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Characterization of Cross-Flow Ultrafiltration Fractions from Maitake Medicinal Mushroom, *Grifola frondosa* (Agaricomycetes), Reveals Distinct Cytotoxicity in Tumor Cells

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ABSTRACT: β-glucans from Basidiomycetes like *Grifola frondosa* (the maitake mushroom) are well known for their health benefits. Polysaccharide preparations from medicinal mushrooms such as *G. frondosa* have been successfully tested in a vast number of studies. Many mushroom extracts have been developed and today are merchandized for use medicinally and commercially. Studies could show that, in particular, chemical structural features such as the molecular size of β-glucans significantly influence their bioactivity. Thus it is highly important to explore the composition and structural properties of β-glucans extracted from medicinal mushrooms and their effects on human tumor cell viability. Our study focuses on the molecular weight cutoff distribution of β-glucans in hot water–based extracts from maitake mushrooms. Cross-flow ultrafiltration was applied to obtain 5 fractions of different molecular size. β-glucan content was quantified using an enzyme-based test kit, specialized to 1,3-1,6-β-glucans. Here we show that only small amounts of β-glucans with a high molecular weight (>100 kDa) could be detected from an aqueous extract of *G. frondosa*. The main compounds encompass substances with a low molecular weight (<5 kDa), composing about 35% of the whole extract. In addition, tumor cell viability studies demonstrate significant cytotoxic potential in 2 different solid cancer cell types for the fraction with a high molecular weight (>100 kDa) and for 1 fraction with a low molecular weight (5–10 kDa). In summary, our experiments prove that cross-flow ultrafiltration serves as a quick and easy method for dividing crude aqueous mushroom extracts into different molecular-weight fractions that inhibit tumor cell viability *in vitro*.

KEY WORDS: medicinal mushrooms, A549, β-glucans, breast cancer, cytotoxicity, extract, *Grifola frondosa*, MCF-7, non-small-cell lung cancer, polysaccharides, tumor cell growth, ultrafiltration

ABBREVIATIONS: MWCO, molecular weight cutoff; **NSCLC**, non-small-cell lung cancer; **SEC**, size exclusion chromatography

I. INTRODUCTION

Medicinal mushrooms like maitake *Grifola frondosa* (Dick.: Fr.) S.F. Gray (Polyporaceae, Agaricomycetes) have a long history of culinary and medicinal use in Asia. For years they have been the focus of international medicinal research. In addition to a wide variety of health beneficial compounds such as phenolic substances, sterols, alkaloids, lactones, terpenes and ceramides, bioactive polysaccharides and polysaccharide-protein complexes are the most studied groups of functional compounds in medicinal mushrooms. Different glucans such as galactomannan and grifolan have been found in *G. frondosa.* These glucans show bioactive properties such as immunomodulating, antitumor, antiviral, and hepatoprotective effects.[1–5] Chemical structure plays an important role in their effectiveness: linkage, ratio of main to side chains, and solubility are important factors. β-glucans can be extracted from the fruiting body or cultured

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mycelia.[6–10] Several studies demonstrated positive results for β-glucans and their respective protein complexes in cancer treatment.[11] Natural immune responses become activated, thereby potentially supporting adjuvant chemotherapy and radiotherapy. Many studies have proven the positive effect of *G. frondosa*, and the increasing incidence of various cancers will require novel and optimized treatments in the future.[11] However, whether polysaccharides with a high molecular weight are particularly effective is still a matter of debate.[11] Recent studies demonstrated inhibition of tumor cell growth in the presence of natural mushroom extracts.[12–16] Moreover, mushroom extracts have been reported to be capable of inducing apoptosis via caspase activation in hepatocellular carcinoma and by inhibiting tumor-specific telomerase activity in breast cancer cells.[12,13] Despite these promising results, the pharmacological mechanism of action of extracts derived from *G. frondosa* still needs to be explored. To date, the positive effects of polysaccharides from *G. frondosa* have not only been used in medicine. For instance, various products containing β-glucans derived from *G. frondosa* as beneficial ingredients are commercialized as dietary supplements, functional foods, and nutraceuticals because of their disease-preventing ingredients.[17–19] However, to achieve pharmaceutical quality for such products, the most important aspects are identity, purity, and amounts of active constituents. Origin and mode of preparation are key factors for the final β-glucan content in the product being sold. In particular, the quantity of effective β-glucans contained in the final product needs to be determined.[9,20–22] Thus the main objectives of our study were to develop an effortless method for extracting water-soluble polysaccharides, to develop fast and effective steps for purification by ultrafiltration, and to establish a standardized method for the quantitative determination of β-glucans of maitake extracts.

Because there is no standard method for determining the quantitative content of β-glucans in mushroom extracts, the phenol–sulfuric acid method (the anthrone reaction method) is commonly used for polysaccharide determination.[23] However, because this method does not distinguish between size, shape, or linking of O-glycosidic bonds and displays only crude information about actual polysaccharide contents, a combination of alternative, more accurate analysis and purification methods is highly recommended to determine bioactive polysaccharides, including β-glucans. In our experiment we used an enzyme-based test kit manufactured by Megazyme Ltd. (Wicklow, Ireland). The previously described method is expected to deliver reliable results when determining β-glucan content.[24–28] In addition, our study addressed the putative cytotoxic effects of the *G. frondosa* fractions in a human breast cancer an in a non-small-cell lung cancer (NSCLC) cell line to explore further the putative mechanism of action of different extracts with respect to their molecular sizes. Previous studies showed β-glucans with a higher molecular weight cutoff (MWCO) to be more efficacious than those with lower ones, and therefore the focus on cell growth inhibition tests was particularly important.[11] Because breast cancer is the most common solid tumor entity among women worldwide, and because lung carcinoma, especially NSCLC, represents the most common tumor entity worldwide among men,[29] our study focuses specifically on the effect of *G. frondosa* fractions on these 2 tumor entities.

II. MATERIALS AND METHODS

A. Extraction

Dried *G. frondosa* 250 g, 3- to 4-cm slices produced in China and purchased from Biofungi GmbH (Hitzkirch, Switzerland) were extracted with 2500 mL of distilled water for 30 minutes at 100°C. This step was repeated twice with 2500 mL of fresh distilled water. Thus, an overall volume of 7500 mL of distilled water was used for the extraction. A yield of 3950 mL (the rest of the water was absorbed by the dried mushroom slices) was prefiltered (8- to 12-μm VWR filter paper, grade 424).

B. Cross-Flow Ultrafiltration

The prefiltered extract was ultrafiltrated with a Sartoflow Slice 200 benchtop crossflow

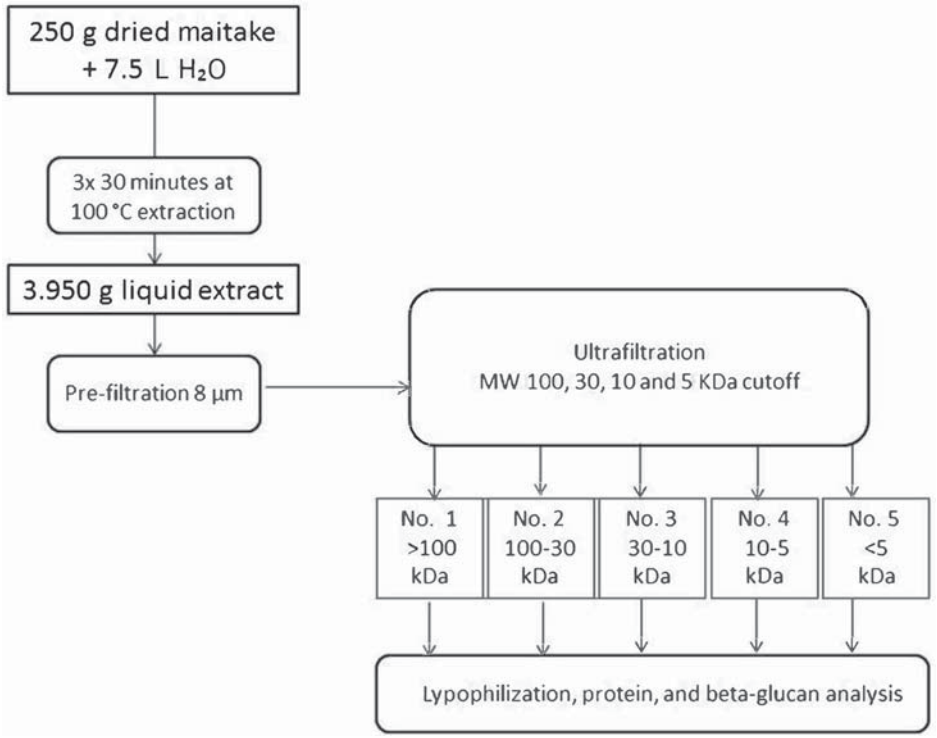

FIG. 1: Description of applied methodical steps of hot water extraction and ultrafiltration for obtaining 5 different fractions from a dried and lyophilized G*rifola frondosa* extract. Slices (250 g) of dried organic maitake mushroom were extracted with 7500 mL deionized water (3 × 2500 mL) at 100°C. A 3950-g hot water extract was obtained, prefiltered, and ultrafiltrated with 4 different membrane cutoffs (Sartorius Stedim Biotech, Germany).

system (Sartorius Stedim Biotech S.A., Goettingen, Germany). Four different membranes (Sartocon Slice 200 cassettes; Sartorius Stedim Biotech S.A.) with MWCOs of 100, 30, 10, and 5 kDa were used. The prefiltered *G. frondosa* extract was first filtrated with the largest slice (100 kDa). The permeate obtained after passing through the 100-kDa slice was subsequently subjected to the membranes with the MWCOs of 30, 10, and 5 kDa. The resulting retentates with >100, 30–100, 10–30, 5–10, and <5 kDa cutoffs were lyophilized and stored at −18°C (Fig. 1).

C. Detection of β-Glucan Content

1,3-1,6-β-glucans were determined using an assay kit (Megazyme Ltd., Wicklow, Ireland), which was used according to the manufacturer's instructions in quadruplicate. The dried extract samples (100 mg in quadruplicate) were weighted into culture tubes and 1.5 mL of concentrated hydrochloride acid (37%) was added. After heating at 30°C for 45 minutes, 10 mL of distilled water were added and the samples were incubated in a boiling water bath for 2 hours. After a neutralization step with 2 M potassium hydroxide, samples were adjusted to 100 mL with a sodium acetate buffer (pH 5.0). To measure total glucan content, 0.1-mL aliquots were mixed with exo-1,3-β-glucanase (20 U/mL) and β-glucosidase (4 U/mL) and incubated in a water bath at 40°C for 60 minutes. A glucose oxide/peroxidase reagent was added and again incubated at 40°C for 20 minutes.

To determine α-glucan content, dried extract samples (100 mg in quadruplicate) were stirred with 2 mL of potassium hydroxide in an ice water bath for 20 minutes. After adding 8 mL of sodium acetate buffer (pH 3.8) and 0.2 mL of amyloglucosidase (1630 U/mL), samples were incubated in a water bath at 40°C for 30 minutes. Aliquots (0.1 mL) were

mixed with 0.1 mL of sodium acetate buffer (pH 5.0) and 3 mL of the glucose oxide/peroxidase reagent and again incubated at 40°C for 20 minutes. For control purposes, β-glucan content of both a yeast standard and a mushroom powder (the internal control) was determined. All samples were measured at 510 nm in a photometer against a reagent blank. β-glucan content was determined by subtracting the α-glucan content from the total glucan content. Both steps measured not only total glucan/α-glucan content but also D-glucose in oligosaccharides, sucrose, and free D-glucose; subtracting α-glucan content from total glucan content provided the β-glucan values.[30]

D. Determination of Protein Concentration

The protein concentration in each fraction was determined according to the Bradford method using a protein assay standard kit (bovine gamma globulin) and Quick Start dye reagent (Bio-Rad, Hercules, CA).[31] Samples were tested in triplicate.

E. Size Exclusion Chromatography

Size exclusion chromatography (SEC) was used to determine the molecular size of the fractions and to confirm the success of the ultrafiltration process. A snap-glass column (90 × 1 cm; Essential Life Solutions) filled with Sephacryl S-200-Gel (GE Healthcare Bioscience AB, Uppsala, Sweden) was run with a phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. SEC was performed using a Smartline Manager 5000, Smartline Pump 1000, and RI Detector 2300 (Knauer, Berlin, Germany). Lyophilized fractions were diluted in a buffer (10 mg/mL) and 1 mL was injected into the column. Dextran standards (Sigma Aldrich, Germany) were used for calibration.

F. Cell Viability Assay

1. Cell Cultures

The human tumor cell lines A549 (lung adenocarcinoma) and MCF-7 (breast adenocarcinoma) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). A549 cells were cultivated in Dulbecco's modified Eagle medium (Gibco, Germany) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. MCF-7 cells were cultivated in RPMI medium (Gibco, Germany) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin, nonessential amino acids, 1 mmol/L sodium pyruvate, and 10 µg/mL human insulin. All cell lines were maintained at 37°C in a 5% carbon dioxide atmosphere. Subconfluent cells were passaged 2 times/week.

2. Cell Viability Assay

To determine the toxicity of the extracts, cell viability was measured by quantitating ATP with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), according to the manufacturer's protocol. In short, cells were seeded at the desired concentration in white 384-well plates (5.55×10^4 cells/mL). After 24 hours, 2 µL of the test compounds were added, each concentration in quadruplicate. After the desired incubation time of 72 hours, the CellTiter-Glo reagent was added and the luminescence was measured with an Infinite m200 microplate reader (Tecan Group AG, Maennedorf, Switzerland).

3. Data Analysis

Data were obtained from 3 or more independent experiments performed in quadruplicate and represent means ± SEM. Statistical data analysis was performed with equal samples of a minimum of 9 biological values using 1-way analysis of variance followed by the Dunnett post hoc test in cases where groups were compared with a control. $P < 0.05$ was used to determine a statistically significant difference. Curve fitting and statistical analysis were performed using nonlinear regression with GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA).

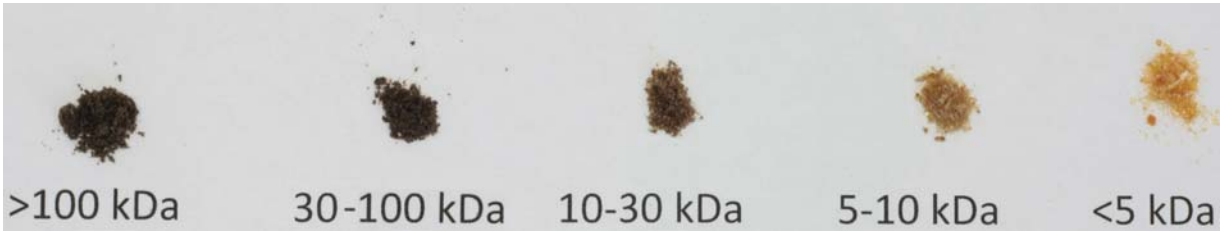

FIG. 2: Photographs of 5 powdered and lyophilized fractions obtained from *Grifola frondosa* extract with different molecular sizes: >100, 30–100, 10–30, 5–10, and <5 kDa.

TABLE 1: Relative content of total glucans, α-glucans, and β-glucans in all 5 ultrafiltration fractions from *Grifola frondosa*

MWCO of Fraction	Total Glucan Content (%)	α-Glucan Content (%)	β-Glucan Content (%)
>100 kDa	13.493 ± 0.984	4.039 ± 0.341	9.454 ± 0.918
30–100 kDa	9.131 ± 0.342	1.617 ± 0.126	7.514 ± 0.325
10–30 kDa	13.728 ± 2.81	1.131 ± 0.023	12.596 ± 2.81
5–10 kDa	20.171 ± 2.137	1.031 ± 0.032	19.141 ± 2.153
<5 kDa	35.694 ± 2.42	0.965 ± 0.103	34.728 ± 2.457

Data are presented as means ± SD from 4 experiments. Values were determined using a 1,3-1,6-β-glucan assay kit (Megazyme, Wicklow, Ireland). MWCO, molecular weight cutoff.

III. RESULTS AND DISCUSSION

A. Extraction and Ultrafiltration

In this study a hot water–based extract of *G. frondosa* was produced and divided into fractions with ultrafiltration membranes. Five fractions with a different MWCO were obtained. They differ in color and texture (Fig. 2); Table 1 shows the results. For optimal comparison, all results are calculated on the basis of 100 g of dried maitake mushroom.

The ultrafiltration process is a quick and easy method to filter crude mushroom extracts and fractionate them based on their MWCO, which has been described previously.[32–34] Because of the importance of the MWCO of β-glucans, which we have discussed, this application could be interesting for separation applications and further experiments, and for the production of extracts with β-glucans of a defined molecular size. Possible disadvantages could be the loss of filtrated material during the process. The cross-flow ultrafiltration system is the best solution in filtration systems, but loss of material cannot be completely avoided. The prefiltration step (using an 8- to 12-μm filter) is necessary to avoid blocking the membrane. It also should be mentioned that the fractions isolated by cross-flow ultrafiltration are defined by their cutoff values. It depends on other factors, for example, molecular conformation. Thus linear molecules with a high molecular weight are able to pass through ultrafiltration membranes, whereas globular molecules with a lower molecular weight are cut off because of their shape. As a consequence, the cutoff does not define an exact MWCO limit, but it can provide approximate limits of separation. Nevertheless, cross-flow ultrafiltration is an energy-saving, gentle, nonthermal method for fractionating and concentrating aqueous extracts.

B. β-Glucan Content

Figure 3 shows the relative amounts of β-glucans. Figure 4 shows the absolute amounts of β-glucan

content found in the 5 different fractions. From 100 g of dried maitake mushrooms, an absolute weight of 12.48 g of dried extract (lyophilized) could be obtained. The total weight of β-glucans in all fractions adds up to 3.24 g, which equals 26% β-glucan content from dried extract mass. This experiment shows that only small amounts of aqueous *G. frondosa* extract contain β-glucans with a high molecular weight. From 3.24 g of total β-glucans found in the extract, 120 mg of β-glucans have a MWCO of 100 kDa or higher. Only 9 mg of the β-glucans have a MWCO between 30 and 100 kDa, whereas 160 mg of the β-glucans are in the MWCO range between 10 and 30 kDa. Finally, 220 mg of β-glucans were in the fraction with an MWCO of 5–10 kDa. Thus most parts of the obtained extract contain small particles (<5 kDa). This fraction (dry weight, 7.64 g) represents the largest part of the extract (about 61%), including smaller molecules such as sugars, salts, smaller peptides, and glucans. (Sugars like D-glucose, sucrose, and oligosaccharides can be detected with the method described, and by subtracting α-glucan content from total glucan content, they are eliminated in the calculation step). Nevertheless, this fraction shows a larger amount of β-glucans (2.65 g) (Fig. 4). The enzymatic assay test for detecting 1,3-1,6-β-glucans in mushrooms is a complete method for the quantitative determination of specially linked β-glucans in yeast and fungi. All glucans are split into their glucose monomers and are measured photometrically. Standard errors approximately <5% are routinely achieved.[30]

C. Protein Content

Protein content was measured using the Bradford method. The fractions differ in protein concentration. It was shown that the largest fraction (>100 kDa) has the highest protein yield (0.45 g), and the protein content decreases with each fraction (Table 2). An overall yield of 1.08 g of protein could be detected by this method. The protein content, determined using the Bradford method, shows a difference in protein concentration among the 5 fractions (Table 2). The fraction of compounds >100 kDa contains the largest amount of protein. The protein content declines with the molecular weight of the different samples.

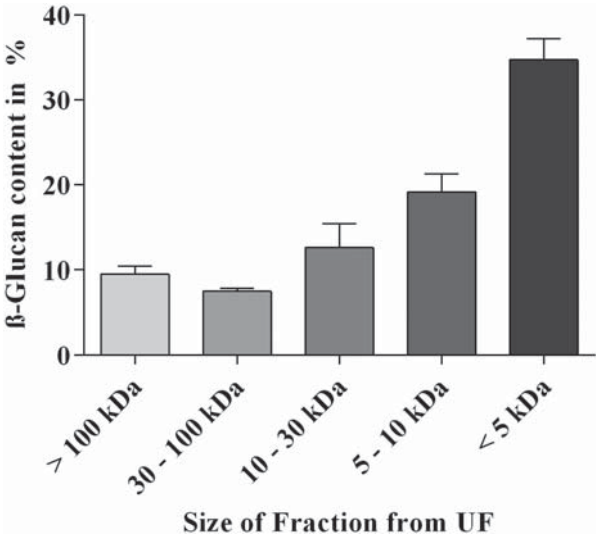

FIG. 3: Relative amounts (percentages) of β-glucan content present in the 5 different *Grifola frondosa* fractions obtained from the ultrafiltration (UF) process. All values are presented as means ± SD of 4 experiments.

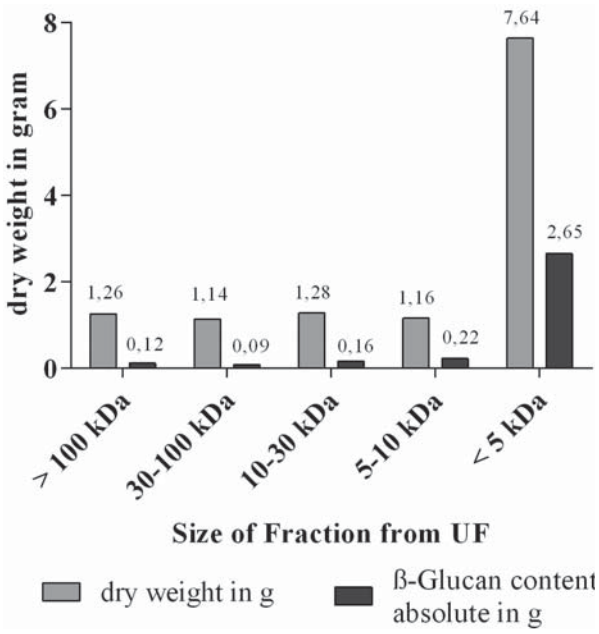

FIG. 4: Dry weights and absolute β-glucan content (grams) of 5 ultrafiltrated (UF) fractions obtained from *Grifola frondosa* (calculated for 100 g dried maitake slices).

TABLE 2: Absolute extract dry weight, absolute yield of β-glucan, absolute protein content, and description of the appearance of 5 *Grifola frondosa* fractions obtained from the ultrafiltration process

MWCO of Fraction	Dry Weight of Fraction (Grams)	Weight of β-Glucans* (Grams)	Protein Content† (Grams)	Appearance‡
>100 kDa	1.26	0.12 ± 0.918	0.45 ± 0.009	Compact fine powder with blackish-brown color, dull
30–100 kDa	1.14	0.09 ± 0.325	0.32 ± 0.008	Very fine and dusty powder with blackish-brown color, dull
10–30 kDa	1.28	0.16 ± 2.807	0.20 ± 0.029	Flaky powder with medium brown color, shiny
5–10 kDa	1.16	0.22 ± 2.153	0.08 ± 0.083	Coarsely flaked powder with amber color, shiny
<5 kDa	7.64	2.65 ± 2.457	0.041 ± 0.002	Very coarsely flaked and fluffy powder with light fawn color, very shiny
Total	12.48	3.24	1.08	

*Data are presented as the mean ± SD from 4 experiments.
†Data are presented as mean ± SD from three experiments.
‡Compare with Fig. 2.
MWCO, molecular weight cutoff.

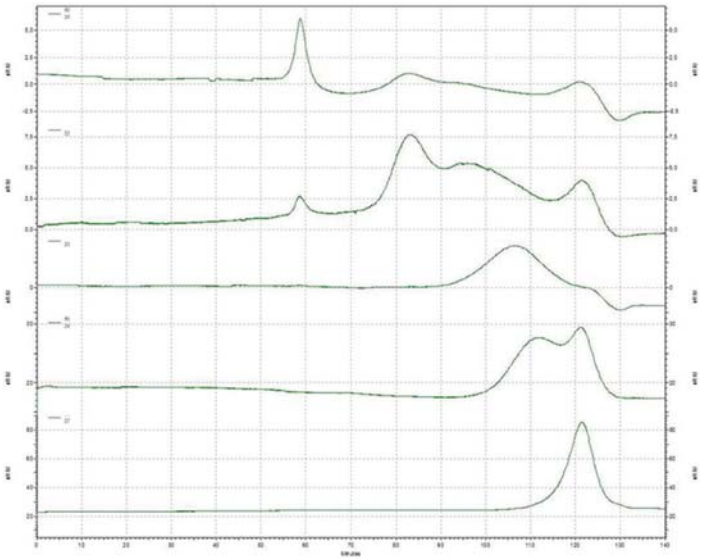

FIG. 5: Size exclusion chromatograms of 5 *Grifola frondosa* fractions obtained from the ultrafiltration process (from top to bottom: >100, 30–100, 10–30, 5–10, and <5 kDa) measured with a refractive index detector 2300 (Knauer, Germany) on a snap glass column (Essential Life Solutions) filled with Sephacryl S-200 (GE Healthcare Bioscience AB, Sweden).

D. Size Exclusion Chromatography

The SEC chromatograms of the 5 fractions show the successful separation of the crude *G. frondosa* extract by cross-flow ultrafiltration (Fig. 5). A rough separation is presented. The fractions may overlap at marginal areas, but the separation is clear to a great extent. Only fraction 1 (>100 kDa) presents

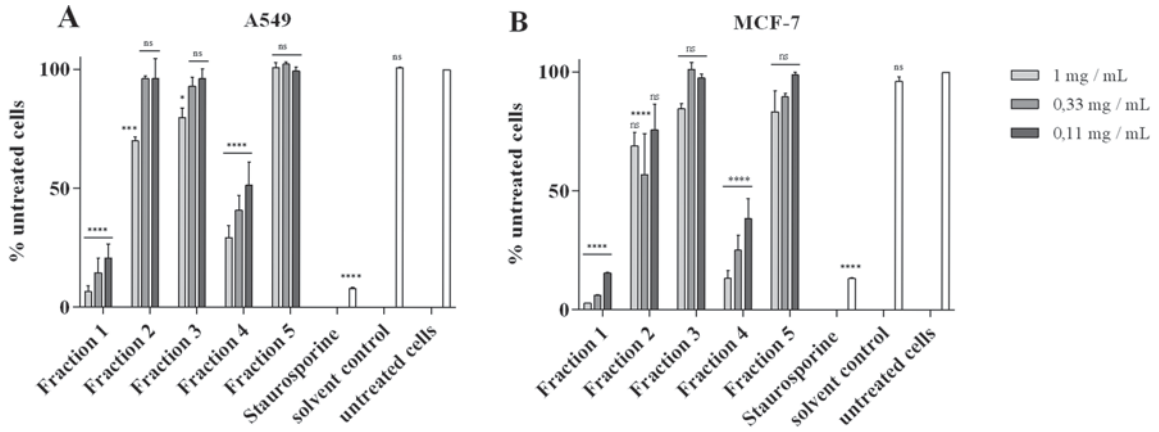

FIG. 6: The cell viability of A549 (A) and MCF-7 (B) cells after a 72-hour incubation with mushroom extracts was measured using CellTiter-Glo assay reagent (Promega). The data represent the mean values ± SEM of 3 independent experiments performed in 4 biological replicates. They were normalized against the untreated cell control. Staurosporine was used as a positive control (50 μmol/L). Curve fitting and data analysis using analysis of variance (ANOVA) were performed in GraphPad Prism software (version 6.05; GraphPad Software, San Diego, CA). Statistical analyses were performed using one-way ANOVA relative to the respective value of the untreated cell control. **P* < 0.05, ****P* < 0.001, *****P* < 0.0001. ns, not statistically significant.

a smaller fraction over a wide range. One explanation could be the degradation of molecules after ultrafiltration (due to storage conditions). Another explanation could be an unfinished ultrafiltration process. Filtration processes with longer durations could be a solution to improve separation.

E. Cell Viability Assay

Our experiment demonstrated pronounced cytotoxic effects for fraction 1 and fraction 4 in NSCLC cells (A549; Fig. 6A) and in breast cancer cells (MCF-7; Fig. 6B). Interestingly, the highest concentration of fraction 1 (1 mg/mL) reduces cell viability by approximately 90%, which is comparable to the effect of the well-described apoptosis inducer staurosporine in this assay format. Even at the lowest concentration of 0.11 mg/mL, fraction 1 demonstrates the ability to induce cytotoxicity in 75% of MCF-7 and A549 cells. Furthermore, fraction 2 at a concentration of 1 mg/mL reduced cell viability by approximately 30%, which was confirmed in the investigated cell lines. Moreover, it is noteworthy that fractions 1 and 4 were statistically significantly dependent on concentration for both cell lines, a finding that can be confirmed by full concentration response curves, as shown in Fig. 7.

IV. CONCLUSIONS

Higher Basidiomycetes have been used in traditional medicine since ancient times. Several products based on medicinal mushrooms have been recently developed. The importance of the production of extracts of medicinal mushrooms like *G. frondosa* has grown rapidly.[11,35,36] Unfortunately, extracts from mushrooms like the maitake are often not further tested because of the quantitative amounts of β-glucans. In conclusion, the results from this study clearly confirm the promising therapeutic potential of fractionated extracts from *G. frondosa*, especially the high-molecular-weight fraction. Cross-flow ultrafiltration and the enzymatic method for separation and quantitative determination of β-glucans were proven to be reliable technological and analytical methods.

Thus further research needs to be carried out to guarantee defined products. Mushroom raw materials and production processes will have to be standardized to retain high-quality products. Moreover, the question regarding structural

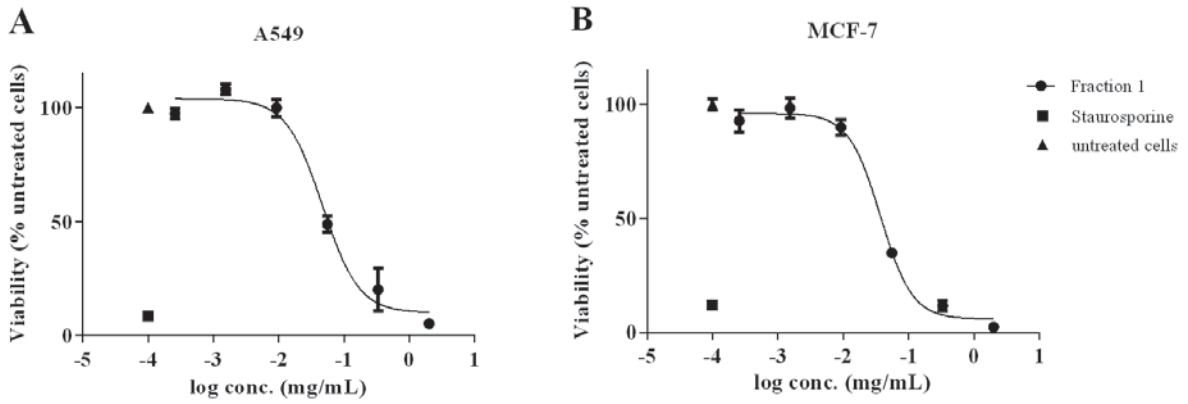

FIG. 7: Dose-response curve of fraction 1 after a 72-hour incubation on A549 (A) and MCF-7 (B) cells measured with CellTiter-Glo Assay (Promega). The data represent the mean values ± SD of 4 biologic replicates. They were normalized against the untreated cell control. Staurosporine was used as a positive control (50 µmol/L). Curve fitting and data analysis using analysis of variance (ANOVA) were performed in GraphPad Prism software (version 6.05; GraphPad Software, San Diego, CA). Statistical analyses were performed using one-way ANOVA relative to the respective value of the untreated cell control.

properties and characteristics in correlation to their therapeutic effectivity needs to be addressed. More clinical and preclinical trials need to be implemented to ensure the quality and effectiveness of the products.[11] Because natural crude extract fractions were used in our experiments, the identification of the active compound/ingredient remains elusive. Finally, future studies will have to address the underlying molecular mechanism of action resulting in the promising cytotoxicity data from the high- and low-molecular-weight fractions in cancers causing great medical need.

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