Determination of biological activity of *suillus granulatus* mushroom extracts

Monika Stojanova¹ · Milena Pantić¹ · Mitko Karadelev² · Vladimir Ivanovski³ · Miomir Nikšić¹

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Abstract

In the last decade, in addition to the study of the nutritional composition of mushrooms, the study of biologically active compounds occupies an important place. However, there is a need to find new and lesser known mushrooms species that have biological activity and potential for application in industrial conditions. The aim of this research is to determine the biological activity of aqueous and ethanolic extracts of *Suillus granulatus* wild mushroom through the determination of the content of total carbohydrates, total, α and β-glucans, as bioactive compounds, as well as determination of cytotoxic activity. Total carbohydrate content was determined using spectroscopic method, total, α and β-glucans were analysed using specific kits, and MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used for cytotoxic activity. IR-ATR (infrared spectroscopy - attenuated total reflection) spectra of mushroom extracts were performed, too. Aqueous extracts had a higher content of total carbohydrates as well as glucan and had better cytotoxic activity against HeLa cells, while ethanolic extract of *Suillus granulatus* was characterized with better cytotoxic activity against HepG2 cells. Based on IR-ATR, the presence of different types of carbohydrates, glucans, proteins, phenols and flavonoids can be observed in aqueous and ethanolic, which is one of the reasons for the differences in their anticancer activity. The analysed extracts are an excellent basis for their further application in various products in order to obtain functional food with enriched biological value. Thus, using natural dietary supplements can significantly affect the positive changes in the health of consumers.

Keywords Extract · Mushroom · Cytotoxic activity

Introduction

Mushrooms are a food rich in proteins and carbohydrates, they have a low fat content, but a very high content of unsaturated fatty acids. Mushrooms are known to be a good source of almost all of the essential amino acids and vitamins, especially vitamins B₁, B₂, B₅, C, and D [1, 2]. Among the minerals, magnesium, selenium, phosphorus, iron, zinc, calcium and others are represented.

Due to their excellent nutritional values, mushrooms are increasingly used in the daily diet, as a food or as a dietary supplement, and their medical efficiency and application arouse significant interest and increased contribution. They have a natural and exotic taste, as well as a pronounced aroma. They are used both fresh and processed [3, 4]. The content of nutrients in mushrooms depends on the origin of mycelium from the substrate, conditions and methods of cultivation. Ingredients with antioxidant, antimicrobial and cytotoxic activity have been found in most edible mushrooms [5]. Even that, mushrooms as well as their extracts also contain many bioactive compounds, which is why they are classified as functional foods [1, 6].

Edible mushrooms contain a source of compounds that “strengthen the host’s defenses” with their stimulating activity on the immune system. Various substances with immunostimulatory action have been isolated from the mycelium...
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The determination was performed according to the key by Horak (2005) in a pine forest (Mountain near the village Sretkovo at an altitude of 1100 m, *Pinus* (L.) Roussel, known as the “weeping bolete”), an edible mushroom collected from the territory of the Republic of North Macedonia (from Bistra Mountain near the village Sretkovo at an altitude of 1100 m, in a pine forest (*Pinus*), on a soil substrate). The determination was performed according to the key by Horak (2005) and from the fruiting bodies of many fungi, mainly lectins and terpenes [7]. These compounds stimulate different cell populations such as macrophages, NK cells (lymphocyte type), neutrophils or lymphocytes and initiate cytokine synthesis [8]. Thus, some polysaccharides or polysaccharide-protein complexes of fungi can stimulate a nonspecific immune system and exert antitumor activity by stimulating host defense mechanisms [9].

The mechanism of immunomodulatory action is different, depending on the molecular weight of polysaccharides extracted from different types of fungi. Low molecular polysaccharides can enter the cells and thus have an enhanced effect on the immune system. When those with higher molecular weights cannot enter the cell, they bind to certain receptors on the cell membrane and thus manifest a response [10].

Polysaccharides achieve their anticancer effect indirectly by activating various defense immune reactions. Immunomodulatory properties of higher fungal polysaccharides include mitogenic activity, stimulation of pluripotent stem cells in hematopoiesis, activation of an alternative complement pathway, and activation of immune system cells such as macrophages, T helper cells (Th cells), and cytotoxic T cells (Tc cells) and B cells [11].

**Suillus granulatus** (L.) Roussel, known as the “weeping bolete”, is an edible mushroom with a white, soon yellowish and non-staining flesh. It has a mild to slightly fragrant odour and tastes mild. Although this species is not one of the species most consumed as a delicacy, such as truffles or morels, it is widely harvested and consumed by the general population, particularly those who traditionally practice mushroom picking. This mushroom is characterized with a low fat content, and it is rich in plant fibers, carbohydrates and other compounds and because of that it is included in the category of functional foods [6].

Therefore, the aim of this research is to determine the in vitro cytotoxic activity of aqueous and ethanolic extract from *Suillus granulatus*, as well as determining the possibility of their use in the food industry for the production of functional food.

**Materials and methods**

**Collection, identification and drying of mushrooms**

In this research, as a work material was used *Suillus granulatus* (L.) Roussel, edible mushroom collected from the territory of the Republic of North Macedonia (from Bistra Mountain near the village Sretkovo at an altitude of 1100 m, in a pine forest (*Pinus*), on a soil substrate). The determination was performed according to the key by Horak (2005) [12]. The collected fresh mushrooms were chopped into thin slices. The mushroom pieces were dried in a chamber dryer with hot air at a temperature of 40 °C for 6 to 7 h, to a constant mass on a dry basis. Dried mushrooms were first ground to a fine powder and then extracted in two ways, with water and ethyl alcohol as extrainets.

**Preparation of aqueous extract**

Aqueous extract was prepared by Sławińska et al. (2013) [13] and Ribeiro et al. (2015) [14] method. The measured mass of dried and finely powdered mushroom sample (10 g) was poured with about 200 mL of distilled water, and after that was extracted on a boiling water bath (BIOBASE, TB-1, Shandong, China) for 1 h. The extract was strained through filter paper, then rinsed once more with boiling water and the sample was filtered again. The resulting supernatant was combined and evaporated on a vacuum evaporator (Resona Technics Labo Rota 300 type B, The Netherlands). The samples are then further dried in a stream of warm air (40 °C) to a constant mass.

**Preparation of ethanolic extract**

Ethanol extract was prepared by Vidović et al. (2011) [15] method. The measured mass of dried and finely powdered mushroom sample (10 g) was poured with 100 mL of 50% ethanol (Alkaloid, 1,006,278, N. Macedonia) and extract was covered for 40 min on the ultrasonic bath (VEVOR, 10 L 490 W Ultrasonic Cleaner JPS-40 A) at 45 °C. The sample was filtered through filter paper. The resulting supernatant extract was evaporated in a vacuum evaporator (Resona Technics Labo Rota 300 type B, The Netherlands) at 60 °C to constant mass. For each sample, the extraction procedure was done in triplicates.

**Determination of total carbohydrates content**

The content of total carbohydrates in the extracts was determined according to the modified method of Monsigny et al. (1988) in microplate [16]. A series of different concentrations of extracts was prepared, and the standard D-glucose was prepared, too, at a different concentration. 50 µL of extract was taken (50 µL of D-glucose was taken to create a standard curve), 15 µL of concentrated H2SO4 and 30 µL of 5% phenol (5 mL of 100% phenol in 100 mL of distilled water) were added. All trials were performed in triplicate. The absorbance was read on a spectrophotometer (JENWAY 6305, United Kingdom) at a wavelength of 490 nm. The content of total carbohydrates was calculated on the basis of the calibration curve (absorbance depending on the concentration) of the standard D-glucose solution.
Eq values glucose/g dry matter in the tested mushroom extracts were obtained according to the following formula:
mg eq. glucose/g d.m. = read conc. glucose (µg/mL)/working conc. x 1000.

**Determination of total, α and β-glucons**

The content of total glucan and α-glucan in aqueous and ethanolic extracts was determined using specific kits Mushroom and Yeast Beta-glucan Assay Procedure, K-YBGL 11, 2019 (Megazyme Co. Wicklow, Ireland). The β-glucan content was calculated as the difference between the total glucan content and the α-glucan content.

**IR-ATR spectra of mushroom extracts**

The spectra of the extracts were recorded on the FT-IR Perkin Elmer System 2000 using Specac Golden Gate ATR accessory. Diamond ATR single crystal was used for the purpose. ZnSe focusing lenses (a part of the accessory), restrained the measurements to 520 cm$^{-1}$ at the lower wavenumber side; the higher wavenumber side was at 4000 cm$^{-1}$. The spectra were recorded using 4 cm$^{-1}$ resolution and 64 scans (both for the background – the N$_2$/air, and the samples). The instrument was purged with 99.999% purity gaseous N$_2$ during the whole measurement process, to avoid the spectrarn contamination with the bands due to air H$_2$O and CO$_2$.

The recorded ATR spectra are presented as measured.

**In vitro determination of cytotoxic activity.**

Two malignant cell lines were used to test cytotoxic activity: HeLa$^{ATCC CCL−2}$ (ATCC, Poland) (cervical cancer) and HepG2$^{ATCC HB−8065}$ (ATCC, Poland) (hepatocellular carcinoma). Cells were grown at 37 °C, in a CO$_2$ incubator (Model 199, LabX, USA) (5% CO$_2$), in nutrient medium DMEM (Dulbecco’s Modified Eagle’s Medium) (HyClone Logan, USA). HeLa and HepG2 were seeded on 96-well microtiter plates (Greiner 655,101, Germany), and then 6 concentrations of tested extracts were added. Final concentrations ranged from 0.01 to 3 mg/mL. The analysis was made according to the method of Kajišarević et al. (2007) [17]. At the end of the incubation time (24 and 72 h) a solution of MTT (MTT Assay Kit, ABCAM, UK) at a concentration of 0.5 mg/mL was added, which was prepared in the medium immediately before the addition. 100 µl of solution was added to each sample, so that the final concentration was 0.05 mg. After treatment, the cells were incubated for 3 h at 37 °C, whereby the activity of mitochondrial dehydrogenase in viable cells transformed MTT into purple-blue formazan crystals. After incubation, the medium was drained and the reaction was stopped by the addition of 100 µl of 0.4 M HCl in isopropanol (Merck, Germany), whereby the formazan crystals are dissolved.

After 10 min at room temperature, the plates were shaken on a shaker then the absorbance was measured at 690 nm using a spectrophotometer (JENWAY 6305, United Kingdom).

Based on the obtained results, the percentage of cytotoxicity (CI - cell inhibition) was calculated by the formula: 

\[
CI(\%) = (1 - \frac{As}{Ac}) 
\]

where As is absorbance of treated cells (sample) and Ac is control absorbance (untreated cells).

The efficiency of inhibition of tumor cell proliferation was quantitatively expressed as the IC$_{50}$ value.

**Statistical analysis**

The obtained results were statistically processed using the software package SPSS 20. The Independent Sample T-test was used to determine statistically significant differences (p < 0.05) between the values.

**Results and discussion**

**Content of total carbohydrates, total, α and β-glucons**

The content of total carbohydrates in the analyzed extracts was higher in aqueous extracts compared to ethanolic extracts (Table 1), which was probably due to the fact that carbohydrates are generally water-soluble compounds [18]. Moreover, aqueous extract from *Suillus granulatus* was characterized with statistically significant (p <0.05) higher content of total, α and β-glucons compared to its ethanolic extracts.

| Mushroom extract | Total carbohydrates | Glucan | α-glucan | β-glucan |
|------------------|---------------------|--------|----------|----------|
| Suillus granulatus aqueous extract | 34.33 ± 8.12 | 18.55 ± 0.04 | 1.19 ± 0.04 | 17.36 ± 0.04 |
| Suillus granulatus ethanolic extract | 3.03 ± 0.03 | 20.62 ± 0.04 | 3.10 ± 0.07 | 17.52 ± 0.03 |

* Values of different extract of the same fungus, marked with different letters, have a statistically significant difference (p < 0.05)
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IR-ATR spectra of mushroom extracts

The spectra of aqueous and ethanolic extract of *Suillus granulatus* are presented in Fig. 1. Spectra can be clearly divided in several general wavenumber regions. The first region between 3500 and 3000 cm\(^{-1}\) is the one where –OH stretching vibrations from the glucane, phenols, water and protein side chains would appear. One can easily recognize spikes on the broad ν(OH) band at 3350 and 3250 cm\(^{-1}\) assigned to the ν(NH) A and B bands from the proteins, respectively (Fig. 1). The second region is the one between 3000 and 2800 cm\(^{-1}\), where stretching bands from the CH\(_2\) groups of the protein side chain and lipids appear. Maxima around 2930, 2886 and 2852 cm\(^{-1}\) speak on the behalf of these findings (Fig. 1). The third wavenumber region is between 1800 and 1200 cm\(^{-1}\). This region can be roughly subdivided in protein and sugar regions [24].

Polysaccharides are one of the most researched components of *Suillus*, *Trametes* and *Phellinus* species, which have shown a wide range of antimicrobial, antioxidant, anticancer, immunomodulatory and hepatoprotective effects [19].

Aqueous extraction is the only clinically proven method that allows the release of carbohydrates from chitin and their extraction. In this way, α and β-glucans are mostly concentrated, which are considered to be the main components when it comes to the medical activity of the fungus [20]. The β-glucans is thought to have the power to regulate the immune system, lower total cholesterol levels and LDL levels, and exhibit a number of other immunomodulatory effects [21].

Several studies have confirmed the activity of aqueous and ethanolic extracts from *Suillus granulatus* against different cancer cells, through different mechanisms: inhibition of cell growth by stopping their development cycle, stimulation of host immune response or by induction of apoptosis [22]. Some hydrosoluble compounds are thought to be responsible for these effects, such as β-D-glucans, β-D-glucans with heterosaccharide bonds of xylose, mannose, galactose, β-D-glucans with protein complexes (proteoglu- cans) and phenols, which indicate an immunomodulatory and therapeutic effect in humans and animals [22, 23].

**Fig. 1** IR-ATR spectra of the samples 1 (aqueous extract of *Suillus granulatus*) and 1’ (ethanolic extract of *Suillus granulatus*). The region between 2380 and 1850 cm\(^{-1}\) where diamond ATR band appear, was removed from the spectra. Figure 1 presents the main characteristics of the mid-IR spectra of the investigated mushrooms with the assignment of the bands.

**IR-ATR spectra of mushroom extracts**

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The protein region is comprised of Amide I band at ca. 1650 cm\(^{-1}\), Amide II band at 1550 cm\(^{-1}\) and Amide III band 1400–1200 cm\(^{-1}\). However, this protein region is further burdened by the δ(H\(_2\)O) band at ca. 1640 cm\(^{-1}\) [25], and bands from phenolic and flavonoid compounds. The strong band appearing between 1658 and 1619 cm\(^{-1}\) can be
Many studies show that proteins and polysaccharides are the major anticancer components in the extract from *P. djamor*, which show cytotoxic activity against HepG2 and MCF-7 cells, and polysaccharides from *P. gilvus* significantly inhibit melanoma growth [29, 30], while proteins isolated from *L. edodes* are the main anticancer components that inhibit leukemia cells [31].

Besides, it is known that the anticancer activity of polysaccharides has a significant correlation with their structure, molecular mass, solubility, monosaccharide composition and extraction method. Hydrophilic polysaccharides have also been shown to demonstrate higher immunomodulatory activity compared to insoluble ones. On the other hand, proteins show a strong cytotoxic effect against HeLa cells [32].

Also, the presence of double or triple helix, as a structural characteristic of biopolymers, or any structure, as well as the degree of degradation of sugar and non-sugar components (especially protein complexes or sulphur bonds), greatly affect the healing properties of fungi [2, 33].

**Cytotoxic activity of tested mushroom extracts**

According to the data presented in Figs. 2 and 3 can be seen that both tested extracts showed moderate anticancer activity at different tested concentrations. Namely, in all samples, with concentration increasing, the cytotoxic effect of the examined cell types increased proportionally. Thus, the samples showed the highest activity at the highest tested concentration of 3 mg/mL.

After 24 h of the treatment of HeLa cells can be noticed that aqueous extracts had better results (33.86 – 66.12%...
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In the case of HepG2 cells, after 72 h of incubation can be noticed that the ethanolic extract from *Suillus granulatus* showed a minimal reduction in the cytotoxic effect compared to the results after 24 h of incubation.

In accordance with the results for the percentage of cytotoxic activity are the IC$_{50}$ values of the analysed extracts (Table 2). IC$_{50}$ values showed a statistically significant difference (p < 0.05) between aqueous and ethanolic extracts. Moreover, in the aqueous extract there was statistically significant difference (p < 0.05) between the incubation time of both tested cells, while in the ethanolic extract statistically significant difference (p < 0.05) showed the IC$_{50}$ values from HeLa cells between 24 and 72 h.

Glucans, phenols, flavonoids and a many other compounds, such as alcohols, esters, aldehydes, coumarins, etc., are thought to contribute to the cytotoxic activity [34].

In the MTT test should also be borne in mind that the results are largely dependent on mitochondrial activity, and if their activity is impaired by other factors, it may be shown that the cells are not alive, which would give false positive results [29].

Santos et al. (2013) [35] found that aqueous and ethanolic extracts from *Suillus luteus* have an IC$_{50}$ > 400 µg/mL against tumor cells NCI-H460 and MFC-7, while methanol extract shows an IC$_{50}$ > 30.33 µg/mL and an IC$_{50}$ > 32.25 µg/mL, respectively. The values have been measured after 72 h of treatment, and the authors concluded that methanol extract had the best antimicrobial properties, but both aqueous and ethanol extracts showed good cytotoxic activity as a basis for their medical use.

### Table 2 IC$_{50}$ values for anticancer activity of tested extracts against HeLa and HepG2 cells

| Extract                  | n  | IC$_{50}$ (mg/mL) |
|--------------------------|----|-------------------|
|                          |    | HepG2 24 h | HepG2 72 h | HeLa 24 h | HeLa 72 h |
| *Suillus granulatus*     | 3  | 0.72±0.00 | 0.90±0.00 | 0.60±0.01 | 0.75±0.01 |
| aqueous extract          |    |          |          |          |          |
| *Suillus granulatus*     | 3  | 0.88±0.00 | 0.50±0.01 | 0.71±0.00 | 0.56±0.01 |
| solvent* extract         |    |          |          |          |          |

- IC$_{50}$ values of different extract of the same fungus, marked with different letters, have a statistically significant difference (p < 0.05)
- IC$_{50}$ values of the same extract of the fungus between the same tested cells on different incubation period, marked with different letters, have a statistically significant difference (p < 0.05)
- * - the extract was obtained using ethyl alcohol

Cytotoxic activity. A statistically significant difference (p < 0.05) was found between the anticancer activity of aqueous and ethanolic extracts.

Furthermore, after 24 h of the treatment of HepG2 cells can be seen that ethanolic extract from *Suillus granulatus* (36.94 – 66.67%) showed statistically significant (p < 0.05) better results compared to its aqueous extracts.

After 72 h of incubation can be observed that all tested extracts had better results compared to 24-hour incubation. Namely, in HeLa cells there was a proportional improvement of the obtained effects in all tested extracts. The effects from aqueous extract were statistically significant (p < 0.05) better than ethanol extracts (33.63 – 67.71% cytotoxic activity).

![Fig. 3 Cytotoxic activity of aqueous and ethanolic extracts against HepG2 cells after 24 and 72 h](image-url)
According to Tomasi et al. (2004) [36] methanol extract from Suillus granulatus showed strong anticancer activity (IC$_{50}$ 4.7 µg/mL) against L1210 cells and against 3LL (IC$_{50}$ 6.8 µg/mL), which are better values compared to methanol extract from Trametes versicolor (IC$_{50}$ > 100 µg/mL and IC$_{50}$ 79.5 µg/mL, respectively). Namely, according to the authors, the cytotoxic effect on the examined cells is reduced in the following order: Suillus granulatus > S. luteus > Strobilomyces strobilaceus > S. bovinus > Tylopilus felleus > Boletus edulis.

On the other hand, Ünyayar et al. (2006) [37] concluded that methanol extract from Trametes versicolor showed a 45% ability to inhibit HeLa cells at a concentration of 10 µg/mL. By reducing the extract concentration, the authors found a reduction of the effect up to 27% at a concentration of 1 µg/mL.

In their study, Knezević et al. (2018) [38] pointed out that the ethanolic extract from Trametes versicolor had an IC$_{50}$ value of 168.54 µg/mL against HeLa cells and an IC$_{50}$> 200 µg/mL against LS174, A549 and MRC5 cells. The authors concluded that fungi of this species showed significant medical potential.

According to research by Lau et al. (2004) [39] the aqueous extract from Trametes versicolor had an IC$_{50}$ value of 269.3 µg/mL against NB-4 cells and an IC$_{50}$ of 147.3 µg/mL against KL-60 cancer cells indicating high anticancer activity.

Samchai et al. (2009) [40] in their study found that methanolic extract from Phellinus linteus has an IC$_{50}$ value of 17.36 against MFC 7, i.e. 19.14 against NCI-H187 cells. In the case of ethanolic extract, the IC$_{50}$ value (µg/mL) was determined to be 27.26 and 40.15, respectively.

Veljovic et al. (2017) [41] in their study, among other parameters, determined the antiproliferative effect of ethanolic mushroom extract from Ganoderma lucidum against HeLa cells, and found that this extracts showed a cytotoxic effect at a concentration of 500 µg/mL, i.e. IC$_{50}$ values range from 223.1 to >500 µg/mL after 24 h and from 119.3 to 391.2 µg/mL after 48 h.

Aqueous and ethanolic extracts from G. lucidum have also been found to give significant results in preventing tumour proliferation in mice, which the presence of polysaccharides is thought to play a key role [42].

On the other hand, beside the cytotoxic activity, aqueous extracts of Suillus granulatus have high content of total phenols, and as a consequence good ability to capture DPPH radicals, ability to reduce iron ions and ability to chelate iron ions. Moreover, ethanolic extracts of these mushrooms have strong activity on the antioxidant test for reducing the presence of conjugated dienes, which is thought to be due to the higher content of flavonoids [43].

### Conclusions

According to the results can be concluded that, both of aqueous and ethanolic extract of Suillus granulatus showed good biological activity. Thus, this mushroom can be classifying as functional food, because it’s beneficial properties. Namely, aqueous extract had better anticancer activity against HeLa cells, while ethanolic extract of Suillus granulatus was characterized with better anticancer activity against HepG2 cells. Nevertheless, after 72 h of incubation, the examined extracts give better results, compared to the 24 h incubation.

Aqueous extract from Suillus granulatus were characterized with higher content of total, α and β-glucans compared to its ethanolic extracts. Aqueous extraction is the only clinically proven method that allows the release of carbohydrates from chitin and their extraction.

Based on IR-ATR, the presence of different types of carbohydrates, glucans, proteins, phenols and flavonoids can be observed in aqueous and ethanolic, which is one of the reasons for the differences in their cytotoxic activity.

Therefore the main conclusion from this research is that aqueous and ethanolic extracts of Suillus granulatus are suitable for use in the food industry as an excellent basis for the production of functional food.

### Author contributions
Monika Stojanova – mushroom collection, performing analysis and writing. Milena Pantic – conceptualization, final reviewing. Mitko Karadelev – mushroom collection. Vladimir Ivanovski – performing analysis. Miomir Niksic – conceptualization, final reviewing. All the authors discussed these results and contributed to the final manuscript.

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### Data Availability
All data analysed during this study are included in this published article.

### Declarations

**Conflict of interest** The authors declare that there is no conflict of interest.

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