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Cytotoxicity of Blended Versus Single Medicinal Mushroom Extracts on Human Cancer Cell Lines: Contribution of Polyphenol and Polysaccharide Content

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ABSTRACT: The use of mushrooms contributes to human nutrition by providing low lipid content of lipids and high dietary fiber content, as well as significant content of other biologically active compounds such as polysaccharides, minerals, vitamins, and polyphenolic antioxidants. This study aimed to determine the content of polyphenols and polysaccharides, as well as the cytotoxic and antioxidative properties of several medicinal mushroom preparations. The content of total phenols and flavonoids of preparations of blended mushroom extracts (Lentifom, Super Polyporin, Agarikon, Agarikon Plus, Agarikon.1, and Mykoprotect.1) was evaluated quantitatively by using ultraviolet–visible spectroscopy spectrophotometric methods. The antioxidant capacity of the preparations was evaluated using the ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and ferric reducing/antioxidant power assays. The content of water-soluble polysaccharides was determined using a specific gravimetric method, based on ethanol precipitation. To determine cytotoxic effects of single and blended mushroom extracts, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and neutral red assays were conducted using human small cell lung cancer, lung adenocarcinoma, colon cancer, and brain astrocytoma cancer cells. The obtained results suggest that due to the significant content of beneficial polyphenolic antioxidants and soluble polysaccharides, use of these mushroom preparations is beneficial in maintaining good health, as well as in the prevention and adjuvant biotherapy of various human pathological aberrations. These results reveal that these extracts exhibit different cytotoxic effects on tumor cells originating from different tissues. In addition, the comparison of investigated blended mushroom extracts with three well-known commercial mushroom products derived from single mushroom species or single mushroom compounds shows that blended mushroom extracts exhibit significantly stronger cytotoxic effects on human tumor cell lines.

KEY WORDS: medicinal mushrooms, cancer, colon cancer, small cell lung carcinoma, lung adenocarcinoma, brain astrocytoma, human tumor cell lines, cytotoxicity, polysaccharides, polyphenols, antioxidants

ABBREVIATIONS: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); **Caco2**, human colon cancer cell line; **CCTG-1**, human astrocytoma cell line; **FRAP**, ferric reducing/antioxidant power; **GAE**, gallic acid equivalent; **H69V**, human small cell lung carcinoma cell line; **MEM**, Minimum Essential Medium Eagle growth medium (Sigma-Aldrich); **MGN**, immunobran MGN-3 arabinoxylan compound; **MTT**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NR**, neutral red assay (3-amino-7-dimethylamino-2-methylphenazine hydrochloride); **PSK**, polysaccharide-K (Krestin); **PSP**, polysaccharide peptide; **ROS**, reactive oxygen species; **SKLU-1**, human lung adenocarcinoma cell line; **TFC**, total flavonoid content; **TPC**, total phenol content

I. INTRODUCTION

Millennial experience of traditional medicine and more than 60 years of scientific research have conclusively proven that medicinal mushrooms inhibit cancer in humans and other mammals.[1–3] A vast number of experimental studies, beginning in the United States in the late 1950s and followed mainly by Japanese and Chinese researchers in the 1970s and 1980s, determine whether the antitumor activity

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of some mushrooms exists and if so, what the tumor inhibition rates and complete regression rates are. Research began on animal and human tumor cell lines and animal models, followed by human trials and clinical studies.[1–4] Following the human clinical trials of single mushroom compounds, the first official antitumor drugs from medicinal mushrooms were registered, including PSK (1977), Lentinan (1985), and Schizophyllan (1986) in Japan, and PSP (1983) in China. Such drugs are limited to the Far Eastern countries, whereas medicinal mushroom supplements are used as oncological therapy support in Western countries.[1]

New studies address interpretation of molecular mechanisms related to the anticancer effects of medicinal mushrooms. In addition to the immunomodulation mainly caused by high-molecular-weight polysaccharides, polysaccharide-protein complexes, and proteins produced during primary metabolism, other compounds (especially low-molecular-weight polysaccharides, polyphenols, flavonoids, triterpenes, tocopherols, and carotenes) and certain secondary metabolic products directly affect cancer cells through interaction with intracellular signaling pathways and changing the course of inflammation, cell differentiation, survival, apoptosis, angiogenesis, tumor progression, and so forth.[4,5]

Since there is a multitude of evidence that single compounds from medicinal mushroom extracts possess certain biological activities, there are tendencies to isolate new, prospective, and synthesizable compounds. Most recent studies focus on single compounds or "the best" single mushroom species; however, for many mushroom species, it is not known whether a single compound or a synergy of multiple ingredients causes the observed effects. Empirical evidence and studies[6] strongly support the superiority of the blends. In addition, the optimal daily dose and extraction conditions for most compounds remain unknown.[7]

In this work, the content of bioactive compounds of mushroom preparations was elucidated, especially in terms of polyphenol and polysaccharide content. The dosages of active mushroom compounds that provide effective cytotoxic action were also determined. Therefore, as part of ongoing research on medicinal mushrooms and their bioactive compounds and biological activities, commercially available preparations of several medicinal mushrooms were screened for their antioxidant properties and biological activity on human tumor cell lines using an array of *in vitro* assays.

II. MATERIALS AND METHODS

A. Medicinal Mushroom Extracts

Dr Myko San–Health from Mushrooms Company (Zagreb, Croatia) supplied their commercial blended mushroom products, which included four liquid extracts (Lentifom, Super Polyporin, Agarikon, and Agarikon Plus) and two in tablet form (Agarikon.1 and Mykoprotect.1). Lentifom is a proprietary extract blend of 3 medicinal mushroom species, whereas Super Polyporin contains 7, Agarikon contains 8, and Agarikon Plus contains 10 mushroom species, including the most well-known ones such as *Lentinus edodes*, *Ganoderma lucidum*, *Trametes versicolor*, *Grifola frondosa*, and *Agaricus brasiliensis* (=*blazei* ss. Heinem.). Agarikon.1 is made from *L. edodes*, *G. lucidum*, *A. brasiliensis*, *G. frondosa*, and *Pleurotus ostreatus*, with 750 mg of mushroom polysaccharides per tablet (the standard daily dosage is three tablets per day). Mykoprotect.1 is made from *L. edodes* and *G. lucidum*, and contains 850 mg of mushroom polysaccharides per tablet (the standard daily dosage is three tablets per day).

For comparison, we included three commercial single mushroom products (extracts produced in a similar manner as described below): PSP (from *T. versicolor*; KunShan Long-Teng Biotech Manufacture, KunShan City, China), ImmunoBran MGN-3 (from *L. edodes*; Daiwa Pharmaceutical, Tokyo, Japan), and β-glucan (from *Saccharomyces cerevisiae*; Transfer Point Inc. Columbia, SC, USA).

We extracted 50 g of dried mushroom fruiting bodies in 1 L of boiling water for 24 h using a Dr Myko San–Health from Mushrooms Company proprietary production method. Insoluble matter

was removed by forcing the solution (in suspension form) through a filter press, and then concentrating it 4-fold. For tablet preparations, hot water extract was precipitated with ethanol and dried.

To measure dry matter content, we used the Association of Official Analytical Chemists method[8] of drying the material in an oven at 105°C until constant weight was achieved. The moisture content was determined by the weight difference before and after drying, whereas the dry matter was the ratio of the final to the initial sample weight percentage.

To determine the phenolic content and antioxidant capacity, liquid extracts were used; tablets were ground using a mortar and pestle and dissolved in distilled water (1 g/25 mL).

To determine the cytotoxic effects, the liquid extracts and dissolved tablets were evaporated until dry and dissolved in corresponding growth medium (MEM and RPMI 1640, respectively). Following the manufacturers' recommendations for treatment doses, the prescribed amounts of bioactive compounds were determined and used in order to prepare the extract concentrations that correspond to 0.1, 1, 10, and 100 times of the prescribed amounts.

The recommended dosages for Dr Myko San products were developed during the company's 23 years of operation. Early dosages were determined from published research and later refined through systematic follow-up and evaluation of product use in several thousand patients. Cancer patients today receive 3.5–7 g/d of active medicinal mushroom compounds.

B. Chemicals

Analytical grade Folin–Ciocalteu, formic acid, potassium peroxydisulfate, sodium carbonate, formaldehyde, ferric chloride hexahydrate, ferrous sulfate heptahydrate, ethanol, and hydrochloric acid were supplied by Kemika (Zagreb, Croatia). Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)diammonium salt), neutral red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma-Aldrich (Steinheim, Germany). Growth media (MEM and RPMI 1640, respectively), fetal bovine serum, and penicillin-streptomycin solution were purchased from Gibco Co. (Carlsbad, CA, USA).

C. Determination of Polyphenolic Compounds

Total phenol content (TPC) of medicinal mushroom preparations was determined spectrophotometrically according to a modified Lachman method.[9] To determine total flavonoid content (TFC), these compounds were precipitated using formaldehyde, which reacts with C-6 or C-8 on 5,7-dihydroxy flavonoids. The condensed products of these reactions were removed by filtration and remaining nonflavonoid phenols were determined according to the previously mentioned procedure for the determination of TPC. Flavonoid content was calculated as the difference between total phenol and nonflavonoid content. Gallic acid was used as the standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per liter. All measurements were performed in triplicate.

D. Determination of Antioxidant Capacity

1. Ferric Reducing/Antioxidant Power

The ferric reducing/antioxidant power (FRAP) assay was carried out according to a standard procedure by Benzie and Strain.[10] The FRAP assay is based on the reduction of the Fe^{3+}-2,4,6-tripyridyl-S-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}). Antioxidant activity of the samples was monitored by measuring the change of absorption at 593 nm. FRAP reagent was prepared by mixing acetic buffer, TPTZ, and $FeCl_3 \times 6 \cdot H_2O$ (20 mM water solution) at a ratio of 10:1:1. Briefly, 3.8 mL of FRAP reagent was added to a 200 μL volume of the sample. After 4 min, the absorbance of blue coloration was measured against a blank sample. All measurements were performed in triplicate.

Aqueous solutions of $FeSO_4 \times 7H_2O$ (100–1000 mmol/L) were used for the calibration and the results are expressed as millimoles of Fe(II) per liter.

2. ABTS Radical Scavenging Assay

The Trolox equivalent antioxidant capacity (TEAC) was also estimated by the ABTS radical cation decolorization assay.[11] This method is based on the scavenging of stable blue-green ABTS radical cations ($ABTS^+$), which are formed either by chemical or enzymatic oxidation of ABTS several hours prior to the analysis. The improved technique for the generation of $ABTS^{\bullet+}$ applied here involves direct production of the blue-green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. Stock solutions of ABTS (7 mM) and potassium peroxydisulfate (140 mM) in water were prepared and mixed together to a final concentration of 2.45 mM potassium peroxydisulfate. The mixture was left to react for 12–16 h at room temperature in the dark. Prior to the analysis, the ABTS radical solution was diluted with ethanol to an absorbance of 0.70 (±0.02) at 734 nm. All measurements were performed as follows: 20 μL of the sample was added to 2.0 mL of the ABTS radical solution, and the absorbance readings were taken after exactly 6 min against the appropriate reagent blank instead of the sample. The results, obtained from triplicate analyses, were expressed as Trolox equivalents and derived from a calibration curve determined for this standard (100–1000 μmol/L).

E. Determination of Soluble Polysaccharides

The content of soluble polysaccharides was determined according to a modified method of Wei et al.[12] Briefly, liquid mushroom extracts were centrifuged (10,000 rpm for 15 min at 20°C) to collect the supernatant, which was subsequently concentrated by vacuum evaporation until approximately 35° Brix was reached. The obtained concentrate was precipitated by the addition of two volumes of ethanol to a final concentration of 75% (v/v). The precipitates collected by centrifugation (10,000 rpm for 15 min at 20°C) were solubilized in deionized water and lyophilized to obtain the crude polysaccharides. The tablet preparations were primarily ground to obtain a fine powder and were then dissolved in distilled water as described above in order to obtain liquid samples. The content of soluble polysaccharides was determined according to the previously described procedure. Polysaccharide yield was expressed as milligrams per gram of dry matter of sample.

F. Human Cell Lines

The cytotoxic effect of mushroom extracts was examined on four human cancer cell lines: human colon cancer cells (Caco-2), human lung adenocarcinoma cells (SKLU-1), human small lung carcinoma cells (H69V), and human astrocytoma cells (CCTG-1). All human cancer cell lines were purchased from the European Collection of Cell Cultures/Sigma-Aldrich. Cancer cell lines were grown as monolayer cultures in MEM (Caco-2 and SKLU-1) and RPMI (H69V and CCTG-1) media (Gibco) and were supplemented with 10% fetal bovine serum (Gibco). Growth medium served as the negative control.

G. Cytotoxicity Assay

NR and MTT assays were used to determine cytotoxic effects of single mushroom extracts and mushroom blends in order to define whether active compounds destabilize the membrane or inhibit mitochondrial activity. Cells were seeded in microtiter plates at a concentration of 6×10^3 cells/well. After 24-hour incubation, cells were treated with mushroom extracts for 72 hours. After treatment, the MTT and NR assays were performed as described by Babich and Borenfreund[13] and Mishchish et al.,[14] respectively. The absorbance intensity was measured at 432 nm and 540 nm, respectively, using a microplate reader (Cecil Instruments Ltd., Cambridge, UK). Each concentration was tested in quadruplicate and each experiment was repeated three times.

TABLE 1. Composition and Antioxidant Activity of Blended Mushroom Extracts

Extract	Dry matter (%)	Soluble polysaccharides (mg/g dry matter)	Total phenols (mg GAE/L)	Total flavonoids (mg GAE/L)	Antioxidant capacity	
					ABTS (mM Trolox)	FRAP (mM Fe(II))
Agarikon	4.02	579.35	839.17	455.83	6.29	7.28
Agarikon Plus	4.60	490.42	908.33	542.08	7.56	10.33
Lentifom	1.71	402.61	339.58	174.17	3.53	2.97
Superpolyporin	3.61	467.09	788.75	305.83	6.53	8.08
Agarikon.1	93.00	1053.88	448.75	240.83	3.53	3.14
Mykoprotect.1	92.94	935.35	635.42	362.92	4.65	6.81

H. Statistical Analysis

Statistical analysis was performed using SPSS software (version 8.0, SPSS Inc., an IBM Company, Chicago, IL, USA). A one-way analysis of variance was employed to determine whether the means obtained with various groups differed significantly from each other. Significance was established using the Schaffer and Tukey post hoc tests. A probability level <0.05 was considered significant. All data are expressed as means ± standard deviations (SD) of the values obtained by three independent measurements.

III. RESULTS

Table 1 presents the dry matter content and bioactive composition of six mushroom preparations. The highest content of soluble polysaccharides was detected in tablet products Agarikon.1 and Mykoprotect.1. The highest content of total polyphenols and flavonoids was measured in the Agarikon Plus mushroom preparation (908.33 mg GAE/L) followed by Agarikon (839.17 mg GAE/L), whereas Lentifom exhibits the lowest content of total phenols and flavonoids (339.58 mg GAE/L). The ranking of mushroom preparations based on their antioxidant potential corresponds to those obtained for the TPC, which is confirmed by a high correlation obtained between the results ($r_{TPC/ABTS} = 0.966$ and $r_{TPC/FRAP} = 0.954$).

Correlation coefficients (r) of all evaluated bioactive and cytotoxic parameters are displayed in Table 2. According to the obtained results, the content of soluble polysaccharides correlated well only with the cytotoxic effect exerted in the SKLU-1 cell line ($r_{SKLU/polysaccharides} = 0.659$), followed by a low correlation in the astrocytoma cell line ($r_{astroMTT/polysaccharides} = 0.415$). A higher correlation was observed between the polyphenolic compounds, antioxidant capacity, and cytotoxic effects, thus linking the potential cytotoxic effects to polyphenolic compounds, rather than polysaccharides. TFC and antioxidant potential of examined extracts showed good correlation to the H69V cell line ($r_{H69V/TPC} = 0.616$, $r_{H69V/ABTS} = 0.718$, and $r_{H69V/FRAP} = 0.568$). A high correlation coefficient was obtained in the case of total flavonoid and polyphenol compounds and astrocytoma cells ($r_{astroMTT/TPC}$, $r_{astroMTT/TFC} = 0.805$ and $r_{astroNR/TPC} = 0.766$). Medium correlation was obtained between SKLU-1 and CaCo2 cells (NR cytotoxicity test; $r_{SKLU-1/CaCo2} = 0.571$) as well as CaCo2 and H69V (MTT assay and NR cytotoxicity test, respectively: $r_{CaCo2/H69V} = 0.571$ and $r_{CaCo2/H69V} = 0.683$).

Human colon cancer cells were resistant to the majority of investigated extracts at the recommended daily doses. The higher concentrations of Agarikon.1 and Agarikon Plus (10× and 100×) significantly decreased cell survival. A slight cytotoxic effect at the therapeutic dose was observed after treatment with Agarikon and Agarikon.1. Super Polyporin decreased cell survival in a dose-response manner, with observable cytotoxic effects at the therapeutic dose (Figure 1).

Agarikon influenced membrane permeability

TABLE 2. Correlation Coefficients (*r*) between the Results of Measurements

	Polysaccharides	TPC	TFC	ABTS	FRAP	SKLU-1_{NR}	SKLU-1_{MTT}	H69V$_{NR}$	H69V$_{MTT}$	Caco2$_{NR}$	Caco2$_{MTT}$	CCTG-1_{NR}	CCTG-1_{MTT}
Polysaccharides	1	–0.288	–0.145	–0.487	–0.344	0.659	0.205	–0.644	–0.850	0.400	0.003	0.263	0.415
TPC		1	0.904	0.966	0.954	–0.638	–0.738	0.224	0.616	–0.327	–0.155	0.475	0.661
TFC			1	0.852	0.884	–0.415	–0.828	0.372	0.353	0.093	0.124	0.766	0.805
ABTS				1	0.960	–0.669	–0.789	0.299	0.718	–0.400	–0.176	0.342	0.471
FRAP					1	–0.481	–0.854	0.300	0.568	–0.262	0.012	0.382	0.560
SKLU-1_{NR}						1	0.105	–0.355	–0.919	0.571	0.366	–0.100	–0.139
SKLU-1_{MTT}							1	–0.223	–0.222	–0.025	–0.091	–0.458	–0.483
H69V$_{NR}$								1	0.445	0.357	0.683	0.303	0.039
H69V$_{MTT}$									1	–0.654	–0.254	–0.138	–0.081
Caco2$_{NR}$										1	0.764	0.580	0.295
Caco2$_{MTT}$											1	0.285	0.122
CCTG-1_{NR}												1	0.872
CCTG-1_{MTT}													1

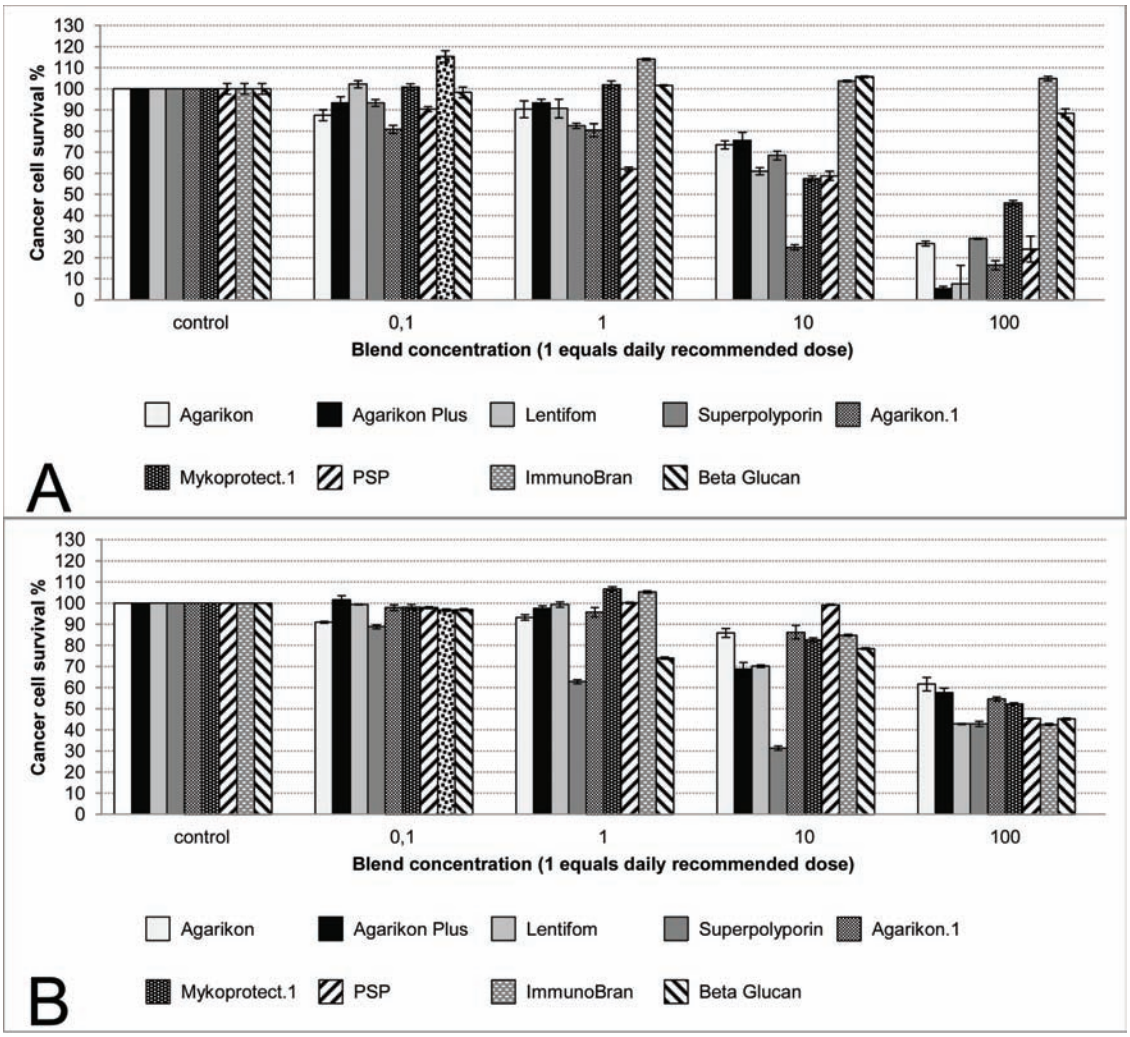

FIGURE 1. Comparison of human colon cancer cell (Caco-2) survival measured by the MTT assay (A) and the NR method (B).

at the therapeutic dose after treatment of human adenocarcinoma cells. No effect on mitochondrial dehydrogenase activity was observed. On the contrary, Agarikon Plus inhibited mitochondrial activity at the therapeutic dose and caused a loss of membrane selectivity to some extent (Figure 2). Super Polyporin decreased membrane stability after treatment of SKLU-1 cells with the therapeutic dose.

Agarikon Plus decreased mitochondrial dehydrogenase activity of small cell lung carcinoma cells in a dose-response manner, while it slightly destabilized cell membranes. Agarikon.1 and Mykoprotect.1 strongly decreased cell survival at the therapeutic dose, affecting both mitochondrial activity and membrane selectivity. In addition, Super Polyporin caused cytotoxic effects in dose-dependent manner. Cytotoxicity was detected on both levels, with decreased membrane and mitochondrial activity (Figure 3). Lentifom and Super Polyporin had strong cytotoxic effects on human astrocytoma cells (Figure 4). All investigated extracts showed significant cytotoxic effects at concentrations 10 or 100 times higher than the

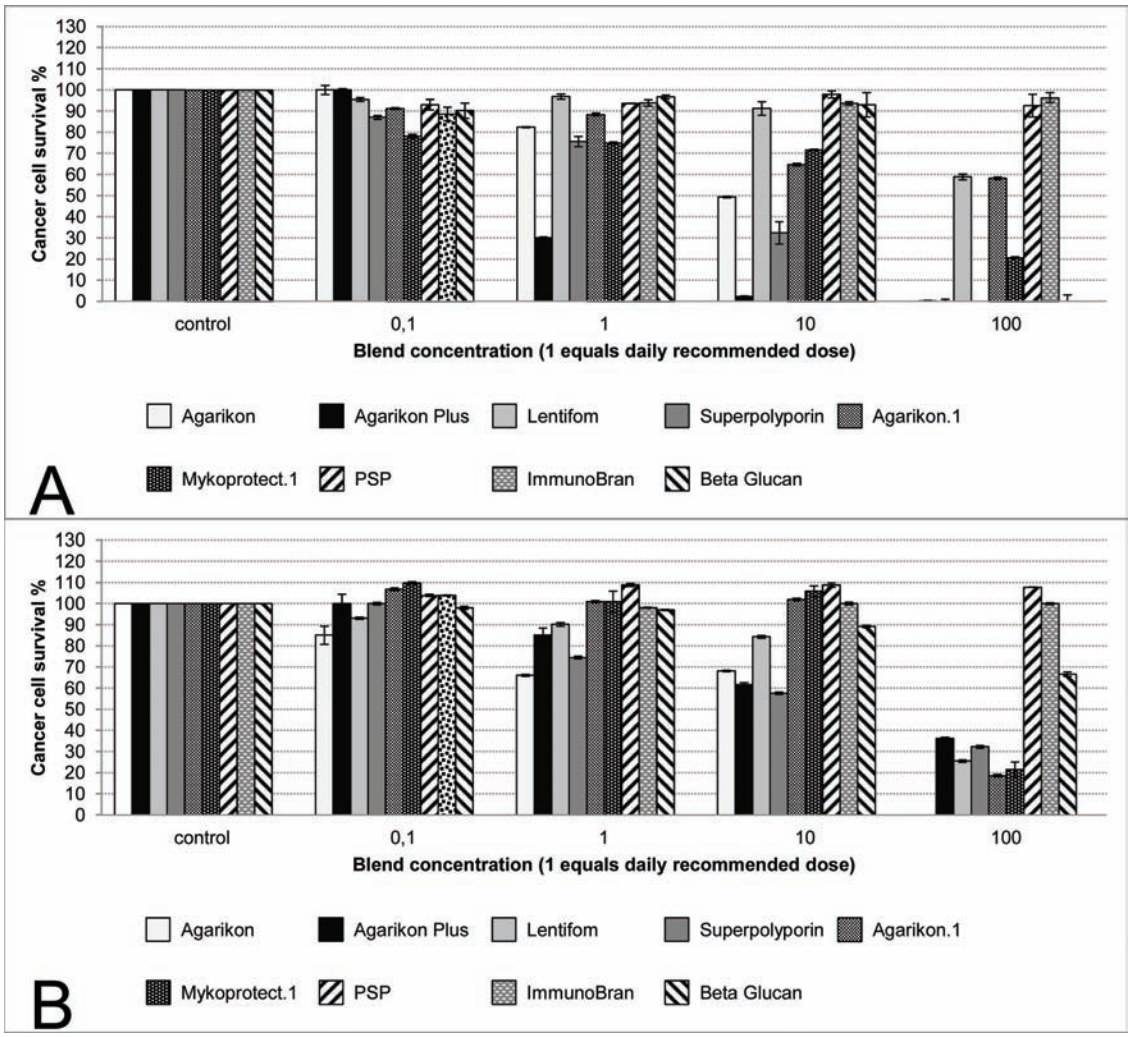

FIGURE 2. Comparison of human lung adenocarcinoma cells (SKLU-1) survival measured by the MTT assay (A) and the NR method (B).

therapeutic concentration. The cytotoxic effect of medicinal mushroom blends was stronger than that of single mushroom species extracts.

IV. DISCUSSION

The use of edible and inedible mushroom preparations in medicinal purposes as a supportive therapy is well known and documented.[15] The compounds in the majority of mushrooms with biological activity against infections (both viral and microbial, high blood pressure, cancer development, oxidative stress–related diseases, consequences of ionizing radiation) are similar for both edible and inedible mushrooms.[15–18]

Polysaccharides are the best known and most potent mushroom-derived antitumor and immunomodulating substances.[19] On the other hand, plant polyphenolic antioxidants are an important group of secondary metabolites because of their contribution to human health and their multiple biological effects, such as antioxidant activity, antimutagenic and/or anticancerogenic activities, and anti-inflammatory action. However, there is a lack of data re-

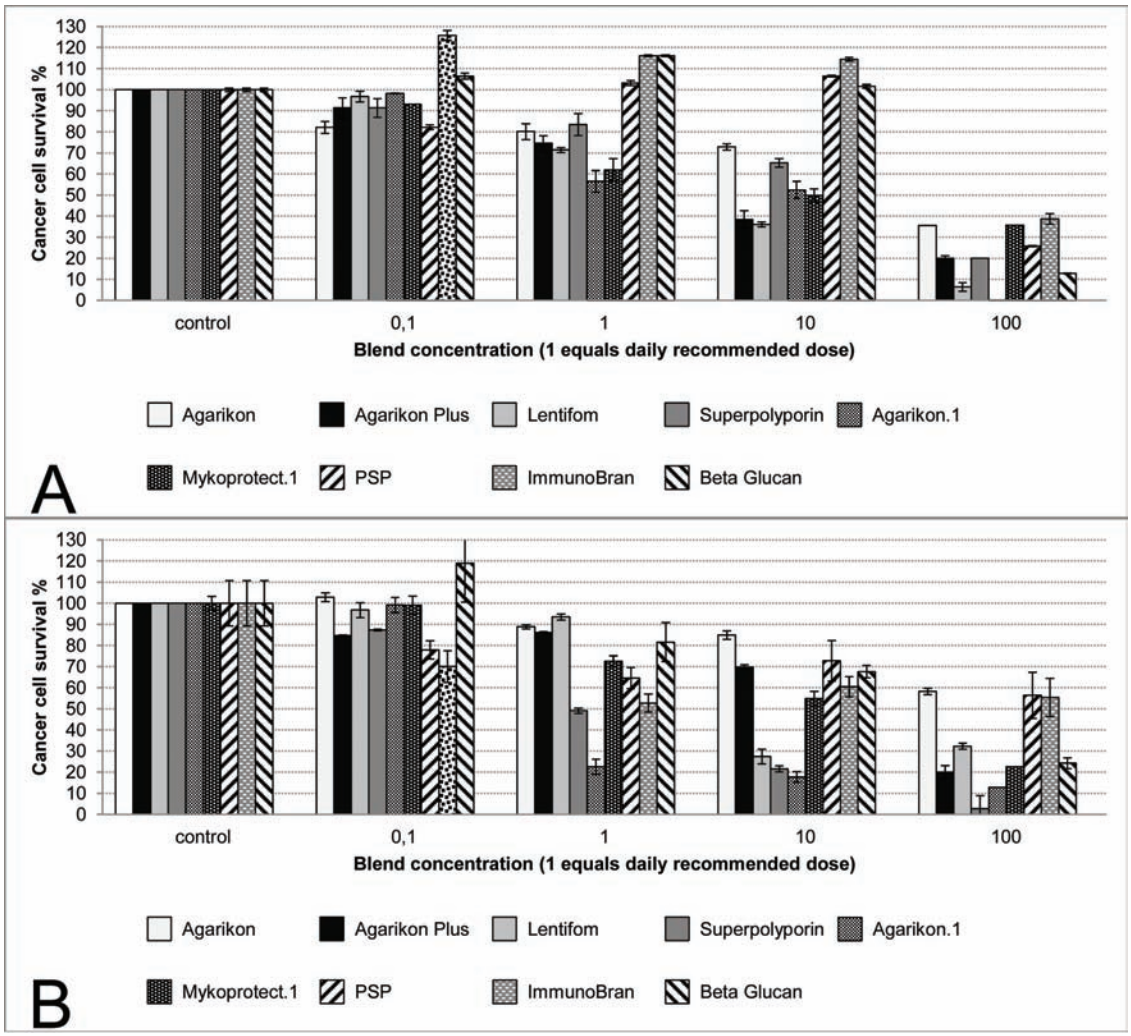

FIGURE 3. Comparison of human small cell lung carcinoma cell (H69V) survival measured by the MTT assay (A) and the NR method (B).

garding their content in mushrooms, especially in blended mushroom extracts.

This work determined the content of soluble polysaccharides, total polyphenols, and flavonoids and the antioxidant capacity of six blended mushroom extracts, four liquid extracts (Agarikon, Agarikon Plus, Lentifom, and Super Polyporin) and two tablet products (Agarikon.1 and Mykoprotect.1).

Compared with single mushroom species, the blended mushroom preparations analyzed in this study contained a higher content of soluble polysaccharides (e.g., 6.8% in *L. edodes* versus 16.0% in *T. fuciformis*[16]) (Table 1), confirming the effective polysaccharide extraction procedure developed by the producer. According to the findings of Song and van Griensven,[21] the polysaccharide content of three mushroom mixtures ranges from 1.2 to 15.0 mg glucose equivalent/mL (mg GE/mL), which is higher than the mushroom blends evaluated in our study.[21] The same authors also evaluated the TPC of several medicinal mushroom species and their mixtures. Their results showed high variability of polyphenolic content in the same mush-

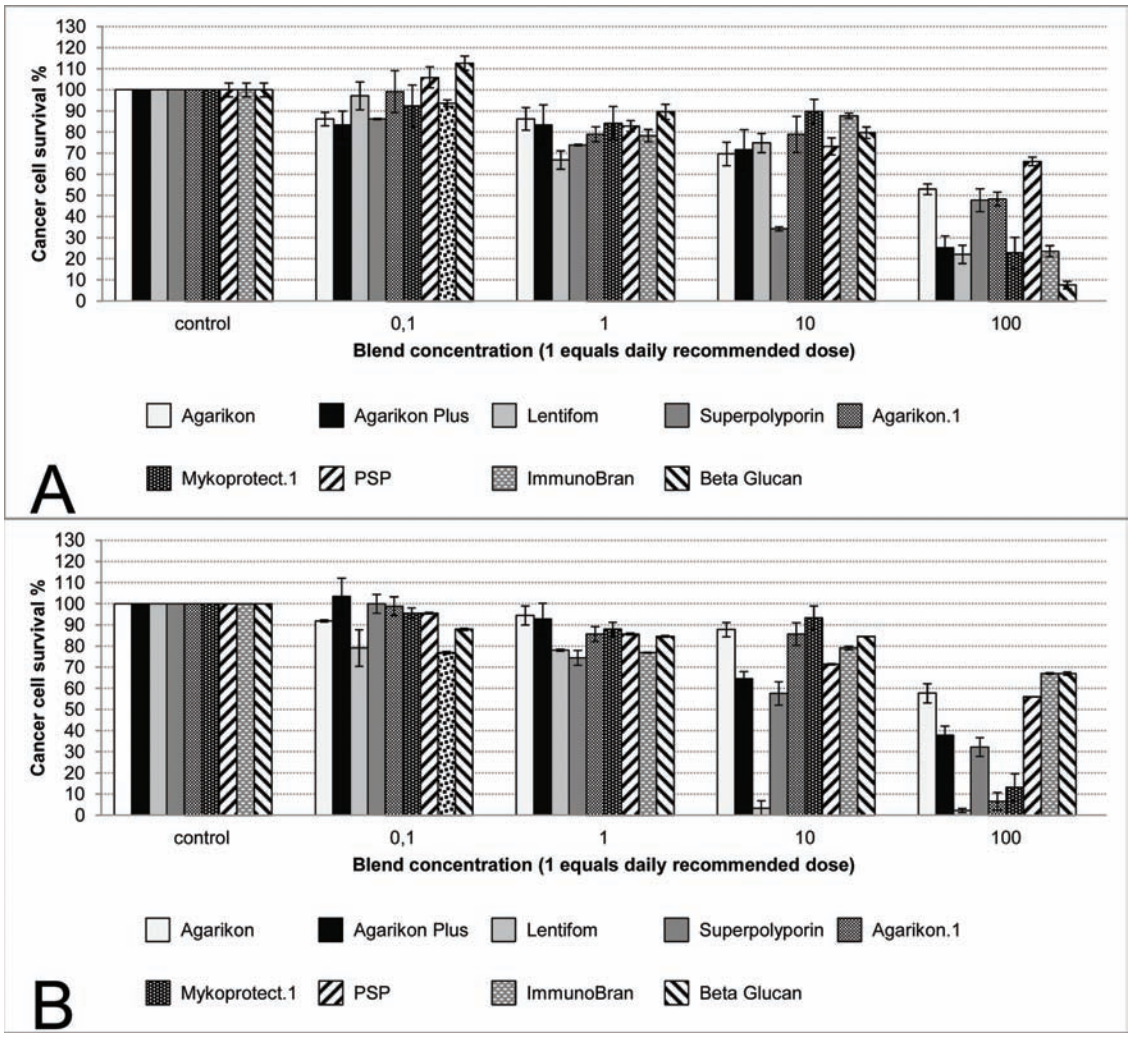

FIGURE 4. Comparison of human astrocytoma cell (CCTG-1) survival measured by the MTT assay (A) and the NR method (B).

room species derived from different geographical origins; the TPC of their three mushroom mixtures ranged from 213.8 to 647.8 μg GAE/mL, which is in accordance with the results obtained in this study. However, the results indicate that both phenolic content and polysaccharides depend primarily on the composition of mushroom blends.[21]

The results displayed in Table 1 also reveal a higher content of total phenols in liquid mushroom blends, rather than in the tablets. This may be due to the use of other substances during tablet forming process, which may interfere in the analytical determination of polyphenols. The difference between the liquid and tablet preparations can also be observed in the content of soluble polysaccharides, since the tablet preparations exhibit almost double the content compared to the liquid ones. It is well known that one of the main characteristics of polyphenols is their propensity to form complexes with proteins, polysaccharides, and alkaloids (e.g., caffeine).[22–24] Therefore, in tablet preparations with higher polysaccharide content, the formation of polyphenol-polysaccharide complexes may have occurred, leading to underestimation of total phenols in these mushroom blends. However, this could also be attributed to the mushroom composi-

tion (species) in each evaluated mushroom blend. In addition, there were no proteins or peptides in the examined extracts because these compounds were degraded during mushroom extract preparation (unpublished data). Therefore, it can be concluded that polyphenols, flavonoids, and polysaccharides play a crucial role in biological activity of the examined extracts.

High correlation coefficients observed between the TPC and antioxidant capacities of mushroom blends indicate that antioxidant activity of the examined mushroom preparations is attributed to the polyphenolic compounds, rather than polysaccharides. Although Song and van Griensven[21] determined a high correlation among the polyphenols and polysaccharides of different medicinal mushroom extracts (R=0.82), our study revealed no connection between these compounds (Table 2). According to Lu and Ding,[25] naturally derived antioxidant compounds of *Coprinus comatus* include total phenols, flavonoids, tocopherols, and polysaccharides. The results of our study revealed no correlation between the content of polysaccharides and antioxidant capacity of mushroom blends.

Tablets contain the highest content of polysaccharides, while Agarikon and Agarikon Plus possess the highest content of polyphenols and flavonoids. The comparison of cancer cell line survival after treatment with the six blends and single compounds reveals a strong cell type–specific cytotoxic effect. Agarikon was the most effective against human lung adenocarcinoma cells (SKLU-1) at the therapeutic dose. This effect was detected through changes in membrane active transport. This compound caused decreased mitochondrial activity in colon cancer cells (Caco2) and small cell lung carcinoma cells (H69V) at the therapeutic dose to some extent.

Agarikon Plus inhibited mitochondrial dehydrogenase activity in human lung adenocarcinoma cells and colon cancer cells, while astrocytoma cells were susceptible to Lentifom at the therapeutic dose through mitochondrial activity deprivation.

Super Polyporin inhibited both mitochondrial and membrane activity of small cell lung carcinoma cells and colon cancer cells in a dose-dependent manner, whereas it caused a slight decrease in cell survival in other cell lines. Polysaccharide-rich extracts, Agarikon.1 and Mykoprotect.1, were the most effective through both mitochondrial and membrane activity against small cell lung carcinoma cells and human lung adenocarcinoma cells. Agarikon.1 decreased cell survival of small cell lung carcinoma and human lung adenocarcinoma cells by 80%. Super Polyporin and Mykoprotect.1 also inhibited growth of small cell lung carcinoma and human lung adenocarcinoma cells at 50% and 30%, respectively. Single pharmaceutical compound PSP decreased cell survival at 20% (Figure 3), pointing out that mushroom blends have stronger cytotoxic effects compared with the single compound.

A possible explanation of observed cytotoxic effects lies in the fact that polysaccharide extracts of various mushrooms are highly active in reactive oxygen species (ROS) generation; however, there is evidence that some 1,4 glucans cannot induce ROS.[12,21] ROS mediate in signal transduction and regulation of diverse processes, such as phagocyte activation, cell proliferation, and migration, and finally apoptosis. Molecular damage caused by ROS in normal cells, induces repair mechanisms, while in tumor cells ROS activate cell death processes through apoptosis.[22] Shnyreva et al.[5] found that ROS-dependent activation of apoptotic cell death is crucial to antitumor activity.

In this work, we showed that Agarikon and Agarikon Plus, which have high polyphenol and flavonoid content, decrease cell survival of human lung adenocarcinoma cells by 70% and 30%, respectively, at the daily recommended dose (Figure 2). Astrocytoma cells were most sensitive to Super Polyporin and Lentifom, which inhibited astrocytoma cell growth by 30% (Figure 4). These blends contain the lowest levels of polysaccharides, polyphenols, and flavonoids. It is possible that the synergistic effect of all three major groups of compounds decreased astrocytoma cell survival. In addition, Lentifom is rich in lentinan polysaccharide, which has been proven to inhibit cancer cell growth. Lentinan causes release of cytokines,

nitric oxide, and other cell signal messengers, potentiating immune cells.[4]

Human colon cancer cells seem most resistant to cytotoxic effects of the examined blends and single mushroom extracts. Super Polyporin decreased cell survival by 40% at the therapeutic dose, while isolated compounds, PSP and β-glucan, decreased cell survival by 30% and 40%, respectively (Figure 1). Lentifom also decreased colon cancer cell survival, but this was only noteworthy at concentrations higher than the daily recommended dose.

There are various events responsible for the biological effects of different mushrooms and their selected components. Postemsky et al.[26] detected that *Grifola gargal* extract inhibits the mutagenic effect of standard promutagen DMBA in the fruit fly (*Drosophila melanogaster*). The extract's bioactive compounds modify detoxification *in vivo*, preventing the promutagen from metabolizing into active intermediates that can damage cellular macromolecules (including DNA). The study excluded analysis of the responsible compound(s).[26] Joseph et al.[18] isolated a polysaccharide–protein complex from *Phellinus rimosus* that prevents gamma radiation–induced oxidative stress. The mushroom also contains important immunomodulatory polysaccharides as well as a protein-bound polysaccharide that inhibits cell matrix-degrading enzyme production, tumor cell-induced platelet aggregation and cell mobility, and angiogenesis by modulating cytokine production, which explain its antimetastatic activity.[27,28] β-glucans from *Agaricus brasiliensis* (=*A. blazei*) showed anti-inflammatory, antiallergic, and antiasthmatic properties in mouse models.[29] Contrary to those findings, Goncalves et al.[30] proved that extracts from *A. brasiliensis* caused induction of $CD4^+$ T cells, natural killer T cells, phagocytes, monocytes, and proinflammatory cytokines, excreting both local and systemic inflammation in mice. One explanation given by the authors was that the proinflammatory action could be a consequence of applying of whole fruit body extract instead of concentrated selected components of *A. brasiliensis*, for which an anti-inflammatory effect was proven. Some higher Basidiomycetes glucans showed a direct antioxidant effect *in vitro*.[4] In *in vivo* conditions, β-glucans rapidly enter the small intestine, where they are partially degraded to smaller units and transferred to the bone marrow and endothelial reticular system. These units are released from macrophages and are consequently taken up by circulating granulocytes, monocytes, and dendritic cells, inducing humoral and cell-mediated immunity that eliminates tumor cells and pathogens.[4]

Smina et al.[31] isolated triterpenes from *Ganoderma lucidum* that cause its strong antioxidative activity with insignificant toxicity. Grifolin, a secondary metabolite from *Albatrellus confluens*, causes deprivation of kinases and G1 arrest.[32]

V. CONCLUSIONS

At the recommended doses, the most effective blended extracts were as follows: Super Polyporin was effective against colon cancer cells (Caco2), human lung adenocarcinoma cells (SKLU-1), small cell lung carcinoma cells (H69 V) and human astrocytoma cells (CCTG-1). Agarikon and Agarikon Plus were most effective against human lung adenocarcinoma cells (SKLU-1). Lentifom was effective against human lung adenocarcinoma cells (SKLU-1), small cell lung carcinoma cells (H69 V), and human astrocytoma cells (CCTG-1). Agarikon.1 and Mykoprotect.1 were effective against small cell lung carcinoma cells (H69 V).

In this work, it was determined that the blends of mushroom extracts consistently elicit greater cytotoxic effects than single species extracts. Antioxidant activity is not correlated with the polysaccharide content of the blends, but is strongly correlated with their TPC. The concentration of any single compound cannot predict the overall cytotoxic effect of the mushroom (or the mushroom blend); the ratio of all components as well as the tumor cell type determine the effectiveness of cytotoxicity of the mushroom extract blend.

Many companies worldwide produce dietary supplements from medicinal mushrooms. Most are made from a single mushroom species and usually

contain many compounds. Isolated, single compound supplements are rare. However, attempts to register mushroom preparations as dietary supplements in Western countries have been almost completely denied.[33]

Although cancer therapies (such as surgery, chemotherapy, and radiotherapy) can inhibit tumor growth and prolong patients' life span to some extent, they usually compromise the immune system (often already damaged by the cancer), induce free radical production, and damage genetic material of healthy cells. Supportive biotherapy, including the use of effective mushroom extracts, is gaining attention since it reduces side effects and helps overcome cancer growth.[4]

The only registered mushroom drugs come from the Far East; Western medicine remains indifferent, and thus medicinal mushrooms are underused. Medicinal mushroom research has discovered many promising compounds that are in need of follow-up (e.g., in clinical studies) and development into medical drugs. Contemporary pharmaceutical drugs are well-defined single compounds with completely defined physical and chemical properties and understood mechanisms of action (pharmacokinetics and pharmacodynamics). Mushroom extracts contain a mixture of active compounds that interact with intracellular signaling pathways, changing the course of inflammation, cell differentiation, survival, apoptosis, angiogenesis, and tumor progression.[15,16]

CONFLICTS OF INTEREST

To avoid any doubt in objectivity, the coauthors affiliated to Dr Myko San–Health from Mushrooms Company were not involved in the laboratory measurements and have not influenced the results in any way.

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