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Cytotoxic activity of proteins isolated from extracts of *Corydalis cava* tubers in human cervical carcinoma HeLa cells

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Abstract

Background: *Corydalis cava* Schweigg. & Koerte, the plant of numerous pharmacological activities, together with the studied earlier by our group *Chelidonium majus* L. (Greater Celandine), belong to the family *Papaveraceae*. The plant grows in Central and South Europe and produces the sizeable subterraneous tubers, empty inside, which are extremely resistant to various pathogen attacks. The *Corydalis sp.* tubers are a rich source of many biologically active substances, with the extensive use in European and Asian folk medicine. They have analgetic, sedating, narcotic, anti-inflammatory, anti-allergic and anti-tumour activities. On the other hand, there is no information about possible biological activities of proteins contained in *Corydalis cava* tubers.

Methods: Nucleolytic proteins were isolated from the tubers of *C. cava* by separation on a heparin column and tested for DNase activity. Protein fractions showing nucleolytic activity were tested for cytotoxic activity in human cervical carcinoma HeLa cells. Cultures of HeLa cells were conducted in the presence of three protein concentrations: 42, 83 and 167 ng/ml during 48 h. Viability of cell cultures was appraised using XTT colorimetric test. Protein fractions were separated and protein bands were excised and sent for identification by mass spectrometry (LC-ESI-MS/MS).

Results: The studied protein fractions showed an inhibiting effect on mitochondrial activity of HeLa cells, depending on the administered dose of proteins. The most pronounced effect was obtained with the highest concentration of the protein (167 ng/ml) - 43.45 ± 3% mitochondrial activity of HeLa cells were inhibited. Mass spectrometry results for the proteins of applied fractions showed that they contained plant defense- and pathogenesis-related (PR) proteins.

Conclusions: The cytotoxic effect of studied proteins toward HeLa cell line cells has been evident and dependent on increasing dose of the protein. The present study, most probably, represents the first investigations on the effect of purified PR proteins from tuber extracts of a pharmacologically active plant on cell lines.

Background

Plants from the family *Papaveraceae* are frequently used in traditional medicine as a remedy for treatment of several diseases. Such plants include *Corydalis cava* Schweigg. & Koerte; syn. *Corydalis bulbosa* (L.) Pers. non (L.) DC. and *Chelidonium majus* L. studied earlier by our group (Greater Celandine). The two species are

closely related and belong to order *Papaverales* [1,2]. Milky sap as well as extracts of the whole *Chelidonium majus* plant has been used to treat papillae, warts, condylomae, which are a visible effect of human papilloma virus (HPV) infection. It has also been found that they have antimicrobial, antitumor, anti-inflammatory, antifungal, and fungistatic properties [3]. The tuber of *Corydalis sp.* contains isoquinoline alkaloids of apomorphine type, e.g. bulbocapnine, corydaline, which manifest analgetic, sedating and narcotic effects [4-7]. The plant has been used for the treatment of severe neurological

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disorders and mental diseases. It was also used in cases of asomnia, tension and anxiety conditions [7]. Some species of *Corydalis* are used in East Asia as analgetic drugs: in the traditional Chinese medicine, the species of *Corydalis yanhusuo* was used to alleviate post-traumatic, colic, abdominal and menstrual pains [7]. Moreover, extracts of the same species showed anti-cancerous metastasis effect *in vitro* [8]. Anti-tumour activity of *Corydalis* species was also reported for Korean *Corydalis turtschaninowii*, which is effective for the treatment of inflammatory, allergic diseases and tumours [9]. Isoquinoline alkaloids contained in alcohol extracts of tubers in many species of *Corydalis*, affect metabolism of neurotransmitters [10]. Active compounds in such extracts include alkaloids, such as bulbocapnine, corydaline and corydine [11]. The similar curing properties of *Chelidonium majus* milky sap were attributed mainly to alkaloids, such as chelidonine, sanguinarine, berberine, coptosine, chelerythrine, and also several flavonoids and phenolic acids [12]. However, different findings show that all of them may be potentially toxic for human either alone or in combination [13,14].

Our earlier studies have shown that biological activity of *Chelidonium majus* milky sap may be related to its protein content. The majority of the identified proteins can be linked to direct and indirect stress and defense reactions, e.g. against different pathogens [15-17]. We have recently discovered that purified plant proteins from *Chelidonium majus* milky sap with nucleolytic activity, which probably belong to pathogenesis-related (PR) protein family, are capable of inducing apoptosis in human cervical cancer HeLa cells [17].

Therefore, the objective of our study was to evaluate the effect of purified proteins from extracts of *Corydalis cava* tubers with nucleolytic activity on HeLa tumour cells and to identify the protein content of such purified fractions using tandem mass spectrometry.

Methods

Plant material

Corydalis cava plants were collected during 2007 and 2008 in the neighbourhood of Poznań, Poland, during flowering, in April. A voucher specimen was deposited at the Department of Molecular Virology, Faculty of Biology, Adam Mickiewicz University in Poznan, Poland.

Protein extract

The protein extract was prepared from tubers, dissolved in 0.1 M Tris-Cl buffer, pH 8.0, containing 10% glycerol (extract : buffer ratio was 1:1). The tuber extract (50% v/v) samples were separated into a supernatant, and a pellet fraction, by centrifugation at 12000 rpm for 20 min at 4°C as described in the protocol [16], with modifications. Supernatants were stored at -20°C for

further analysis. Protein concentration was determined according to Lowry et al. [18].

Isolation and purification of proteins

For isolation and purification of proteins, crude extracts from *C. cava* tubers collected in April were used. About 0.5-µg protein was loaded on HT Heparin column (GE Healthcare) (0.7 × 2.5 cm) equilibrated with 0.1 M Tris-HCl, pH 8.0, 10% glycerol. The column was eluted with a linear gradient of 0 to 2 M NaCl in the same buffer. The absorbance at 280 nm and DNase activity of all fractions (volume 1 ml) were determined.

Analysis by SDS-PAGE

In order to verify the protein composition of chromatographic fractions, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a slab mini-gel apparatus according to Laemmli 1970 [19], using 10% polyacrylamide as the separating gel and 5% polyacrylamide as the stacking gel. The proteins were reduced by heating them at 100°C in the presence of 2-mercaptoethanol for 5 min. After SDS-PAGE, the gels were fixed and stained with silver according to Shevchenko et al. [20].

In-gel nuclease assay

An in-gel DNase assay was performed according to Thelen and Northcote [21], with modifications [22]. For in-gel DNase assay, *C. cava* tuber extracts or fractions after purification were dissolved in SDS-PAGE sample buffer without a reducing agent, incubated at 37°C for 10 min, and subjected to SDS-PAGE in 10% polyacrylamide gel containing denatured calf thymus DNA (40 µg/ml). After the electrophoresis and removal of SDS, the gels were washed with reaction buffer (10 mM Tris-HCl, pH 8.0, containing 10 mM CaCl₂). DNase activity was visualized by staining the gel with ethidium bromide.

Cell culture and viability assay

HeLa cells were cultured in RPMI 1640 supplemented with 5% FCS, 2 mmol/l L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Viability of cell cultures was appraised using XTT colorimetric test, based on a dynamics of XTT (tetrazoline 2,3-bis(2-methoxy-4-nitro-5-sulfofenylo)-2H-5-carboxyanilide) stain reduction by viable cells with the formation of a colored product. Intensity of the fluorescence was measured at the wavelength of 450 nm. Percentage of mitochondrial metabolism activity inhibition was calculated according to the following equation: $100 - (\text{OD drug treated cells} - \text{OD medium alone}) / (\text{OD untreated cells} - \text{OD medium alone}) * 100$. Each experiment was conducted independently in 8 cultures and in each culture at least 200 cells were scored. Statistical analysis of the results

involved the *t*-test for unpaired data using the STATISTICA ver.6.1 software. *P* value < 0.05 was considered to represent threshold of significance.

LC-ESI-MS/MS analysis

Stained protein bands were excised from the gel and analyzed by liquid chromatography coupled to mass spectrometer in the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Samples were concentrated and desalted on RP-C18 precolumn (LC Packings, UK) and further peptide separation was achieved in a nano-HPLC RP-C18 column (LC Packings, 75 mM i.d.) of a UltiMate nano-HPLC system, using a 50-min linear acetonitrile gradient. Column outlet was directly coupled to Finnigan Nanospray ion source of LTQ-FT (Thermo, USA) mass spectrometer working in the regime of data dependent MS to MS/MS switch. An electrospray voltage of 1500 V and a cone voltage of 30 V were used.

MS/MS data analysis

The data were analysed automatically by database matching against the NCBI protein database (NCBI, Bethesda, USA) with a Viridiplantae filter, using the MASCOT database search engine (Matrix science, London, UK; <http://www.matrixscience.com>) [23].

Results

Isolation and purification of proteins from *Corydalis cava* tuber extracts on a heparin column

In order to evaluate if biological activity of *Corydalis* tuber extracts is related to the proteins contained in them, the proteins of nucleolytic activity were isolated from the tubers by purification on a HT Heparin column (Figure 1A) using ÄKTA Explorer™ chromatographic system (Amersham Biosciences). Following the application of the extract to the column (0.7 × 2.5 cm), molecules which did not bind to the column were eluted using elution buffer (buffer A: 100 mM Tris, pH 8.0, 10% glycerol). The first eight fractions of 1.5 ml each represented the flow-through fractions. The subsequent fractions were eluted using a linear gradient of NaCl (from 0 to 2 M) (19 fractions of 1 ml each). In all the fractions, absorbance was measured at 280 nm (Figure 1A), indicating the presence of purified proteins in fractions 16-18. Protein fractions purified on heparin column were tested for DNase activity using in-gel DNase assay (Figure 1B) according to Thelen and Northcote [21]. Nucleolytic activity was demonstrated in fraction numbers 16, 17 and 18. All the fractions were also separated in SDS-containing polyacrylamide gel (SDS-PAGE), which was stained with silver according to Shevchenko et al. [20] (Figure 1C). Fraction numbers 16

and 17 each contained five protein bands of molecular weights (MW) approx. 30, 32, 35, 38 and 68 kDa, while the fraction number 18 contained an additional fraction of MW around 140 kDa.

Analysis of mitochondrial activity of HeLa cells

All the fractions with nuclease activity were tested for cytotoxic activity in human cervical carcinoma HeLa cells. For the analysis, proteins contained in fraction numbers 16, 17, 18 and 19 were used, from three different rounds of purification. The buffer used for tuber extract isolation (0.1 M Tris, 10% glycerol), at the concentration of 26.7 μl/ml served as a negative control. For each fraction, 48 h cultures of HeLa cells were conducted in the presence of three protein concentrations: 42, 83 and 167 ng/ml from three purification rounds. HeLa cells on the plates were subjected to the action of XTT/PBS solution (1 mg XTT/ml in PBS + 1.53 mg PMS/ml in PBS) in order to estimate cell viability. Following incubation, the results were recorded using a small plate ELISA reader (Labsystems Multiskan MCC/340) with readout at 450 nm. The results are presented in Table 1.

The cytotoxic effect of studied proteins in relation to HeLa cell line was clearly marked and it depended on the applied dose of protein: the most pronounced effect was obtained with protein fractions administered at the highest concentration (167 ng/ml), while at the intermediate concentration (83 ng/ml), the effect was appropriately lower compared to the highest concentration. Fraction 18 exerted the highest cytotoxic effect at the concentration of 167 ng/ml: 43.45 ± 3% cells underwent mitochondrial activity inhibition (Table 1). Protein fractions at the lowest protein concentration (42 ng/ml) manifested the least pronounced cytotoxic effect (e.g., fraction 16: 4.55 ± 3.5%, table 1). In the experiments, the control was provided by the pure elution buffer (0.1 M Tris, 10% glycerol), added to HeLa cells at the concentration of 26.7 μl/ml, which did not exert any effect on mitochondrial activity of HeLa cells.

Identification of proteins in purified fractions by mass spectrometry

The protein bands were numbered 1 to 6 (Figure 1C), excised from the gel stained with silver [20] and sent for identification by mass spectrometry (LC-ESI-MS/MS) at the Laboratory of Mass Spectrometry, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (IBB PAN) in Warsaw, Poland. The data was analyzed using MASCOT (<http://www.matrixscience.com>).

Results of protein identification using tandem mass spectrometry analysis (LC-ESI-MS/MS) are presented in Table 2. It contains a list of identified proteins from fractions of *Corydalis cava* tuber extracts. Results of

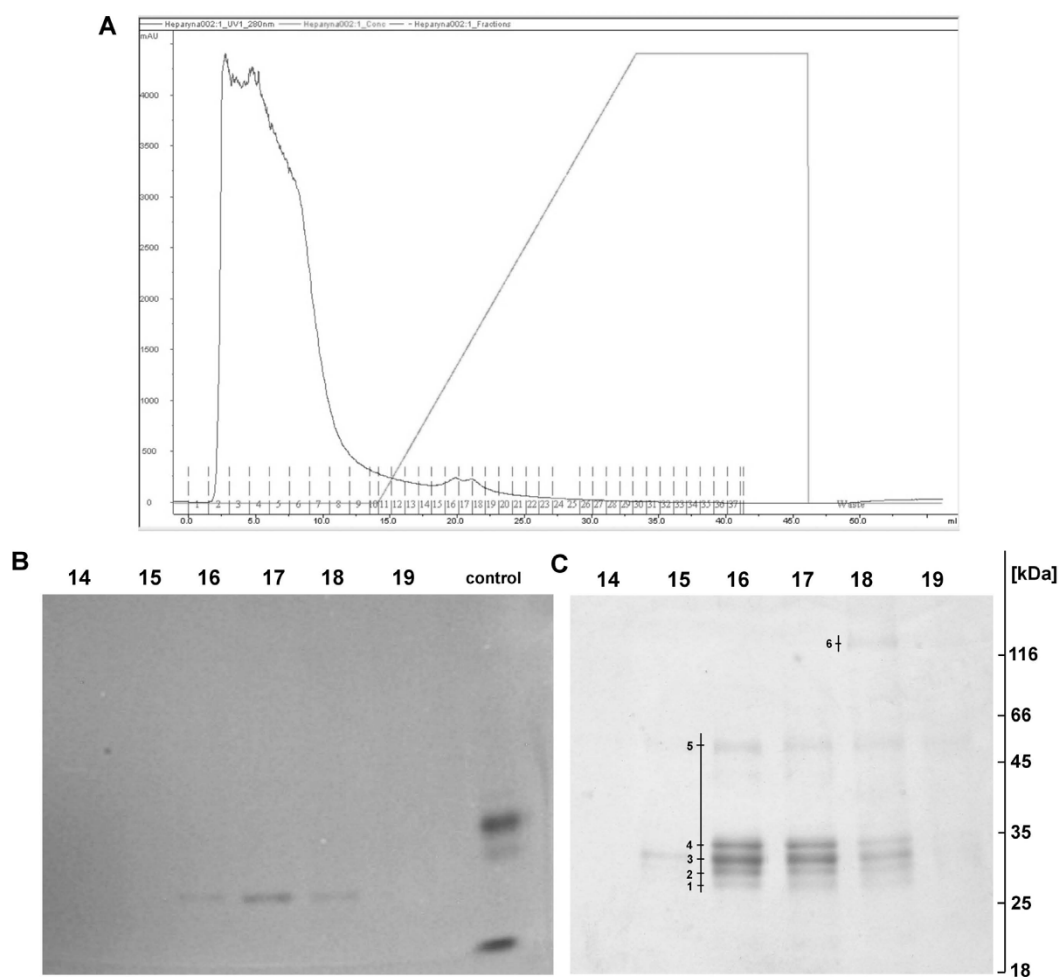


Figure 1 Protein purification and electrophoretic analysis. (A) Chromatographic profile of protein purification from *C. cava* tuber extracts in HT Heparin column (GE Healthcare). Fractions 1 to 9 represented flow-through fractions, 10 to 27 were eluted with a linear NaCl gradient (from 0 to 2 M). The absorbance of all fractions was measured at 280 nm and their DNase activity was estimated using in-gel assay. Proteins present in fractions 16, 17 and 18 were identified using LC-ESI-MS/MS. Fractions 16-19 following three rounds of purification were used in tests on HeLa tumour cell line. (B) In-gel DNase pattern of the gel in which DNase activity of protein fractions originating from *C. cava* tuber extracts was estimated. ssDNA containing 10% SDS-PAGE, following electrophoresis and incubation (12 h) in 10 mM Ca^{2+} buffer, pH 8.0, was stained with ethidium bromide and viewed under UV light. Nucleolytic activity was noted in fractions Nos.16, 17 and 18 of MW around 30 kDa. Control: purified nucleases from *Chelidonium majus* milky sap served as positive control. (C) 10% SDS-PAGE following electrophoresis of fraction-contained proteins and silver staining according to Shevchenko et al. [20]. The separated fractions were identical to those in the gel in Fig. 1B. Fractions nos. 16 and 17 each contained 5 protein bands of MW around 30, 32, 35, 38 and 68 kDa, while fraction no. 18 contained an additional band of MW around 140 kDa. The protein bands were numbered 1-6, excised from the gel and sent for identification by mass spectrometry (LC-ESI-MS/MS). M - Protein Molecular Weight Marker (Fermentas).

the identification show that proteins contained in bands of fraction numbers 16-18 belong to plant pathogenesis- (PR) and defense-related proteins. Most of the identified proteins is directly or indirectly involved in defense reactions of the plant against various stresses, e.g. against attack of a pathogen (Table 2).

Analysis of DNase activity has demonstrated the presence of a band with nucleolytic activity in fractions 16, 17 and 18 of molecular weight around 30 kDa (Figure 1B).

Comparison of SDS-PAGE gel following the separation of the same fractions and staining with the silver method (Figure 1C), demonstrates an analogous band of around 30 kDa molecular weight (No. 1) identified as DNA-binding protein from *Zea mays* with a very similar molecular weight of 27.6 kDa (Table 2). Thus, the studied fraction contains a number of proteins with defense and metabolic significance for the plant.

Table 2 lists identified defense-related proteins with plant species in which the proteins were detected as well

Table 1 Mitochondrial activity inhibition in neoplastic HeLa cells under the effect of purified protein fractions from extracts of *C. cava* tubers.

| Fraction no. | Protein concentration [ng/ml] | Mean value of HeLa cells inhibition [%] | Standard deviation [%] |
|--------------|-------------------------------|---|------------------------|
| 16 | 42 | 4.55 | 3.53 |
| 16 | 83 | 15.57 | 2.42 |
| 16 | 167 | 35.31 | 4.32 |
| 17 | 42 | 8.66 | 5.19 |
| 17 | 83 | 22.35 | 2.40 |
| 17 | 167 | 39.15 | 3.34 |
| 18 | 42 | 12.59 | 5.37 |
| 18 | 83 | 32.42 | 3.13 |
| 18 | 167 | 43.45 | 3.06 |
| 19 | 42 | 5.90 | 5.43 |
| 19 | 83 | 15.18 | 7.10 |
| 19 | 167 | 21.78 | 6.06 |

Proteins from three rounds of purification were administered at concentrations of 42; 83; and 167 ng/ml. The data was analyzed using STATISTICA (version 6.1) software. [%] - inhibition of mitochondrial activity of HeLa cells under effect of examined proteins as compared to the control.

as protein scores and sequence coverages. The number of compatible peptides for individual results ranged between 1 and 43, sequence coverage in a single case reached the maximum value of 26%. In the Table 2, numbers of protein bands were indicated in which particular proteins were identified (Figure 1C).

Discussion

Plants that belong to family Papaveraceae, like Greater Celandine (*Chelidonium majus* L.) and *Corydalis cava* Schweigg. & Koerte, are a rich source of various biologically active substances with strong pharmacological activity. The mechanism of this activity is still unknown, but very important compounds are the proteins contained in the plants. Our earlier studies showed that biological activity of *Chelidonium majus* milky sap may be related to its protein content. The protein fractions which contained two nucleases induced apoptosis in human cervical cancer HeLa cells [17].

Most probably, the present study represents the first investigations on the effect of purified PR proteins from

Table 2 Defense-related proteins identified in fractions after purification of nucleases from extracts of *Corydalis cava* tubers using LC-ESI-MS/MS.

| No. of protein band ^{a)} | Identified protein ^{b)} | Accession No. ^{c)} | Matched peptides ^{d)} | Score ^{e)} | Mol. mass (Da) ^{f)} | pI ^{g)} | Sequence coverage (%) ^{h)} |
|-----------------------------------|--|-----------------------------|--------------------------------|---------------------|------------------------------|------------------|-------------------------------------|
| 1, 2, 3, 4, | peroxidase [<i>Nicotiana tabacum</i>] (PR-9) | gi 14031049 | 16 | 560 | 39495 | 5.99 | 13 |
| 5, 6 | heat shock protein 70 [<i>Chlorella zofingiensis</i>] | gi 18482472 | 4 | 180 | 70112 | 8.29 | 8 |
| 1 | pectinesterase [<i>Phaseolus vulgaris</i>] | gi 21060 | 2 | 112 | 23967 | 9.52 | 4 |
| 2 | SODP_PETHY Superoxide dismutase (ISS) [<i>Ostreococcus tauri</i>] | gi 116056311 | 2 | 74 | 42244 | 11.68 | 4 |
| 4 | GRP-like protein 2 [<i>Gossypium hirsutum</i>] | gi 110559491 | 3 | 69 | 41333 | 6.01 | 8 |
| 5 | disease resistance protein (TIR-NBS-LRR class), putative [<i>Arabidopsis thaliana</i>] | gi 15229962 | 1 | 64 | 117364 | 7.49 | 0 |
| 3 | ribosomal protein S12 [<i>Mesostigma viride</i>] | gi 11466414 | 1 | 55 | 13815 | 11.32 | 9 |
| 1 | DNA-binding protein [<i>Zea mays</i>] | gi 195658581 | 1 | 54 | 27597 | 6.49 | 4 |
| 4 | chloroplast nucleoid DNA-binding protein-related [<i>Arabidopsis thaliana</i>] | gi 18391062 | 1 | 53 | 48429 | 7.48 | 2 |
| 4 | RPP13-like protein [<i>Arabidopsis arenosa</i>] | gi 46410122 | 1 | 52 | 70976 | 5.51 | 2 |

a) Assigned numbers of protein bands as indicated in Fig. 1C.

b) Identified homologous proteins and organism from which it proceeds.

c) Database accession numbers according to: NCBI (nr); trEMBL (trm).

d) Number of matched peptides with Mascot Search data (<http://www.matrixscience.com>).

e) Mascot Search Probability Based Mowse Score. Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 48 indicate identity or extensive homology ($p < 0.05$).

f) Theoretical mass (kDa) of identified proteins. The values were retrieved from the protein database.

g) Theoretical pI of identified proteins. The values were retrieved from the protein database.

h) Amino acid sequence coverage for the identified proteins.

tuber extracts of a pharmacologically active plant on cell lines. The cytotoxic effect of studied proteins toward HeLa cell line cells has been evident and dependent on increasing dose of the protein.

Results of protein identification in fractions from purification of *C. cava* tuber extracts using LC-ESI-MS/MS analysis have shown that proteins contained in bands of fractions 16-18 belong to plant pathogenesis- and defense-related proteins, indirectly or directly involved in plant defense reactions against stresses of various kind. Many of them belong to the PR protein family involved in plant defense against the pathogen attack. The family includes 17 protein classes of variable activities [24]. *C. cava* tubers are exposed to the attack of fungal, viral, bacterial and other pathogens during the entire vegetation season as well as during winter time [25]. Accumulation of high number and variability of defense proteins in tubers together with several secondary metabolites, such as isoquinoline alkaloids, provides an effective and long-time protection against attack of pathogens. Moreover, defense-related proteins contained in *C. cava* tubers resemble 21 proteins identified earlier by our group in *Chelidonium majus* milky sap [15].

The studies represent preliminary demonstration of the effect exerted by tuber proteins from a plant of pharmacological significance on cells of a tumour cell line. The investigated protein fractions comprise a mixture of plant defense-related proteins. In subsequent studies, an effort will be made to isolate the individual proteins and examine their effect on tumour cells individually and in combinations as well. Nevertheless, the synergistic action of all the compounds present in plant protein extracts is very important for their activity. Many effective and extensively studied compounds of plant origin are, in fact, crude protein mixtures, such as phytotherapeutical drug bromelain, which is crude, aqueous extract from the stems and immature fruits of pineapples (*Ananas comosus* from the family Bromeliaceae), constituting a complex mixture of different thiol-endopeptidases and other components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins, carbohydrates and several proteinase inhibitors [26]. Also, *Viscum album* L. extracts (VAE, European mistletoe) are composed of pharmacologically relevant compounds like: mistletoe lectins (ML I, II and III), viscotoxins and other low molecular proteins, VisalCBAs (*Viscum album* chitin-binding agglutinin), oligo- and polysaccharides, flavonoids, vesicles, triterpene acids, and others. Whole VAE as well as several of the compounds are cytotoxic and the mistletoe lectins have strong apoptosis-inducing effects [27].

Although the mechanism of presented activity of *C. cava* proteins is unknown, their defensive role for the plant could be very important. Many plant defense-related

proteins belong to small (14-40 amino acids), linear, cationic peptides. These peptides have membrane lytic properties and potent activity against a broad spectrum of microorganisms. They organize into ordered secondary structures (α -helix and β -sheet) in the membrane [28]. Above a threshold concentration, peptides disturb the cell membrane and cause cell death due to membrane disintegration [29].

Conclusions

Presented studies confirm that biological activity of *C. cava* extracts may also be related to proteins contained in the extracts. For possible further applications, the biologically active plant proteins should be separated from alkaloids and other secondary metabolites, which in higher doses might be toxic [14]. The studies represent preliminary demonstration of the effect exerted by tuber proteins from a plant of pharmacological significance on cells of a tumour cell line. In the subsequent studies, an effort will be made to isolate the individual proteins and examine their effect on tumour cells individually and in combinations.

Acknowledgements

This work was financed by the AMU-UMS-ULS inter-universities grant no. 512-00-073 (2009, Poznań, Poland) and Polish Minister of Science and Higher Education grant no. 0872/B/P01/2008/34.

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Authors' contributions

RN: Performed the study, collected plant material, evaluated the MS/MS data and prepared the manuscript; MWC: Carried out the study on cell lines, performed the statistical analysis and helped to draft the manuscript; WB: Isolated and purified proteins from plant extracts on a heparin column; DW: Participated in protein isolation and purification and in cell line analyses, helped in MS/MS data analysis; SB: Participated in design and coordination of the study, helped to collect the plant material; AGJ: Supervised the work, participated in its design and coordination and corrected the manuscript for publication. All the authors have approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 5 June 2010 Accepted: 17 December 2010

Published: 17 December 2010

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Pre-publication history

The pre-publication history for this paper can be accessed here:
<http://www.biomedcentral.com/1472-6882/10/78/prepub>

doi:10.1186/1472-6882-10-78

Cite this article as: Nawrot et al.: Cytotoxic activity of proteins isolated from extracts of *Corydalis cava* tubers in human cervical carcinoma HeLa cells. *BMC Complementary and Alternative Medicine* 2010 **10**:78.

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