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Original Article

CHEMICAL COMPOSITION AND ANTIOXIDANT ACTIVITY OF BEEBREAD, AND ITS INFLUENCE ON THE GLIOBLASTOMA CELL LINE (U87MG)

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Abstract

Beebread is processed pollen stored in the cells of the honeycomb, with the addition of various enzymes and honey, which undergoes lactic acid fermentation. Ethanolic extracts (EBBs) were obtained from three different samples of beebread from Poland. Assays were carried out for the determination of chemical composition (GC/MS), for the total phenolic content, and for the antioxidant and cytotoxic activities. The effects of beebread extracts (10, 20, 30, 50, 100 µg/mL) on the viability of the glioblastoma cell line (U87MG) were studied after 24 h, 48 h, and 72 h. Our results indicated a time-dependent inhibitory effect on the viability of U87MG cells treated EBB. The main inhibitory effect of EBB was observed after 72 h; EBB treatment decreased cell viability to 49 - 66%.

Keywords: antioxidant activity, beebread, chemical composition, cytotoxicity, *glioblastoma multiforme*.

INTRODUCTION

Beebread is a fermented mixture of plant pollen, honey, and bee saliva that worker bees use as food for the larvae, and for young bees to produce royal jelly. Pollen collected by bees is mixed with a small amount of honey and saliva and packed into the cells of the honeycomb where it undergoes a chemical change to form a product called beebread (Gilliam, 1979).

Products of *Apis mellifera* have been widely used for centuries in traditional medicine all over the world due to their nutritional and medical properties. Beebread has a positive effect on the immune system of healthy people. It also has antibiotic and antioxidant properties (Audisio et al., 2005; Mutsaers et al., 2005; Baltrušaitytė et al., 2007a). Abouda et al. (2011) studied the antibacterial activity of beebread extracts against some pathogenic bacteria. The results revealed that all the samples showed strong antimicrobial activities on the bacterial strains. Moreover, the Gram positive bacteria were more sensitive to beebread than Gram negative bacteria. Studies assessing the efficacy of treatments with beekeeping products for patients with atherogenic dyslipidemia showed that a significant hypolipidemic effect was registered in patients taking honey in combination with beebread (total cholesterol decreased by 15.7%, LDL cholesterol by 20.5%) (Kas'ianenko et al., 2011).

Glioblastoma (GMB) is the most common and lethal primary brain tumor which demonstrates a high proliferation rate and an aggressive growth pattern and is largely resistant to chemotherapy (Gangemi et al., 2009; Agnihotri et al., 2013).

Researchers are seeking new substances that may reduce the viability of cancer cells, slow tumor growth, and extend life expectancy. Among the apicultural products, the anticancer activity of propolis and honey have been widely

presented in various culture cell lines (Barbarić et al., 2011; Borges et al., 2011; Da Silva Frozza et al., 2012). However anticancer activity of beebread has not yet been analyzed. Our work presents, for the first time, a cytotoxic effect on tumor cell line (*glioblastoma multiforme* – U87MG).

This study is expected to expand the existing information on the chemical characterization, and the antioxidant and anticancer activity of beebread and to assist in a more focused design of further research, e.g. aiming at more specified applications of this product as a natural adjuvant treatment.

MATERIAL AND METHODS

Reagents

Folin–Ciocalteu's phenol reagent, gallic acid, methylthiazolyl diphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The Randox kit for determination of Total Antioxidant Status (TAS) was from Randox Laboratories (London, UK). Minimal Essential Medium Eagle (MEM) with L-glutamine (292 mg/L), trypsin-EDTA, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from PAA Laboratories GmbH (Pasching, Austria); calcium-free phosphate buffered saline (PBS) was from Biomed (Lublin, Poland). The high purity water was prepared in a Simplicity 185 UV water purification system (Millipore, Austria). All other chemicals were of ultrapure grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of the extracts

Three samples of beebread were obtained from different apiaries of north-eastern Poland (the Podlasie region). Samples were collected in the summer (August-September) of 2010. Vacuum dried beebread ethanolic extracts (EBB1, EBB2, EBB3) were prepared in the Department of Bromatology, Medical University of Białystok, Poland. Each of dry samples of beebread were crushed and 20.0 g were extracted on a shaker with 80.0 g of 95% (v/v) ethanol for 12 h. The top layers were decanted (Extract A) and rest of sediments were re-extracted in a shaker with 40.0 g of 95% (v/v) ethanol (Extract B). Extracts A and B of each beebread were pooled together and centrifuged at 3,000 rpm for 30 min at 20°C. Each of the ethanolic extracts of beebread were evaporated (40°C) in a rotary evaporator (Rotavapor R-3, Buchi, Switzerland). The obtained residues were weighed and stored at -20°C in the dark. The yield of prepared extracts (% w/w) in terms of the starting material was: EBB1 – 46.0, EBB2 – 43.5, EBB3 – 42.5.

Into each vial with samples (5 - 10 mg), 220 µL of dry pyridine and 80 µL of BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) with an addition of 1% trimethylchlorosilane were added. The reaction mixture was sealed and heated for 0.5 h at 60°C to form trimethylsilyl (TMS) derivatives.

Gas chromatography/mass spectrometry (GC/MS) analysis and component identification

The extracts from different beebread samples were analyzed by gas chromatograph HP 6890 with mass selective detector MS 5973 (Agilent Technologies, USA) fitted with a HP-5MS fused silica column (30 m x 0.25 mm; 0.25 µm film thickness), with an electronic pressure control (EPC) and split/slitless injector, and checked by gas chromatograph Clarus 680 with Clarus 600 MS (PerkinElmer, USA) according to the method described by Isidorov et al. (2009) and Borawska et al. (2010).

Analysis of total phenolic content

The total phenolic content (TPC) was measured using the Folin–Ciocalteu colorimetric method (FC). The absorbance versus a prepared blank was read at 760 nm using a Cintra 3030 (GBC Scientific Equipment, Australia). The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of dry extract (Djeridane et al., 2006). The concentration of samples equaled 2 mg/mL (extract dissolved in Et-OH). Assays were carried out in triplicate. Data were expressed as mean ± SD and range.

Antioxidant activity

The total antioxidant status (TAS, mmol/L) of the extracts was measured spectrophotometrically

using a chemical Randox test on a Cintra 3030 (GBC Scientific Equipment, Australia). Extracts were dissolved in double deionized water in a concentration of 2 mg/mL. Assays were carried out in triplicate. Data were expressed as mean ± SD and range.

Cell culture

Human glioblastoma cell line U87MG (HTB-14) purchased from American Type Culture Collection, (Rockville, MD) were cultured in MEM supplemented with 10% FBS; 50 U/mL penicillin and 50 mg/mL streptomycin in a humidified incubator at 37°C and 5% CO_2 atmosphere. Subconfluent cells were detached with trypsin-EDTA solution in PBS and counted in a hemocytometer.

Cytotoxicity assay

The effects of EBB1, EBB2, EBB3 (10, 20, 30, 50, 100 µg/mL) on the viability of glioblastoma cell line (U87MG) were studied after 24 h, 48 h, and 72 h of treatment. Extracts were dissolved in 100 µl DMSO and prepared as 1 mg/mL stock solutions (calculated on the dry extract) by dilution in a medium (MEM supplemented with 10% FBS and Pennicillin-Streptomycin). Cells were seeded into 96-well plates in a volume of 200 µL per well at a density of 2 x 10^4 cells per well, and grown for 22 h at 37°C in a humidified 5% CO_2 incubator. Cell viability was measured by quantitative colorimetric assay using MTT, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Carmichael et al., 1987). Water insoluble MTT-formazan crystals formed inside the living cells were dissolved in the DMSO. The absorbance at 570 nm proportional to the number of living cells was measured on a Multimode Plate Reader Victor X3 (PerkinElmer, Singapore). There were cells used from passage 5 to 7. Each experiment was performed in triplicate and independently repeated at least three times.

Statistical analysis

Statistical analyses were performed using Statistica, version 10.0 for Windows. Metric data were tested for normal distribution by the Kolmogorov-Smirnov and the Shapiro-Wilk tests. All data were normally distributed, therefore, they were given as the mean and standard deviation (SD) and the Student's t-test was used to calculate the value significance (p values <0.05 were accepted as statistically significant).

RESULTS

Table 1 contains data on the composition of ethanol extracts from the investigated beebread samples. There are 64 compounds, 37 of which are registered in all three beebread samples. Fatty acids and their derivatives were the main components of the examined beebread samples (Tab. 1). Aliphatic acids were the predominant components of these extracts (62.32 ± 7.0%), and unsaturated, α-linolenic, linoleic, oleic and 11,14,17-eicosatrienoic acids formed more than a half of them (40.63 ± 4.5%).

Carbohydrates were found in all extracts, with the average being 19.46 ± 5.4%. The relative content of other groups of organic compounds was not high: the contents of glycerol and glycerides, sterols, alkanes, polyphenols are, on the average: 7.26 ± 2.9%, 3.92 ± 0.3%, and 2.38 ± 0.4%, 0.58 ± 0.2%, respectively.

The total phenolic content of the different EBB was investigated using the FC assay and ranged from 32.78 to 37.15 mg GAE/g. The mean values and standard deviation are shown in Table 2. It was observed that TPC showed significant differences between the samples of beebread.

The total antioxidant status consisting of all the antioxidants present in EBB were analyzed (Tab. 2). The method was based on the bleaching causing a characteristic colour of a more stable ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation by antioxidants.

Figure 1 shows the results from cell viability after a 24, 48, and 72 hour incubation using a beebread extract concentration range of 10 - 100 µg/mL. The data was expressed as a percentage of the control. The highest inhibitory effect after the 24 h incubation was noted for EBB3; significant differences were observed versus the control and EBB1, EBB2. Viability of U87MG was significantly inhibited by EBB2 in a concentration of 100 mg/mL compared to the control, but EBB1 showed no

Table 1.

Chemical composition of ethanolic extracts from three beebread samples (EBB1, EBB2, EBB3)

No.	Compound, TMS*	Retention parameters		Relative composition,%		
		$LTPRI^{Exp}$	$LTPRI^{Lit}$	EBB1	EBB2	EBB3
1	Lactic acid	1069	1070	0.11	Trace	Trace
2	Phosphoric acid	1289	1289	Trace	Trace	nd
3	Glycerol	1293	1293	1.53	1.64	0.94
4	Proline	1305	1304	0.13	Trace	Trace
5	Succinic acid	1323	1325	0.16	Trace	Trace
6	2-Methylglutaconic acid	1461	1459	Trace	nd	nd
7	5-Oxoproline (pyroglutamic acid)	1527	1527	nd	Trace	nd
8	Arabinoic acid	1658	1657	0.30	0.19	nd
9	Dodecanoic acid	1660	1660	nd	Trace	nd
10	Pentitol	1789	-	0.08	nd	nd
11	α-Methylfructofuranoside	1815	-	0.06	Trace	nd
12	Methylfuranoside	1840	-	0.17	0.10	nd
13	α-Fructofuranose	1845	1843	0.85	0.74	1.01
14	ß-Fructofuranose	1853	1854	7.30	7.88	12.19
15	Methyl galactofuranoside	1862	1865	0.15	nd	Trace
16	α-Glucofuranose	1886	1888	0.10	Trace	Trace
17	Gluconic acid, δ-lactone	1920	1917	0.31	0.17	Trace
18	α-Glucopyranose	1931	1930	3.78	3.59	5.79
19	Glucitol	1980	1980	0.99	0.77	0.99
20	Ethyl hexadecanoate	1995	1993	0.55	0.48	0.42
21	Ethyl tartrate	2010	-	2.49	1.91	1.24
22	ß-Glucopyranose	2031	2030	2.01	2.43	5.37
23	Gluconic acid	2045	2045	0.40	0.35	0.32
24	Hexadecanoic acid	2053	2052	18.73	18.92	14.31
25	Methyl linolenate	2099	2098	0.27	nd	nd
26	*n*-Heneicosane	2100	2100	nd	0.24	0.88
27	Ethyl linoleate	2161	2163	0.21	0.17	nd
28	Methyl-11,14-eicosadienoate	2164	-	nd	nd	0.29
29	Ethyl linolenate	2168	2168	1.62	1.41	0.79
30	Linoleic acid	2215	2215	6.40	5.89	11.50
31	Oleic acid	2222	2222	nd	0.50	0.45
32	α-Linolenic (ω-3) acid	2225	2225	36.59	37.05	23.40
33	Octadecanoic acid	2249	2250	1.66	2.60	2.63
34	7-Tricosene	2272	2271	0.13	nd	nd
35	*n*-Tricosane	2300	2300	0.53	0.70	1.10

Table 1. Continued

Chemical composition of ethanolic extracts from three beebread samples (EBB1, EBB2, EBB3)

No.	Compound, TMS*	Retention parameters		Relative composition,%		
		LTPRI^{Exp}	LTPRI^{Lit}	EBB1	EBB2	EBB3
36	11,14,17-Eicosatrienoic (ω-3) acid	2424	-	0.22	nd	nd
37	Eicosanoic acid	2448	2448	0.57	1.13	0.30
38	7-Pentacosene	2474	2474	0.10	Trace	nd
39	*n*-Pentacosane	2500	2500	0.35	0.46	0.33
40	2-Monopalmitin	2580	2577	0.09	Trace	Trace
41	1-Monopalmitin	2612	2611	1.64	2.36	4.33
42	3-Hydroxyeicosanoic acid	2636	2635	0.08	nd	nd
43	Docosanoic acid	2647	2646	0.35	0.23	0.30
44	7-Heptacosene	2675	2673	0.06	Trace	nd
45	*n*-Heptacosane	2700	2700	0.19	0.21	Trace
46	2-Monostearyl glycerol	2771	2772	nd	nd	Trace
47	β-Linolenate glycerol	2784	2786	0.42	0.41	nd
48	1-Monostearyl glycerol	2805	2806	1.44	2.39	4.59
49	Squalene	2828	2823	0.08	nd	nd
50	Tetracosanoic acid	2844	2844	1.32	0.63	1.27
51	*n*-Nonacosane	2900	2900	Trace	nd	nd
52	Hexacosanoic acid	3040	3045	0.14	Trace	Trace
53	9-Hentriacontene	3073	3075	0.22	0.02	Trace
54	7-Hentriacontene	3080	3082	0.22	Trace	Trace
55	NN (73,517,217,532)	3146	-	0.37	nd	nd
56	Kaempferol	3112	3114	Trace	Trace	nd
57	Apigenin	3159	3159	0.32	Trace	0.47
58	Octacosanoic acid	3240	3240	0.08	Trace	nd
59	3-Hydroxyergosta-7,22-diene (22E)	3249	3248	1.79	1.48	3.00
60	Stigmasterol?	3266	3274	0.37	0.90	0.69
61	9-Tritriacontene	3274	3275	0.45	0.47	0.48
62	ß-Sitosterol	3345	3345	0.41	0.39	0.50
63	Avenasterol	3362	3358	0.95	0.67	0.35
64	7-Sitosterol	3404	3402	0.10	0.50	nd

*TMS – trimethylsilyl derivatives
LTPRI^{Exp} – Linear temperature programmed retention indices – measured values
LTPRI^{Lit} – Linear temperature programmed retention indices – literature data
Trace – below 0.02% of TIC
nd – not detected

Table 2.

Total phenolic content (TPC – milligrams of gallic acid equivalent [GAE] per gram dry extract) and antioxidant activity (TAS – milimol per litr) of beebread (EBB)

Lp.	Extract	TPC [mg GAE/g]		TAS [mmol/L]	
		Mean ± SD (Min - Max)	p*	Mean ±SD (Min-Max)	p*
1.	EBB1	35.18 ± 0.1 (35.06 – 35.30)	$p_{1/2}$<0.01 $p_{1/3}$<0.02	0.56 ± 0.06 (0.49 – 0.64)	$p_{1/2}$<0.001 $p_{1/3}$<0.001
2.	EBB2	33.43 ± 0.7 (32.78 – 34.09)	$p_{2/3}$<0.01	1.11 ± 0.09 (0.99 – 1.21)	
3.	EBB3	36.52 ± 0.6 (35.90 – 37.15)		1.00 ± 0.09 (0.93 – 1.11)	

*p – significant differences

activity at any concentration after 24 h. After 48 h, viability of U87MG incubated with all concentrations of the examined extracts was between 60% to 82%. Differences were statistically significant compared to the control. The main inhibitory effects of EBB were observed after 72 h, and EBB treatment decreased cell viability to 49 - 66%. These values were statistically significant compared to the control.

DISCUSSION

Recently there have been a lot of publications and reviews that deal with the methodology of studying the chemical composition of apicultural products such as honey and propolis (Gómez-Caravaca et al., 2006; Sulaiman et al., 2011; Da Silva Frozza et al., 2012; Markiewicz-Żukowska et al., 2012). But, the chemical composition and antioxidant profile of beebread samples has not been studied uniformly. Beebread has a different composition and nutritional value than the field collected pollen pellets. Mutsaers et al. (2005) reported that beebread is a source of proteins with essential amino acids, fats, minerals, vitamins, and flavonoids. There are only a few publications detailed studies of the chemical composition of this product (Baltrušaityte et al., 2007a; Kaškoniene et al., 2008; Isidorov et al., 2009).

In study of Isidorov et al. (2009), fatty acids were determined in beebread samples. The fatty acids were investigated with the help of a successive extraction with organic solvents of different polarity. Saturated and unsaturated (α-linolenic, linoleic acids) fatty acids were predominant components of ether extracts. Noticeable amounts (9%) of C_{16}–C_{18} aliphatic acids and their esters were identified in hexane extracts. In methanol extracts of beebread, small quantities of hexadecanoic, linoleic and α-linolenic acids were noted. Čeksterytė et al. (2008) identified twenty-two fatty acids in beebread. On average, arachidonic and oleic acids constituting 16% and 15%, respectively, were the major ones, while the content of arachidic acid was 12%, EPA - 8%, α-linolenic acid - 5% and DHA - 5%.

Our research showed that carbohydrate content in EEB3 was the highest. The main part of this fraction is constituted by monosaccharides, among which anomers of fructose and glucose are presented in the largest quantities. Carbohydrates were detected by Isidorov et al. (2009) as the main compounds (80%) of methanol extracts of beebread.

We determined a small or trace amount of phenol compounds in beebread samples. It was kaempferol and apigenin that were detected. The phenolic fractions of beebread were analysed using the HPLC method by Baltrušaityte et al. (2007b). They identified p-coumaric acid, kaempferol, apigenin, and chrysin present in tested samples of beebread after thermal processing. The concentrations were expressed by using peak area units only. Apart from the compounds mentioned above, Isidorov et al. (2009) also detected isorhamnetin and trace amounts of ferulic and caffeic acids, and flavonoids naringenin and quercetin in ether extracts of five beebread samples.

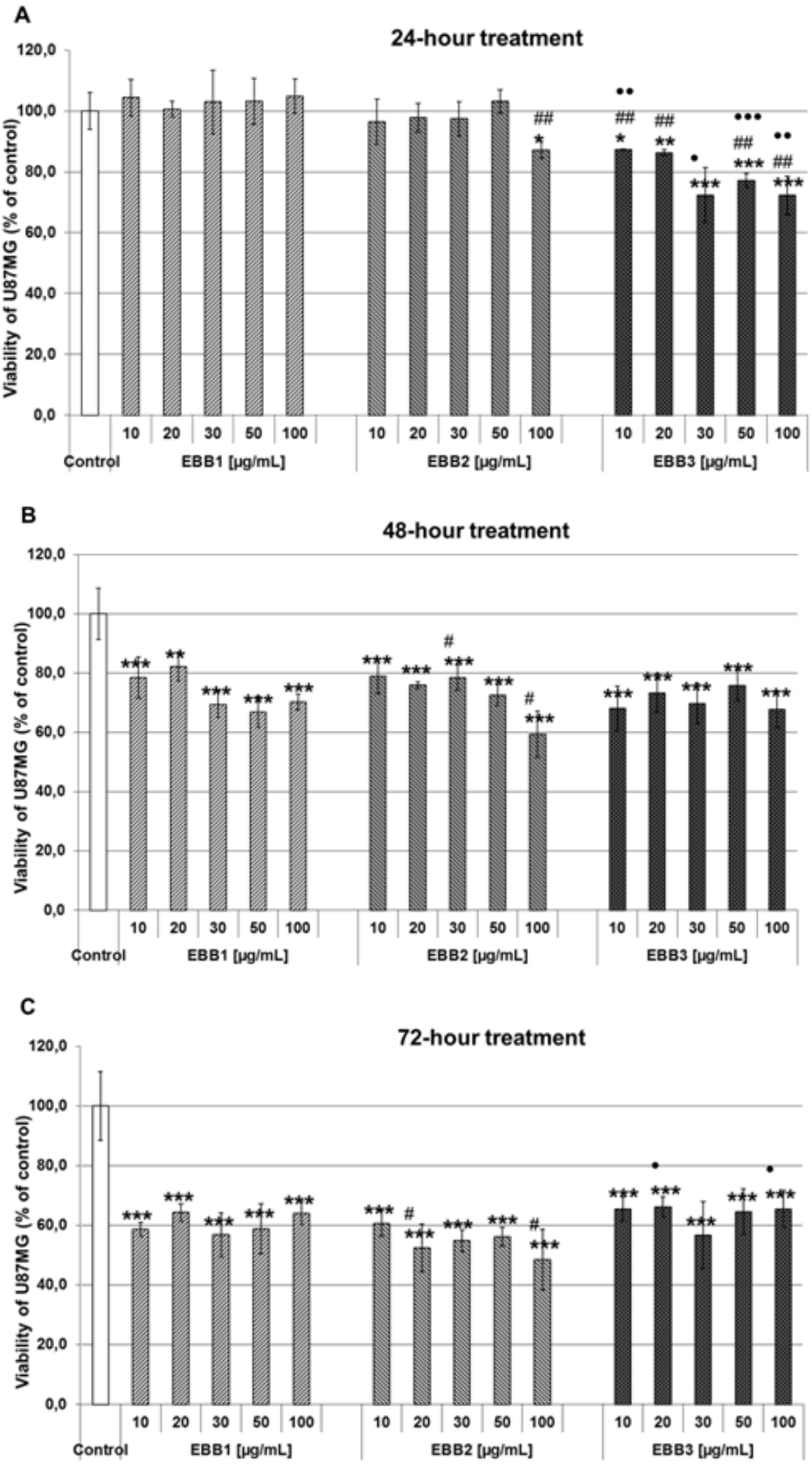

Fig. 1. Viability of U87MG (% of the control) after incubation with beebread (EBB). The results are presented as a percentage of the control after 24 (A), 48 (B), and 72 (C) hours of incubation with EBB1, EBB2, EBB3. Significant changes obtained from the Student-t test are indicated: *p<0.05, **p<0.01, ***p<0.001 vs. the control; #p<0.05, ##p<0.01, ###p<0.001 vs. equivalent concentration of EBB1; ▪p<0.05, ▪▪p<0.01, ▪▪▪p<0.001 vs. equivalent concentration of EBB2.

Polyphenols are part of the chemical composition found in beebread that varies according to the year and location of collection. The examined extracts were characterised by different total phenolic contents compared to other bee products. Higher TPC was noted in propolis: 151 mg/g (Da Silva Frozza et. al., 2012), 232 mg/g (Alencar et al., 2007) and 257 mg/g (Cabral et al., 2009) while pollen was lower: 10.5 - 16.8 mg/g (Morais et al., 2011). It was reported that phenolic compounds are the main components responsible for the antioxidant effects, however, non-phenolic antioxidants are also involved (Gheldof et al., 2002; Aljadi and Kamaruddin, 2004).

We estimated TAS in beebread because there is no data in this field in research literature. In EBB1, a significantly lower TAS value compared to EBB2 and EBB3 was found. In our work (Tab. 2), there was no correlation between TPC and TAS ($r = -0.2634$; $p = 0.830$).

Radical scavenging activity of beebread phenolic extracts was assessed by Baltrušaityte et al. (2007b). They reported that after thermal processing, beebread had comparable inhibition of $ABTS^{\bullet+}$ radical cation and higher antioxidant activity in the $DPPH^{\bullet}$ reaction system (94%) than samples of honey and beebread mixed with honey.

In regard to functional properties, such as antioxidative ability, it can be predicted that this apicultural product will apply more and more as a health food, as a supplement, and in medicine. Anticancer activity of beebread has not been analysed yet. Effects of other apicultural products, especially, propolis and its compounds (e.g. CAPE, chrysin, propolin G), on the viability of glioma cell lines, was presented in a few publications (Guarini et al., 1992; Huang et al., 2007; Borges et al., 2011; Huang et al., 2011; Watanabe et al., 2011).

We have found a time-dependent (from 24 to 72 h) decrement in a viability of U87MG cells treated each of EBB (Fig. 1). In our study, cytotoxic activity of the examined extracts of three beebread samples was similar. Only after the 24 h treatment with EBB3 was the highest inhibitory effect observed. Activity of the analyzed extracts may depend on the chemical composition. Compared to the other two extracts, TPC of EBB3 was significantly higher. Moreover, we observed differences between the ratios of n-6 to n-3 fatty acids in estimated extracts: about 1:6 in EBB1 and EBB2; and 1:2 in EBB3. EBB3 characterized the highest content of linoleic acid. Both n-6 and n-3 polyunsaturated fatty acids (PUFA) have diverse functions in living cells and influence membrane composition and function, eicosanoid synthesis, cellular signaling, and regulation of gene expression (Benatti et al., 2004). There are publications about the importance of fatty acids in gliomas. Linoleic acid and conjugated linoleic acid – CLA (geometrical and positional stereoisomer) have an impact on tumor development. In gliomas linoleic acid, different effects were exerted, ranging from inhibitory to neutral (Maggiora et al., 2004). Cimini et al. (2005) studied the effects of CLA on cell growth, differentiation, and death of a human glioblastoma cell line (ADF). These researchers demonstrated that CLA strongly inhibits cell growth and proliferation rate, and induce apoptosis. Leaver et al. (2002) suggest that intraparenchymal infusion of PUFA may be effective in stimulating glioma regression.

CONCLUSIONS

The antioxidant effect of the analyzed extracts of beebread depends not only on phenolic compounds but also on non-phenolic antioxidants. The main group of the compounds of the estimated extracts were fatty acids and their derivatives, among which were the dominant unsaturated fatty acids. Ethanolic extract of beebread has cytotoxic activity on the U87MG cell line. It would be interesting to know which of the components of the extracts has the strongest anticancer activity. Further study is required in order to know the answer.

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