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Cytotoxicity, antioxidant activity and an effect on CYP3A4 and CYP2D6 of *Mutellina purpurea* L. extracts

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ABSTRACT

Mutellina purpurea is an aromatic Apiaceae plant known as Alpine lovage. Its polar extracts consist of phenolic acids, tannins and flavonoids. The cytotoxic effect of methanolic and aqueous extracts from *M. purpurea* was studied on the most frequently used cell lines: HeLa and BHK-21. Taking into account that the natural products are often used with other medicines there is a risk of reciprocal interaction on the metabolic level. Thus, the influence of *M. purpurea* extracts was investigated on the activity of CYP2D6 and CYP3A4, which are the most important P450 isoenzymes from the pharmacological and toxicological points of view. Additionally, because *M. purpurea* contains phenolic compounds, the antioxidative properties of this plant extracts were also studied and compared.

1. Introduction

According to the World Health Organisation, traditional or folk medicine has been used until now by 75–80% of the world population. What is more, about 80% of the world population use phytotherapy as a one of the major elements of health care. Bioresources have a great potential in providing bioactive compounds for the development of new leading candidates for pharmaceuticals, nutraceuticals, and agrochemicals. At least 7000 chemical compounds used in the official phytotherapy are derived from medicinal plants (Holmes, 2005).

Our attention was drawn by *Mutellina purpurea* Thell. (syn. *Ligusticum mutellina*), which is a perennial, aromatic plant typical of the Carpathian mountains and the Tatra mountains. It grows on Alpine pastures, among the mountain pine trees and on the glades (Bojnanský and Fargašová, 2007). The *M. purpurea* foliage is a parsnip substitute, whereas dried leaves can be used as stomachic and calcium and potassium supplementing tea (Krebs, 1990; Uphof, 1968; Usher, 1974). The roots are the parts used for food flavouring (Seidemann, 2005). Dudek et al., (2008) described *M. purpurea* as an antituberculosis plant listed among *Cetraria islandica*, *Glechoma hederacea*, *Mentha arvensis*, *Plantago lanceolata*, *Plantago major*, *Polypodium vulgare*, *Pulmonaria officinalis* or *Verbascum thapsus* (Dudek et al., 2008). *M. purpurea* is named Alpine lovage and considered as a forage for animal nutrition together with *Plantago alpina* or *Plantago atrata* (De Noni and Battelli, 2008; Fischer and Wipf, 2009; Grabherr, 2009). *M. purpurea* belongs to the Apiaceae family and, like other plants in this group, it produces coumarins, phenolic acids, flavonoids, tannins and volatile compounds (Passreiter et al., 2005; Sieniawska et al., 2010; Sieniawska and Głowniak, 2011). In our previous study of polar extracts of this plant the chlorogenic, ferulic, caffeic, gallic, *p*-OH–benzoic, *p*-coumaric acids and their derivatives were described (Sieniawska et al., 2012).

Botanicals such as herbal products and nutraceuticals are often regarded as low risk because of their long history of human use. However, many of them revealed a very strong and even toxic activity in humans, which especially refers to extracts, concentrates or pure compounds obtained from plants. For this reason, it seems very important to conduct screening tests to assess the toxic and beneficial effects of plant materials. There is no golden means for systematic screening of untested plant extracts. However, it seems reasonable to investigate first of all the toxic effect and safety in usage. Thus, the purpose of this work was to study

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); CYP2D6, cytochrome P450, isoenzyme 2D6; CYP3A4, cytochrome P450, isoenzyme 3A4; DMF, dimethylformamide; DMSO, dimethylsulphoxide; FBS, fetal bovine serum; MEM, eagle's minimal essential medium; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate.

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the effect of extracts from *Mutelina purpurea* on the most frequently used cell lines: HeLa-derived from cervical cancer cells and BHK-21-normal fibroblasts. Taking into account that the natural products are often taken with other medicines, there is a risk of reciprocal interaction on the metabolic level. Thus, the influence of *M. purpurea* extracts was investigated on the activity of CYP2D6 and CYP3A4, which are the most important P450 isoenzymes from the pharmacological and toxicological points of view. Additionally, because *M. purpurea* contains phenolic compounds, the antioxdative properties of this plant extracts were also studied.

2. Materials and methods

2.1. Plant material

The herb of *M. purpurea* was collected in Poland, in the Medicinal Plant Garden of the Medical University in Lublin, in June 2010. Voucher specimen No. ES17/18-19/2010 was deposited in the herbarium of the Department of Pharmacognosy, Medical University of Lublin. The plant material was dried at room temperature, powdered and subjected to extraction.

2.2. Extraction

Methanolic and aqueous extracts from the herb of *M. purpurea* were prepared using the classical extraction technique under reflux (100 g of plant material per each extract). After 24 h extraction the solvents were evaporated to dryness and the residues were used for further investigation.

2.3. Cell cultures

Cell cultures from the American Type Culture Collection, BHK-21 (C-13) (ATCC CCL-10™)–hamster kidney fibroblasts and HeLa (ATCC CCL-2)–cervix adenocarcinoma epithelial cells were used. The cells were cultivated in Eagle's Minimal Essential Medium containing L-glutamine (MEM, Sigma) with a 10% addition of Fetal Bovine Serum (FBS, Sigma) and an addition of penicillin (100 μg/mL) and streptomycin (100 μg/mL) (Polfa-Tarchomin). The cells were grown at 37 °C and the atmosphere of 5% CO_2 to produce the monolayer.

2.4. Cytotoxicity evaluation

Microculture tetrazolium (MTT) assay (also known as mitochondrial reduction assay), a colorimetric assay developed by Takenouchi and Munekata was used to evaluate cell vitality (Takenouchi and Munekata, 1998). This test is based on the ability of succinate dehydrogenase enzymes present in mitochondria of viable cells to reduce the yellow water soluble substrate 3-(4,5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, purple formazan product which is further dissolved with the use of SDS/DMF solution [20% SDS in 50% DMF: a 40% solution of sodium dodecyl sulphate (SDS) was first prepared in phosphate buffered saline (PBS), this solution was diluted by half with dimethylformamide (DMF)] and after 24 h the absorbance is measured spectrophotometrically. Since this process may occur only in viable, metabolically active cells the level of activity is the measure of viability.

Briefly, the monolayer was trypsinized and the cells were seeded in 96-well plates at the density of 3×10^4 cells/well (100 μL/well) in a culture medium containing 10% FBS. Following 24-h incubation and attachment, the cells were incubated for 72 h with different concentrations of plant extracts (25–2000 μg/mL) in culture media containing 2% FBS. The initial series of dilution used for preliminary assessment of cytotoxicity was as follows: 25, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 2000 μg/ml. After preliminary analysis, the highest toxic concentrations for particular samples were eliminated and, if it the results were inconclusive, e.g. IC_5 or IC_{50} values appeared to be between the two tested concentrations, medium concentrations were prepared. Stock solutions of extracts were obtained by dissolving samples in water or DMSO to the concentration of 80 mg/mL, followed by filtration through syringe filters (pore diameter 0.2 μm). Stock solutions were further diluted in culture media to obtain necessary concentrations for the experiments. Simultaneously, the cytotoxicity of DMSO in the concentrations present in dilutions of stock solutions was evaluated. This was achieved by preparing series of dilutions of DMSO in the culture media containing 2% PBS. Dilutions were prepared with the use of the same volumes of DMSO as volumes of stock solutions used to prepare dilutions of the tested samples. Thanks to this approach, the quantity of DMSO in the control dilution was the same as that present in the dilution of stock solutions and it allowed to objectively assess cytotoxicity of the tested samples. Control cells were supplemented only with a medium containing a 2% addition of FBS. All samples were incubated at 37 °C and the atmosphere of 5% CO_2. After 72 h all culture media were removed from the plates, the cells were washed with PBS, and 100 μL of the cell media containing 10% of MTT solution (5 mg/mL) was added to each well, after which the plates were incubated for the next 4 h at 37 °C. Then, 100 μL SDS/DMF solution per well was added and after an overnight incubation the absorbance was read. Absorbance was measured at 540 and 620 nm using a microplate reader (Epoch, BioTek Instruments, Inc., USA). Data assessment was performed with the use of Gen5 software (ver. 2.0 BioTek Instruments, Inc.). The assessment of cytotoxicity was based on a comparison with untreated cells and expressed as IC_{50} (the concentration of the sample required to inhibit 50% of cell proliferation) and IC_5 (concentration inhibiting 5% of cell proliferation), calculated from the dose–response curve (curve fit–nonlinear regression, four parameter). The values are presented as means of triplicate analyses.

2.5. Antioxidant activity

The ABTS spectrophotometric method (Cai et al., 2004) was applied. The ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) radical cation solution was prepared by the reaction of 7 mM ABTS and 2.45 mM potassium persulphate after incubation in the dark for 16 h. The ABTS + solution was then diluted with methanol to obtain the absorbance of 0.70 ± 0.02 at 745 nm. Different aliquots of a methanolic extract (10, 20, 30, 35, 40 μL) were added to ABTS + solution (3.99; 3.98; 3.97; 3.965; 3.96 mL), then they were sampled and mixed thoroughly. After one minute the absorbance was recorded. The concentration of ABTS + at the steady state was plotted as a function of the molar concentration ratio to determine the extract Inhibition Concentration (IC_{50}). The data were reported as mean for at least three replications. A standard curve was obtained by using Trolox standard solution at various concentrations (ranging from 0 to 15 μM) in methanol. The absorbance of the reaction samples was compared to that of the standards.

2.6. Luminescent cytochrome P450 assay

The P450-Glo™ Screening System from Promega (Madison, USA) for CYP2D6 and CYP3A4 was used. The assay was described by (Cali et al., 2006). According to Cali, the CYP reaction is performed by incubating a luminogenic CYP substrate with a CYP enzyme and NADPH regeneration system. The luminogenic P450-Glo™ substrates are derivatives of beetle luciferin [(4S)-4,5-dihydro-2-(6-hydroxybenzothiazolyl)-4-thiazolecarboxylic acid], a substrate of firefly luciferase. The P450-Glo™ substrate does not react with luciferase but is converted by CYP enzymes to a luciferin product, which in turn reacts with a luciferin detection reagent and emits light. Emited light is used to monitor CYP activity because it's intensity is proportional to the amount of D-luciferin produced after the CYP reaction. The assay is ideal for testing the effects of drugs and new chemical entities on CYP enzyme activities.

3. Results and discussion

3.1. Cytotoxicity study

The cell lines from rodents and humans are those which are most commonly encountered in research and fingerprinted. A *HeLa* is an immortal and oldest human cell line most commonly used in medical research (Sharrer, 2006), while BHK-21 is a Syrian golden hamster kidney, a normal fibroblastic tissue. The IC_{50} values of methanol and aqueous *M. purpurea* extracts on two cell lines are shown in Table 1, whereas the concentration values below whose samples proved to be nontoxic are shown in Table 2.

The highest cytotoxicity was observed for methanolic extracts, both on BHK-21 and HeLa cell lines, with IC_{50} values of 245 and 475 μg/mL, respectively. It was impossible to measure IC_{50} values of aqueous extracts dissolved in DMSO because at concentrations above 680 μg/mL for BHK-21 and above 810 μg/mL for HeLa, toxicity of DMSO used as a solvent for stock solutions could be observed. Below those values no cytotoxic effect of DMSO was

Table 1
The IC_{50} values of methanol and aqueous *M. purpurea* extracts.

Cell lines	IC_{50} ± SD (μg/mL)[a]		
	M/DMSO	W/DMSO	W/water
BHK-21	245 ± 17	n/a	918 ± 21
HeLa	475 ± 20	n/a	731 ± 32

[a] IC_{50} values were expressed as mean, determined from the results of MTT assay in triplicate: M, methanolic extract; W, aqueous extract; M/DMSO, M dissolved in DMSO; W/DMSO, W dissolved in DMSO; W/water, W dissolved in water; n/a, not applicable.