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Original Article

ANTIPROLIFERATIVE ACTIVITY AND APOPTOTIC EFFECTS OF *FILIPENDULA ULMARIA* POLLEN AGAINST C26 MICE COLON TUMOUR CELLS

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Abstract

Honeybee collected pollen exhibits high nutritional and pharmaceutical benefits for the human diet and medicine. Pollen's antioxidant, anti-ageing, anti-inflammatory, anti-atherosclerosis, and cardioprotective activity, depending on the floral origin, are well known. Recent studies proposed that pollen may also be an excellent cancer-fighting candidate, as pollen harbours high amounts of phenolic substances. In our study, *Filipendula ulmaria* pollen (bee collected) was methanol-water extracted and used to verify its *in vitro* pharmacological activities on C26 mice cancer tumour cells. Three different concentrations of the extract were tested in antitumour assays. Monitoring was done after 6, 12, 24, and 48 hours. Promising results were obtained for antiproliferative and apoptotic activity of the pollen extracts, with high efficiency for the highest concentration (1 mg/mL). For both activities, time and concentration-dependent effects were observed. Pollen extracts or bee collected pollen has a high potential as an antitumour agent for use in human medicine, because they are both rich in bioactive compounds.

Keywords: Antioxidant activity, antitumour activity, apoptosis, bioactive compound, *Filipendula ulmaria*, floral origin

INTRODUCTION

Bee collected pollen is not only the major proteinous and health enhancing food source for honeybees (reviewed in Erler & Moritz, 2015), it is also used in the human diet as a valuable and highly nutritional food supplement. Bee collected pollen is also used in traditional human folk medicine. The complex chemical composition is comprised of sugar, lipids and fatty acids, proteins and amino acids, vitamins, natural pigments, plant species-specific polyphenols - mainly flavonoids, and minerals and also enzymes added by the bees (Campos et al., 1997; Bogdanov, 2004; Komosinska-Vassev et al., 2015). Phenolic substances in bee pollen add up to 5% of the total pollen composition, including flavonoids (aglycones and glycosides), secondary plant metabolites with different important physiological and pharmacological activities, phenolic acids, anthocyanins, and tannins (Rzepecka-Stojko et al., 2015). All these substances together exhibit a wide range of biological activities including antioxidant, anti-ageing, anti-carcinogen, anti-inflammatory, anti-atherosclerosis, cardioprotective and the ability to improve the endothelial function, depending

on the floral source origin (Furusawa et al., 1995; Komosinska-Vassev et al., 2015).

Another important bioactive group of pollen compounds are the phytosterols. Among several bioactivities, the most prominent is their blood cholesterol-lowering effect, possible anti-atherogenic effects, as well as immune stimulating and anti-inflammatory activities, carried out mainly by beta-sitosterol (Komosinska-Vassev et al., 2015). Furthermore, there is emerging evidence suggesting that particular plant sterols may have beneficial effects suppressing the development of different types of cancers (e.g., colorectal, breast and prostate cancer) (Wu & Lou, 2007). However, it is not clear whether mechanisms other than the established cholesterol-lowering action of phytosterols also contribute to these potential health benefits.

Cancer is still one of the leading causes of death and colon cancer is the second most commonly diagnosed cancer worldwide. Colon cancer is responsible for more than half a million related deaths annually. This high human mortality is because of colon cancer's high metastatic ability. Matrix metalloproteinases (MMP) are key factors in the metastatic process. Therefore, agents with the ability to inhibit MMP activity can potentially help in the treatment of colon cancer (Aulino et al., 2010). Conventional cancer treatments harm the patient's immune system. It is important to find novel antitumour substances, preferably of natural origin, to improve immunity without damaging the immune system and the patient's metabolism. Flavonoids are prime-candidate antitumour substances, showing promising results in *in vitro* colon cancer cell assays (Cárdenas et al., 2006). Almost all anti-cancer drugs come from natural resources (Newman & Crag, 2012). Here, we want to characterise and test the bioactive properties, and antitumour and apoptotic effects of *Filipendula ulmaria* (L.) pollen, collected by honeybees.

MATERIAL AND METHODS

Pollen samples, palynological determination, and extract preparation

Two samples of bee pollen were collected in August 2010, using a bottom-fitting pollen trap placed in the front of the hives. Until further analysis, and to prevent degradation of biologically active substances, the pollen samples were cleaned of impurities and immediately frozen at -20°C. Physicochemical composition was determined from ground pollen powder samples. Palynological identification was made according to Mărgăoan et al. (2014).

Pollen extracts were prepared by dissolving 2 g ground pollen in 15 mL methanol:water solution (80:20) (Carpes et al., 2007; Morais et al., 2011). The mixture was sonicated for 45 min using a Bandelin Sonorex Ultrasonicator water bath (BANDELIN electronic, Berlin, Germany), and centrifuged 15 min at 1683×g. The supernatant was collected and the remaining sediment was re-extracted two times using the same protocol. All supernatants were reunited and stored for further analysis.

Phytochemical analysis and antioxidant activity

The physicochemical composition of the pollen samples was determined by water content (moisture), ash, total lipid (Soxhlet method), and total protein (Kjeldahl method) content (Mărgăoan et al., 2014). Also, the sugar profile (Dezmirean et al., 2012), vitamin C, fatty acids, and carotenoid profiles, were determined using high performance liquid chromatography and gas chromatography (Mărgăoan et al., 2014). The total phenolic content was measured using the Folin-Ciocâlteu method according to Mărghitaș et al. (2009). Total flavonoid content was determined using 2.5% $ZrOCl_2$, modified for methanolic extracts (Stanciu et al., 2011). Briefly, 250 µL methanolic extract was mixed with 10 µL 2.5% $ZrOCl_2$ in methanol for 30 sec. Following a 10 min incubation, the absorbance was read at 425 nm using a microplate reader (Synergy 2 Microplate Reader - BioTek Instruments, USA). The reference standard for total flavonoid quantification was quercetin. Antioxidant activity was characterised by two different methods, measuring the antiradical activity (scavenging of DPPH radical) and antioxidant power of the pollen extracts, according

to Campos et al. (2003), Duda et al. (2015), and Mărghitaș et al. (2009).

Cell line and culture conditions

C26 is an aggressive murine colorectal cancer cell line. The C26 model system has been used over the last three decades for research on carcinoma biology and antitumour therapy (Aulino et al., 2010). The C26 line (Interdisciplinary Research Institute on Bio-Nano-Sciences, Cluj-Napoca, Romania) was cultured in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% (v/v) FBS without antibiotics in 25 cm^2 culture flasks, at 37°C in an atmosphere of 5% CO_2 in the air and 95% relative humidity. After reaching ~ 90% confluence, C26 cells were detached using 0.05% trypsin/EDTA, and were seeded in 96-well microplates at a concentration of 5×10^4 cells per well in 200 µL culture medium. After a 24 h cultivation; the methanol pollen extract was added at the final concentration of 0.25, 0.5, and 1 mg/mL to each experimental group and the cells were again incubated 6, 12, 24, and 48 h for proliferation and for apoptosis assays, under the same conditions. All experiments, including the controls, were performed in five to six wells for each experimental group. At the end of each time interval, the cells of each well were examined using contrast phase microscopy (Olympus IX51, Tokyo, Japan).

Cell proliferation and apoptosis assay

Antiproliferative effects of methanol pollen extract on colon carcinoma C26 cells were assessed using MTT cell proliferation reagent (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, St. Louis, USA). After three PBS washing steps, C26 cells were incubated with a 2.5 mg/mL MTT solution for 2 h. The reaction was stopped by removing the MTT solution. The absorbance of the colour change was read at 550 nm and 630 nm (for background) with a microplate reader (HT BioTek Synergy, BioTek Instruments, USA). The antiproliferative effect of the tested pollen extracts was calculated in relation to the methanol controls (cells incubated with the methanol:water solution (80:20)).

Apoptosis was assessed with the Cell-APO-Percentage Apoptosis Assay kit (Biocolor Ltd., Carrickfergus, UK), using a dye being selectively imported by cells that are undergoing apoptosis, not necrosis. After exposure to the methanol pollen extract for each time interval, the culture medium was replaced with 100 µL fresh medium containing 5% APOPercentage Dye Label and incubated for 30 min. A hydrogen peroxide treatment (100 mM) was used as apoptosis positive control. The cells were optically inspected for apoptosis, with an Olympus IX51 microscope. Both, total numbers of cells and number of apoptotic cells (coloured in bright pink) were counted. An apoptotic index was calculated in percent apoptotic cells of the total cell count.

Statistical analysis

Data analysis was performed using STATISTICA 8.0 (StatSoft, Tulsa, Oklahoma, USA). Statistical differences between pollen extract samples and treatment groups were estimated using General Linear Models (GLM), with pollen sample (pollen 1, 2), concentration (0.25, 0.5, and 1 mg/mL), and time (6, 12, 24, and 48 h), as fixed variables. In a second step, data were pooled for pollen 1 and 2, if the first GLM did not show significant differences for the pollen samples.

RESULTS

Filipendula ulmaria (L.) (Rosaceae) was found to be the predominant pollen in the honeybee collected pollen samples with 70% (pollen 1) and 80% (pollen 2) of the counted total pollen grains. Table 1 summarises the botanical origin (families and species of the pollen samples) following Louveaux, Maurizio, & Vorwohl (1978). Both samples were harvested in the same geographic location. Both samples did not differ much in botanical origin. These minor differences are mainly explained by their different secondary pollen type (Tab. 1). All physicochemical parameters and main nutritional components of the pollen samples are summarised in Table 2 and do not show any salient characteristics.

Table 1

Pollen composition (predominant, secondary, and minor pollen) of the analysed pollen samples. (*, F.H. Wigg; **, Scop.)

	Predominant pollen (> 45%)		Secondary pollen (16-45%)		Important minor pollen (3-15%)		Minor pollen (< 3%)	
	Family	Species	Family	Species	Family	Species	Family	Species
Pollen 1	Rosaceae	*Filipendula ulmaria* (L.)	Ericaceae	*Calluna* sp. (L.)	Asteraceae	*Artemisia* sp. (L.)	Asteraceae	*Taraxacum officinale**
					Hyperi-caceae	*Hypericum* sp. (L.)	Geraniaceae	*Geranium* sp. (L.)
							Onagraceae	*Epilobium* sp. (L.)
Pollen 2	Rosaceae	*Filipendula ulmaria* (L.)	Hyperi-caceae	*Hypericum* sp. (L.)	Asteraceae	*Artemisia* sp. (L.)	Asteraceae	*Taraxacum officinale**
					Lamiaceae	*Thymus* sp. (L.)	Ericaceae	*Calluna* sp. (L.)
					Onagraceae	*Epilobium* sp. (L.)	Fabaceae	*Medicago sativa* (L.)
								*Onobrychis viciifolia***
							Rosaceae	*Rubus* sp. (L.)

Table 2

Physicochemical and nutritional characterisation of bee collected pollen samples. (Values showing means ± SD of three independent analyses)

	Moisture (%)	Ash (%)	Lipids (%)	Sugars (%)			Proteins (%)	Vitamin C (mg/100 g)	Total polyphenols (mgGAE/g)	Total flavonoids (mgQE/g)
				Fructose	Glucose	Sucrose				
Pollen 1	22.09 ± 0.05	2.84 ± 0.07	4.82 ± 0.21	17.64 ± 0.14	10.29 ± 0.23	0.06 ± 0.02	23.79 ± 2.36	45.6 ± 5.30	4.52 ± 0.14	6.33 ± 0.16
Pollen 2	24.37 ± 0.16	2.62 ± 0.01	6.34 ± 1.16	16.99 ± 0.04	8.72 ± 0.06	0.86 ± 0.03	24.47 ± 1.96	58.62 ± 2.51	7.24 ± 0.23	5.70 ± 0.22

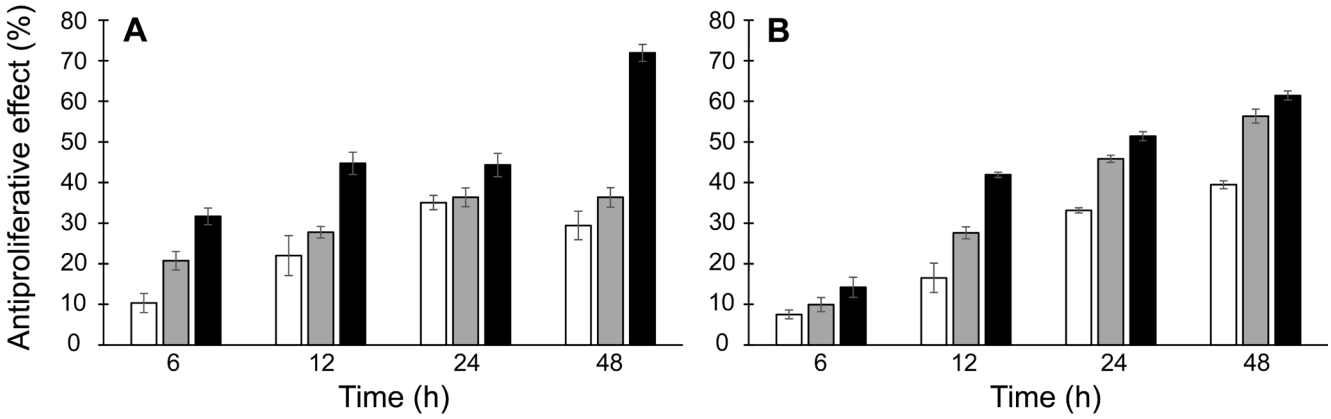

Fig. 1. Antiproliferative effects of *Filipendula ulmaria* pollen methanolic extracts (A: pollen 1, B: pollen 2) tested at different incubation intervals (6, 12, 24, and 48 h) and extract concentrations (white: 0.25 mg/ml, grey: 0.5 mg/ml, black: 1 mg/ml).

Phenolics are important plant secondary metabolites with high antioxidant activity. The average values ranged from 4.38 to 7.47 mg GAE/g sample for the total polyphenolic content (Tab. 2), and quantities between 5.48 and 6.49 mg QE/g pollen for the total flavonoid content. These amounts were similar to those found in other studies of pollen from the same region (Mărghitaș et al., 2009; Stanciu et al., 2011).

The antioxidant activity of bee collected pollen is species-specific (being different according to the botanical origin of the pollen, Campos et al., 2003), and is not influenced or not much influenced by geographical origin. Different methodologies are known to determine the antioxidant activity. Here, the free radical scavenging activity (DPPH) and the reducing capacity (FRAP) were used. The free radical scavenging activity can be expressed in different ways (% Inhibition of DPPH radical, mM Trolox/g sample or EC50) (Lopes et al., 2011). All values are given as the mean ± SD of three independent analyses. The pollen samples had radical scavenging activity of DPPH radicals ranging from 64.25 ± 1.13% (pollen 1) to 83.57 ± 1.39% (pollen 2) for the different pollen extracts. This means that these pollen extracts have a high ability of DPPH neutralisation. Expressed as mM Trolox equivalents (TE) the activity ranged between 1.15 ± 0.03 (pollen 1) and 1.65 ± 0.03 mM TE/g. The very low EC50 values (0.60 - 0.78%) reveal the low concentration of pollen extract needed for the depletion of 50% of the free radicals. As both pollen samples have the same species as predominant pollen, not many differences were detected. FRAP values for the different analysed extracts ranged between 0.60 ± 0.04

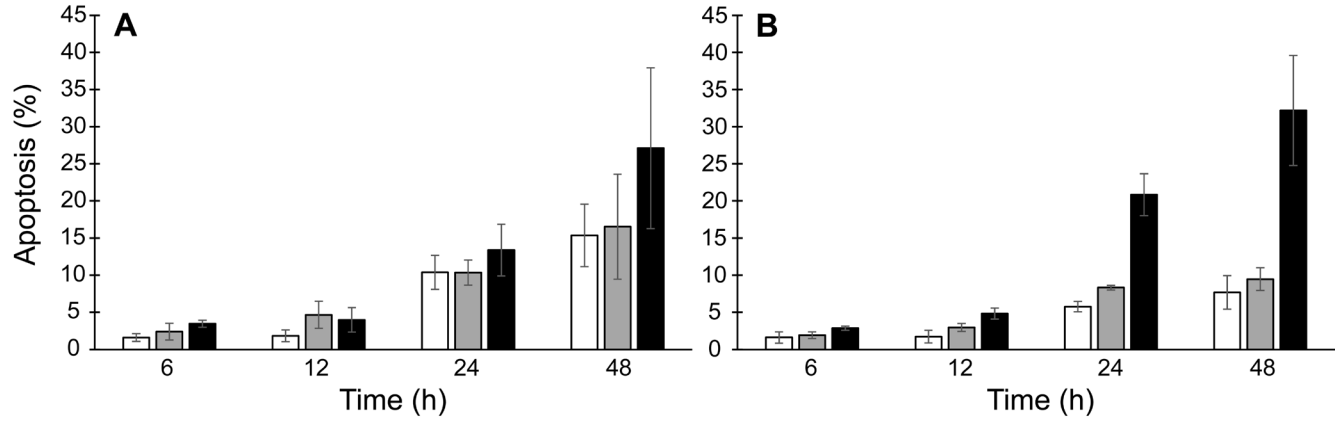

Fig. 2. Apoptotic effects of *Filipendula ulmaria* pollen methanolic extracts (A: pollen 1, B: pollen 2) tested at different incubation intervals (6, 12, 24, and 48 h) and extract concentrations (white: 0.25 mg/ml, grey: 0.5 mg/ml, black: 1 mg/ml).

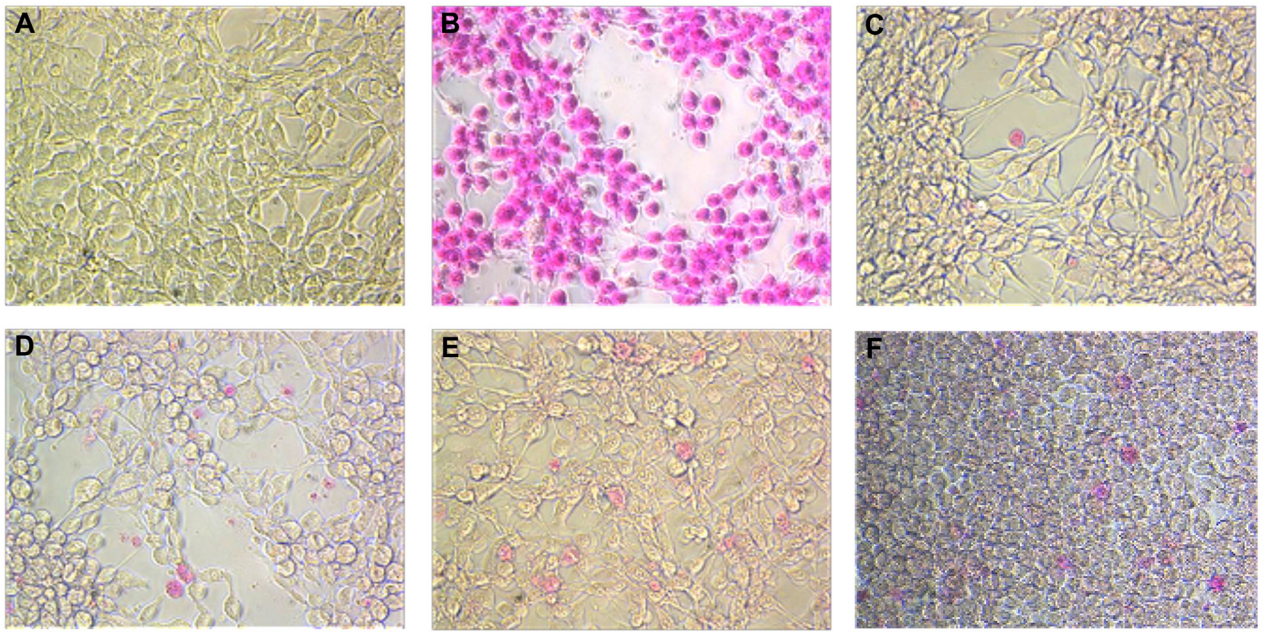

Fig. 3. Morphological changes and apoptosis (coloured in bright pink) observed after 12 hours (A-E) of exposing C26 tumour cells to pollen extract at different concentrations. A: the negative control, B: the positive control (cells treated with H_2O_2), C: cells treated with 0.25 mg/mL pollen extract, D: cells treated with 0.5 mg/mL pollen extract, E: cells treated with 1 mg/mL pollen extract, F: cells treated for 24 hours with 1 mg/mL pollen extract. Images (20× magnification) are representatives of more than five fields of view per sample.

and 1.16 ± 0.05 mM Fe^{2+}/g sample.

The antiproliferative effect of the methanolic pollen extracts strongly increased with an increasing concentration of the extract, corrected for the methanol control, (GLM, df = 2, F = 148.8, p<0.001) and exposure time (GLM, df = 3, F = 225.8, p<0.001) (Fig. 1), by reaching a maximum of ~ 60 to 70% inhibition. These results were independent of the pollen samples (p = 0.24). The apoptotic index revealed very low apoptotic effects (< 10%) for the 6 and 12 h treatment groups, regardless of the concentration of the extracts. After 24 and 48 h of treatment, the values increased slightly for the 0.25 and 0.5 mg/mL pollen extract, without reaching the threshold of 10-15% (Fig. 2). The highest apoptotic index was measured with 1 mg/mL extract at 24 and 48 h treatment (~ 30% on average). Overall the apoptotic index was significantly influenced by all 3 parameters (GLM - pollen sample: df = 1, F = 7.1, p = 0.01; concentration: df = 2, F = 78.1, p<0.001; time: df = 3, F = 282.4, p<0.001), but was strongest by incubation time.

DISCUSSION

The physicochemical parameters were within the standard ranges of previously analysed pollen samples without any obvious outliers (Bogdanov, 2004; Carpes et al., 2007; Szczęsna, 2007; Campos et al., 2008; Mărghitaș et al., 2009), guaranteeing the freshness and high quality of the used pollen. Sample characterisation was completed by fatty acid profiles, carotenoid spectrum, and total carotenoid content (previously published in Mărgăoan et al., 2014). Running a principle component analysis revealed that *Filipendula* pollen had a very unique and distant profile from all other pollen samples tested in the previous study (Mărgăoan et al., 2014).

The phenolics content was in the range of previous pollen studies from the same region (Stanciu et al., 2011), but lower than known for multifloral pollen (Kroyer & Hegedus, 2001; Mărghitaș et al., 2009; Morais et al., 2011). The total flavonoid content can be determined following several methods and specific reagents: aluminium chloride, sodium nitrite,

sodium hydroxide, and zirconium oxychloride. Comparative studies showed that the last method (reagent), used in this study, quantifies the highest amount of flavonoids. This means that this specific reagent is reacting with more flavonoid-like entities, giving the best result in the analysis. Thus, total flavonoid values for bee pollen are difficult to compare with other studies, as different methods and also different reference standards for calibration curves were used, depending on which compounds were quantified. In our study, quercetin was used for the calibration curve, whereas Serra Bonvehí et al. (2001) used rutin (the recommended standard), and Pascoal et al. (2014) used catechin, mainly quantifying tannins. These substances have different absorbencies at the same wavelength and concentration, and will give different amounts of equivalents in analysed pollen. If the phenolic profile of the samples is not available, several reference standards should be used for measuring the total flavonoid content.

Screening literature showed that direct comparisons of pollen antioxidant activity with other studies are quite difficult, as mostly different methods, solvents, and ways of expressing results were used (Kroyer & Hegedus, 2001; LeBlanc et al., 2009; Lopes et al., 2011; Pascoal et al., 2014). Nevertheless, similar activities were obtained for pollen samples of other plant origins (LeBlanc et al., 2009; Mărghitaș et al., 2009), indicating the high antioxidant activity of methanolic pollen extracts in general. Similar or even higher results were obtained for FRAP values of pollen samples of other plant origins (LeBlanc et al., 2009; Mărghitaș et al., 2009). The antioxidant power was well correlated with the total polyphenolic and flavonoid content, as is known for other bee product like honey and royal jelly (Dezmirean et al., 2012; Pavel et al., 2014).

The antitumour effect of methanolic pollen extracts was tested *in vitro*, on murine colorectal cancer cell cultures (C26). This cell line was previously used as the standard model system to study antitumour therapy, efficacy of matrix metalloproteinase inhibitors hampering neoangiogenesis and limiting tumour growth and metastases. Furthermore, studies using this model cell line demonstrate the antitumour effect of interleukin-18, chemokine CCL21/SLC (Leng et al., 2003; Hisada et al. 2004), and those developed novel karyotype analysis approaches, to verify the origin and evolution of tumour cell lines. In this study, different concentrations of pollen extract (0.25, 0.5, and 1 mg/mL) and four different time intervals of exposure (6, 12, 24, and 48 hours) were tested for each pollen extract. The observed antiproliferative activity of the *Filipendula ulmaria* pollen extract was much higher than the antiproliferative activity of sulphated polysaccharides from *Pinus massoniana* (Lamb.) pollen on HepG2 cells (Chu et al., 2013), beebread extract on glioblastoma cells (Markiewicz-Żukowska et al., 2013), and stingless bee pollen extract against five human cancer cell lines (Kustiawan et al., 2014). Only a single study reached comparable or higher antiproliferative values. These values were reached by applying propolis fractions and isolated single substances on human colorectal adenocarcinoma cells (Catchpole et al., 2015).

However, the low apoptotic index values clearly show the minor importance of the methanolic extract as an antitumour agent, in terms of apoptosis, which is also known for hot water extracts (Chu et al., 2013). Whereas a steroid fraction of *Brassica campestris* (L.) pollen, extracted using chloroform, is a much more promising antitumour agent inducing high levels of apoptosis in several cancer lines - mainly prostate cancer (Wu & Lou, 2007). As the apoptotic index was not normalised on a methanol control, the very low apoptotic index observed in this study might also be a combination of pollen extract activity and methanol treatment using pollen extract. Additive effects cannot be excluded. Nevertheless, the major antitumour activity of the extracts originates from their antiproliferative effects.

The apoptosis assay also showed cellular modifications, for instance; concerning shape. At the early time points (6 and 12 h) and at low pollen extract concentrations (0.25 and 0.5 mg/mL), most of the cells did not suffer visible modifications. Most of the cells exhibited complete cell layers and uniform cell distribution per well

(Fig. 3). However, using the highest concentration (1 mg/mL) led to intra-cytoplasmatic vacuolisation and granulation. These most noticeable morphological changes have been observed with longer cell exposure to the pollen extract (24 and 48 h) (Fig. 3).

The APOPercentage test revealed a much lower apoptotic activity of the tested pollen extracts, in comparison with the antiproliferative activity determined by the MTT cell proliferation assay. It may be said that methanolic pollen extracts exhibit very high antiproliferative activity as major antitumour activity. The antiproliferative activity is well in line with the anti-mutagenic, anti-microbial, anti-inflammatory and anti-oxidant activity of different commercial bee pollens (Fabaceae, Cistaceae, Ericaceae, Boraginaceae, and Brasicaceae) tested in previous studies (Eraslan, Kanbur, & Silici, 2009; Pascoal et al., 2014). Altogether, pollen extracts or bee collected pollen has a very promising potential as an antitumour agent for human medicine. Bee collected pollen contains substantial nutrients and bioactive compounds, harbouring high antioxidant properties. The chemical composition of the methanol extract, as well as the plant origin of predominant, secondary, and minor pollen species, may exert a synergistic activity on tumour cells. Further studies must be conducted in order to elucidate the action mechanisms of potential candidate substances (e.g. flavonoid compounds: quercetin, galangin, resveratrol, and kaempferol) underlying these beneficial biological properties.

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