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The effect of selected plant extracts on the mitochondria of leukemic cancer cells in vitro

Wpływ wybranych wyciągów roślinnych na mitochondria białaczkowych komórek nowotworowych in vitro

Abstract:

Individual plant extracts from horse chestnut, arnica and hops have been applied in vitro on Jurkat type leukemic cells from which mitochondria were isolated. Each sample of the isolated experimental mitochondria were monitored by the determination of proteins and by monitoring the fluorescence of endogenous fluorophores of experimental mitochondria compared with control mitochondria isolated from intact leukemic cells by means of the synchronous fluorescence fingerprint method. The horizontal cut of excitation-emission matrices at (λ = 240 nm) demonstrated the final graphical and mathematical fluorescence of mitochondria. The result is the presence of two fluorescent excitation-emission zones (the result of the overlapping effect of very similar fluorescence maxima values), which were subsequently compared with standard fluorophores. A reduction of protein content in all experimental mitochondria was observed, the most after the effect of arnica and the least after the influence of horse chestnut. The decrease of fluorescence of the first fluorescent zone was compared to control mitochondria. The decline of mitochondrial fluorescence confirmed the cytotoxic effect of the substances studied on the mitochondria in vitro.

Streszczenie:

Oceniono wpływ in vitro wyciągów wybranych roślin (kasztanowiec, chmiel, arnica) na mitochondria wyizolowane z nowotworowych komórek białaczkowych typu Jurkat. Każdą próbkę wyizolowanych eksperymentalnie mitochondriów monitorowano określając produkowane białka i oceniając fluorescencję endogennych fluoroforów z użyciem metody the synchronous fluorescence fingerprint i porównano z mitochondriami kontrolnymi uzyskanymi z komórek nie poddanych oddziaływaniu wyciągów. Odczyty wskazań emisji macierzy falą o długości (λ = 240 nm) ukazały fluorescencję mitochondriów. Stwierdzono dwie emisje, które porównano ze standardowymi fluorophorami. Zobrazowano redukcję zawartości białka we wszystkich doświadczalnych mitochondriach, największą po wyciągach z arnica i najmniejszą po kasztanowcu. Obniżenie fluorescencji pierwszej strefy było porównywalne z mitochondriami kontrolnymi. Obniżenie mitochondrialnej fluorescencji potwierdza cytotoksyczny efekt badanych substancji na mitochondia in vitro.

Keywords: *synchronous fluorescence fingerprint, fluorescence, mitochondria, arnica, hops, horse chestnut, cytotoxic effect, leukemic cells*

Słowa kluczowe: *synchronous fluorescence fingerprint, fluorescencja, mitochondria, arnica, chmiel, kasztanowiec, efekt cytotoksyczny, białaczkowe komórki nowotworowe*

Introduction

The treatment connected with using medicinal plants and their extracts is increasingly used due to a lower incidence of side effects. The effect of medicinal plants depends on the presence of a mixture of several biologically active substances (primary and secondary metabolites of plants). The main active substance of horse chestnut seeds is escin which is a mixture of triterpenoid saponins. It occurs in two forms α and β and β - escin is presented in many medicinal preparations. At present, the extract of horse chestnut seeds is being used for the treatment of hemorrhoids [1] and edema and chronic venous insufficiency [2, 3]. *In vitro* studies have also confirmed the antiproliferative and apoptotic effect of β - escin [4, 5] against leukemic cells [6]. The antitumor impact of β - escin have been studied *in vivo,* and *in vitro* in the case of hepatocellular carcinoma [7].

The most important substances present in the blooming mountain arnica (Arnica montana, Asteraceae) are oils of bitter taste. The parts of these essential oils are sesquiterpen lactones, which have a toxic effect during oral application, but arnica used locally can treat inflammatory diseases [8] and hematomas [2].

The prenylated chalcones in hop cones are responsible for an anticancer response (Humulus lupulus, Cannabaceae). Some of them, especially the xanthohumol, induced apoptosis in breast cancer cells [9], colon and leukemia cells *in vitro* and *in vivo* [10, 11].

The various plant extracts from horse chestnut, arnica and hop were applied to the Jurkat leukemic cell type *in vitro* in this study. The cytotoxic effect of plant extracts on Jurkat leukemic cells have been studied on isolated mitochondria by monitoring the endogenous fluorescence of mitochondrial fluorophores.

Material and methods

The horse chestnut, arnica and hop extracts were obtained from CALENDULA a.s. (Nová Ľubovňa, Slovak Republic).

The dipotassium hydrogen phosphate (K_2HPO_4), ethylenediaminetetraacetic acid (EDTA), magnesium chloride ($MgCl_2$), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium succinate ($NaOOCCH_2CH_2COONa \times H_2O$) and Tris HCl were obtained from Sigma-Aldrich, Germany. The respiration medium (pH 7.4) for mitochondria containing EDTA (0.78 mM), $MgCl_2$ (6 mM), Tris HCl (15 mM), KCl (0.08), K_2HPO_4 (0.3 M) and KH_2PO_4 (0.3 M) was prepared using bidistilled water, 1 mM sodium succinate.

The Jurkat cancer cell lines (human acute T-lymphoblastic leukemia) used for this study were kindly provided by Dr. Hajduch (Olomouc, Czech Republic). The Jurkat cells were cultured in RPMI 1640 medium with Glutamax-supplemented with 10% fetal calf serum, penicillin (100 IU/mL) and streptomycin (100 µg/mL) (all from Invitrogen, USA), in an atmosphere of 5% CO_2 in humified air at 37°C. Cell viability, estimated by trypan blue exclusion, was greater than 95% before each experiment.

Mitochondria isolation and determination of proteins

The Jurkat cancer cell lines were divided into two groups: control (without plant extracts) and experimental (with plant extracts). The individual plant extracts (horse chestnut (0.125 mg/ml), arnica (0.5 mg/ml), hop (2 mg/ml) dissolved in an RPMI medium) were added into the experimental Jurkat cancer cells (16 500 000) and the cells were cultured in RPMI medium 72 hours at 37 °C in air. Mitochondria from the control and experimental cells were isolated using the method of Mela and Seitz [12]. The protein concentration given in $\mu g.ml^{-1}$ of the isolated mitochondria was determined by the Bradford method [13].

Fluorescence synchronous fingerprint measurement

The synchronous technique SFF consists of varying simultaneously both excitation and emission wavelengths, while keeping a constant wavelength interval $\Delta\lambda$ between them. Mitochondrial synchronous fluorescence spectrum is a method, in which a simplistic form defines the outer mitochondrial membrane. It is considered to be a characteristic 'fingerprint', as it is specific for a given mitochondrial suspension after isolation and this method enables the analysis of the multifluorescent mixture of mitochondria and its interaction with the drug [14]. The synchronous fluorescence fingerprint (SFF) of mitochondrial suspension diluted to a ratio of 1:100 was analyzed in a respiration medium (pH = 7,4) containing a 1 mmol/l substrate succinate at the constant difference of $\Delta\lambda$ = 10 nm between excitation and emission monochromators. The spectra were run in range λ = 200 - 400 nm on a Perkin-Elmer Model LS 55 Luminiscence spectrometer using a 1 cm pathlength quartz cuvette with a volume of V = 3 ml at room temperature t = 25°C. The scan speed of both monochromators was 1200 $nm.min^{-1}$. The setting of the instrument´s excitation slit was 10 nm and the emission slit was 15 nm. Data processing was managed using the Winlab (Perkin – Elmer) software package. A topographic map of SFF was created from 20 scans of simple synchronous fluorescence spectra of mitochondria placed in a space with an increment of 10. The horizontal cut at $\Delta\lambda$ = 50 nm of SFF were simple synchronous fluorescence spectra (SFS, Fig. 2). The intensity of fluorescence of individual experimental SFS spectra were evaluated mathematically by statistical analysis.with a control SFS spectra (Fig. 3) The practical advantages of fluorescence technique and spectra (SFF) application on mitochondria are high sensitivity, reproducibility, and a minimal quantity of mitochondria (30 µl) and rate of measurement (5 minutes).

Statistical analysis

The statistical significance between control and experimental plant extract-treated mitochondrial groups was calculated by the nonparametric Mann-Whitney U test by use of STATISTIKA 6.0 software (Base StatSoft, Inc., 2001).

Results

The topographic SFF graphs providing detailed information were compared visually (Fig. 1) and mathematically (Fig. 2) and showed differences between experimental mitochondrial groups compared with the control group.

The evaluation of simple synchronous spectra resulting from a three-dimensional horizontal cut of SFF at $\Delta\lambda = 50$ nm revealed two maxima, which suggest the existence of two fluorescent areas (Fig. 2 - 3). The endogenous fluorophores (NADH + H^+, tyrosine, tryptophan, phenylalanine) of the mitochondrial outer membrane were responsible for the final fluorescence of the mitochondria. These results were compared with the results of determining the proteins in control and experimental mitochondria (Fig. 3).

The fluorescence SFF maximum of the control mitochondria was observed at the longest wavelength $\lambda = 70/283$ nm, F = 566. The intensities of fluorescence of mitochondria in the presence of horse chestnut and arnica were increased, but hop decreased autofluorescence of mitochondria compared with the control group of mitochondria (Fig. 2). The maximal intensity of fluorescence (Fig. 1) in all the experimental groups of mitochondria were observed at shorter wavelengths. The mitochondria exposed to horse chestnut had a maximum of $\lambda = 70/277$ nm, F = 663 at similar wavelengths to the experimental group of mitochondria in the presence of hops $\lambda = 30/276$ nm, F = 636, while the experimental group of mitochondria with arnica showed a similar maximum fluorescence of 70/282 nm, F = 778 to the control mitochondria $\lambda = 70/283$ nm, F = 566 (Fig. 1 - 2).

The horse chestnut (c = 0.338 mg / ml) and hop (c = 0.26 mg / ml) decreased protein content in mitochondria, while arnica reduced the amount of protein in mitochondria the most (c = 0.039 mg / ml) when compared to the control group (c = 0.52 mg / ml) of mitochondria.

The proteins and their interaction with plant extracts showed the 1st fluorescent zone and the presence of NADH + H^+ exhibited the 2nd zone in Figures 1 - 3. The changes of fluorescence intensity of both zones were observed during interaction with the plant extracts. The arnica and horse chestnut increased the fluorescence of proteins, the hops decreased fluorescence (there is possible binding of these compounds on protein) and other fluorophores in mitochondria in zone 1 compared to the control mitochondria (Fig. 1 - 3). This 1st fluorescent zone is strongly affected by light scattering, which should be taken into account for interpretation.

The arnica exhibited the highest intensity of fluorescence in zone 2 (fluorescence of NADH + H^+ increases in the absence of oxygen) of experimental mitochondria in comparison with all experimental groups and the control group (Fig. 3), which may be the result of the cytotoxic effect of arnica observed *in vitro* [15, 16].

The hop, but mainly horse chestnut, displayed decreased intensity of fluorescence in zone 2 (fluorescence of NADH + H^+ decreases in the presence of oxygen and increased metabolism) of experimental mitochondria in comparison with the control group, (Fig. 3) which could be the next mechanism of the cytotoxic effect of some plant extracts by metabolic burn up. The comparison of fluorescence intensities of two fluorescent zones (Figure 3) showed the cytotoxic effect of the plant extracts studied *in vitro*, which was also observed *in vivo* [17, 18].

The results presented are in agreement and followed previously published studies of the cytotoxic effect of β-escin and xanthohumol [4, 19, 20] and horse chestnut extract on different cancer cell lines confirmed by MTT test [21, 22]. Based on our observations of endogenous mitochondrial fluorescence, we suggest the use of fluorescence spectroscopy as a quantitative and rapid technique that has the potential to provide important diagnostic information about the mitochondrial hypoxia of cancer cells.

Conclusion

Our results showed that NADH + H^+ fluorescence was the marker of mitochondrial respiration and it could investigate the different cytotoxic effects of plant extract on the mitochondria of leukemia cells *in vitro*. The highest intensity of NADH + H^+ fluorescence as the result of inhibited mitochondrial respiration was detected in the presence of arnica in experimental mitochondria. While the hop and horse chestnut displayed increased the metabolism of experimental mitochondria as the result of possible metabolic burn up.

The protein content of all experimental mitochondria decreased in the presence of the plant extracts studied, the most in the samples with arnica. This was also confirmed by the results of fluorescence analysis.

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Figures

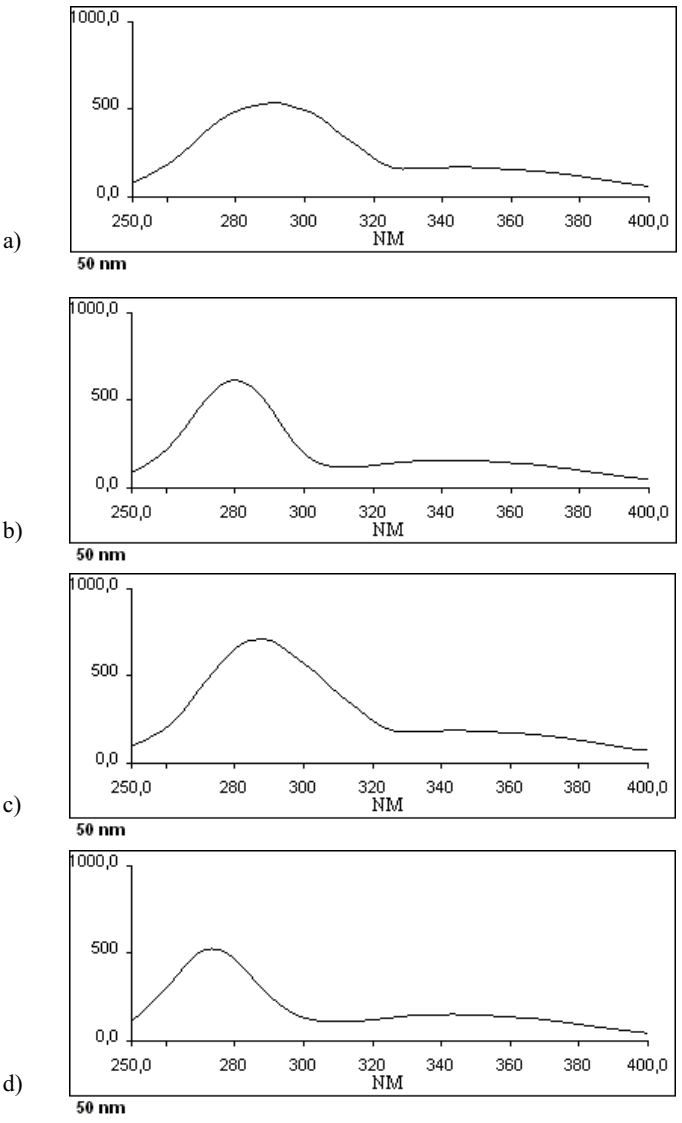
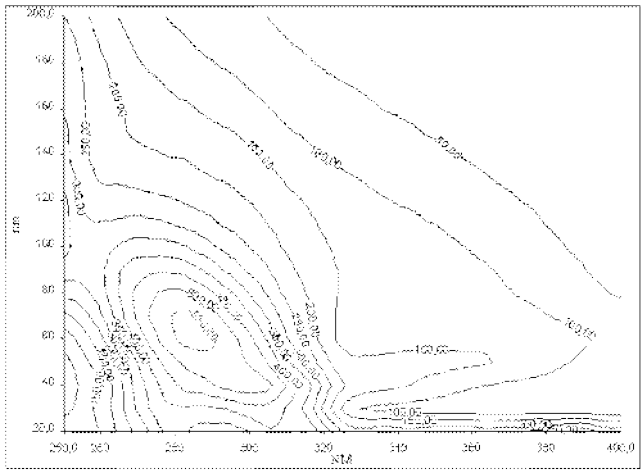
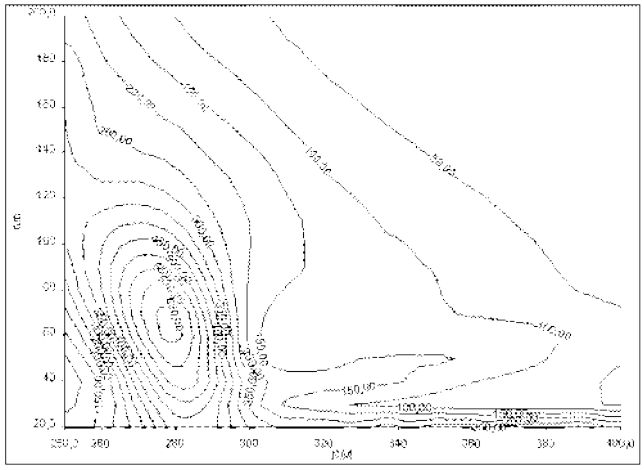


Fig. 1. The comparison of three - dimensional topographic maps of SFF a) control mitochondria and the experimental mitochondria affected by plant extracts administration of b) horse chestnut, c) arnica and d) hop

a)



b)



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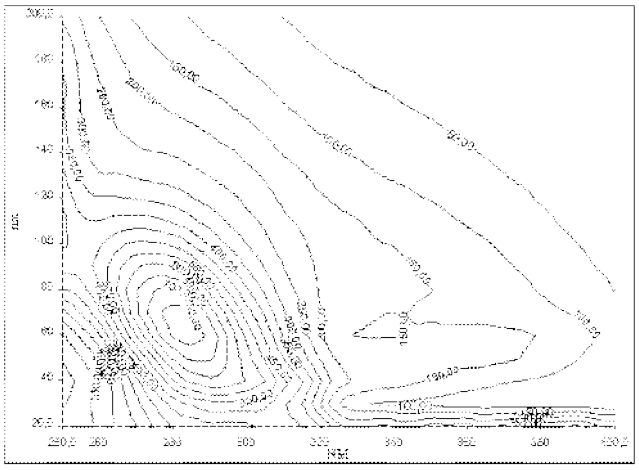

d)

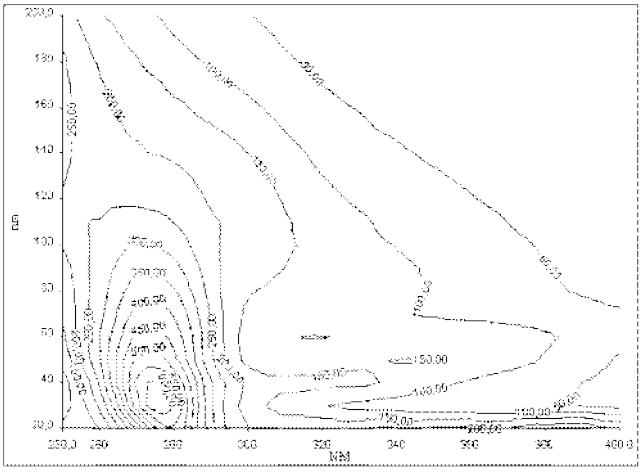

Fig. 2. The comparison of simple synchronous fluorescence spectra (the result of horizontal cut at ($\Delta\lambda = 50$ nm) of SFF) a) control mitochondria ($\lambda = 291$ nm, F = 538; λ = 344 nm, F = 171) and experimental mitochondria altered by plant extracts b) horse chestnut (λ = 280 nm, F = 614; λ = 343 nm, F = 159), c) arnica (λ = 288 nm, F = 710; λ = 344 nm, F = 189) and d) hop ($\lambda = 273$ nm, $F = 526$; $\lambda = 344$ nm, $F = 152$)

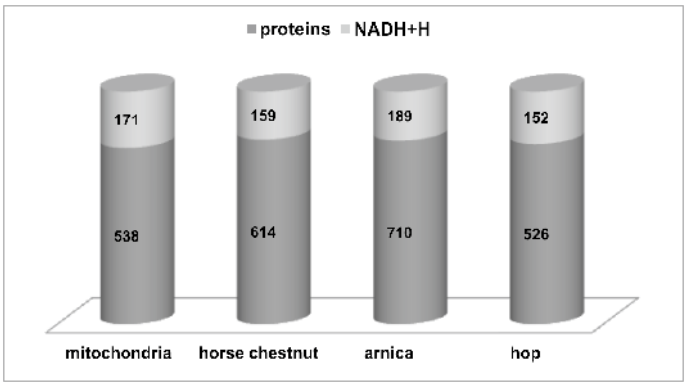

Fig. 3. The comparison of the fluorescence intensities of two fluorescent zones (the results of all fluorophores) of control and experimental mitochondria in Fig.1 and 2