to 16% ($P < 0.05$, $df=5$, $F=12.8$) (Figs 5B & 6). The DNA damage induced by Sw and Gp was more than 6- to 5-fold higher than control, while loganic acid (La), swertiamarin (Sm) and gentian root extract (GE) effect was 4-fold higher than control. Significant negative correlation between percentage of DNA in comet tail and number of viable cells in primary unstimulated treated cells was noted ($r=-0.488$, $P < 0.05$).

CBMN assay showed that GE had no influence on MNi frequency, while all single compounds elevated them in comparison to control ($P < 0.05$, $df=5$, $F=39.7$). Gp, Sw, La and Sm treatments increased MNi frequencies 3.5-fold, 2.7-fold, 2.4-fold and 1.9-fold, respectively (Fig. 5C). As illustrated in Fig. 5D, proliferative capacity of stimulated cells (CBPI), was significantly reduced by all compounds and GE, compared to control ($P < 0.05$, $df=5$, $F=101.5$), in range from 16% for Sw to 11% for GE (Fig. 5D). CBPI negatively correlates with MNi frequency ($r=-0.715$, $P < 0.05$).

Discussion
Quantitative analysis of gentian root extract (GE) showed high content of gentiopicroside (Gp) (20%) (Table 2) which is in an accordance with the other studies that also reported its high concentration. All investigated compounds showed high stability considering temperature and time of exposure ($37^\circ$C, 48 h), presented as overlap of chromatograms recorded in control medium with GE at 0 and 48 h (Fig. 3). Chromatograms of medium after 48 h of PBMCs cultivation showed that all compounds were present although unsuitable for quantification, most likely due to chromatograms complexity (Fig. 4B). To gain precise overview of cell culture activity more extensive analysis is required.

In primary unstimulated PBMCs, upon treatment with single compounds, number of viable cells was lowered compared to control as in PHA-stimulated PBMCs. GE lowered the number of viable cells in primary cell cultures, while in PHA-stimulated its effect was in range of control (Fig. 5A). Disparity in response to treatments, presented as higher number of viable cells in stimulated cultures, might be due to different repair capacity between metabolically dormant and active cells. Several studies reported that genes involved in repair had more than 2-fold increase of transcripts in the presence of PHA-stimulus.

All treatments on unstimulated PBMCs resulted in elevated percentage of DNA in comet tail, and lowered number of viable cells, pointing that accumulation of DNA damage over the treatment period, consequently induced cell death (Fig. 5A & B). Also, observed negative correlation of proliferative capacity presented as CBPI value and MNi frequency, suggests that cell death and/or cytostasis could be due to DNA damage (Fig. 5C & D) which is in accordance with previous study.

Interestingly, gentiopicroside (Gp) concentration in gentian root extract was 10 times higher than in Gp treatment with significant toxic effect (Table 2), indicating that single compounds have different effect than in crude extract. Mildest toxic effect of crude extract than its single constituents previously was explained as antagonistic effects of single compounds when they are present in the same mass concentration. Phenolic, flavonoid and flavone fractions of GE and extracts of plant, in general, display significant antioxidant capacity. They could modify damaging influence of single compounds present in complex extracts, as are secoiridoids present in GE. Mihailovic et al. suggested that bioactive compounds of GE act as bio-antimutagens expressing protective effects in a presence of mutation agent. This mode of action does not exclude potential toxic influence of GE compounds when they are individually applied.
Prior testing of IRFMN/ISSCAN-CGX 1.0.0 showed that this model achieved highest accuracy (75%) and sensitivity (82.6%), of all carcinogenicity models in VEGA-QSAR platform\textsuperscript{39,40}. High sensitivity indicates its strong ability to accurately predict carcinogenic effects of chemicals\textsuperscript{40}. IRFMN/ISSCAN-CGX 1.0.0 model marked Sw and Gp compounds as carcinogens with high reliability prediction (ADI >0.8), as confirmed by our experimental results (Table 1). La that was predicted as mutagen and carcinogenic, with medium reliability, in our study induced higher frequency of MNi than Sm and GE treatment. Swertiamarin (Sm), estimated as carcinogen with low reliability, indeed showed lowest toxic influence compared to other tested compounds. \textit{In silico} analysis marked Gp as a compound with numerous SAs, which is in a compliance with detected level of Gp toxicity (Fig. 1). Sweroside (Sw) and swertiamarin (Sm) have the same structural alerts (SAs) but different level of predicted as well as displayed toxicity, indicating that SAs could be used as indicators, rather than predictors, of potential toxicity\textsuperscript{15}. Apart from the SAs, toxicity potential might be based on other biological or physical property influenced by small structural difference of those molecules\textsuperscript{41}.

Conclusion

In the current study, toxicity of tested monoterpene compounds, documented by \textit{in silico} analysis and \textit{in vitro} testing, marked them as potential cause of GE detrimental effects. Treatment with these single compounds elevates DNA damage, seen as increment of micronuclei and percentage of DNA in the comet tail. This might be linked to the initiation and progression of cell death signalling cascade, although additional studies would elucidate precise mechanism of DNA strand brakes generation. Even though higher concentration of Gp in GE was present compared to single compound treatment, the effects on the cellular survival and DNA damage were milder. These results suggest that GE complex chemical composition could attenuate single compounds’ toxic effects, but their extent depends on cell repair ability.

Informed consent

Blood samples, used in this study, were obtained from healthy volunteers with informed consent. The study conformed to Declaration of Helsinki and was approved by Ethical Committee of the VINCA Institute of Nuclear Sciences, National Institute of the Republic of Serbia, University of Belgrade: Ethics committee approval no. 1109/1.

Acknowledgement

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 173046 and TR 37021).

Conflict of interest

Authors declare no conflict of interests.

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