https://doi.org/10.31925/farmacia.2019.1.24 **ORIGINAL ARTICLE**

INVESTIGATION OF BIOLOGICAL ACTIVITIES AND SECONDARY METABOLITES OF *HYDNUM REPANDUM* ACETONE EXTRACT

JOVANA TUBIĆ [1], DARKO GRUJIČIĆ [1], MARINA RADOVIĆ JAKOVLJEVIĆ [1], BRANISLAV RANKOVIĆ [1], MARIJANA KOSANIĆ [1], TATJANA STANOJKOVIĆ [2], ANDRIJA ĆIRIĆ [1], OLIVERA MILOŠEVIĆ-DJORDJEVIĆ [1,3*]

[1]*University of Kragujevac, Faculty of Science, 34000 Kragujevac, Serbia*
[2]*Institute of Oncology and Radiology of Serbia, 11000 Belgrade, Serbia*
[3]*University of Kragujevac, Faculty of Medical Sciences, 34000 Kragujevac, Serbia*

**corresponding author: olivera@kg.ac.rs*

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Abstract

This study aimed to evaluate the biological activities and polyphenolic contents of the acetone extract of *H. repandum* mushroom. Polyphenolic compounds were evaluated by high-performance liquid chromatography (HPLC). The antioxidant activity was assessed by radical scavenging activity assays and reducing power. The antimicrobial activity was established based on the values of the minimum inhibitory concentration (MIC) using microdilution method. Cytotoxic activity was determined by 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT). The genotoxic and antimutagenic activities were tested using cytokinesis block micronucleus (CBMN) assay on human peripheral blood lymphocytes *in vitro*. Among the determined polyphenolic compounds, ferulic acid and quercetin were mostly found. The extract showed high free radical scavenging activity, while the reducing power was less emphasized and concentration-dependent. The MIC fluctuated in a range of 0.009-10 mg/mL. The cytotoxic activity (based on IC_{50}) ranged from 116.5 to 158.33 µg/mL, when HeLa cells were the most sensitive. The highest tested concentrations of the extract showed significant genotoxic activity, while against mitomycin C, the extract caused protective activity. The results indicated that *H. repandum* acetone extract contained secondary metabolites which showed biological activities such as antioxidant, antimicrobial, cytotoxic, genotoxic and protective against chemotherapeutics, indicating that their inclusion in nutrition could be of great importance in the prevention and treatment of various pathological conditions.

Rezumat

Acest studiu a urmărit evaluarea activităților biologice și conținutul polifenolic al extractului acetonic din ciuperci din specia *H. repandum*. Compușii polifenolici au fost evaluați prin cromatografie de lichide de înaltă performanță (HPLC). Activitatea antioxidantă a fost evaluată prin testele specifice. Activitatea antimicrobiană a fost stabilită pe baza valorilor concentrației inhibitorii minime (MIC) utilizând metoda microdiluției. Activitatea citotoxică a fost determinată cu ajutorul bromurii de 3-4,5-dimetiltiazol-2-il-2,5-difeniltetrazoliu (MTT). Activitățile genotoxice și antimutagene au fost testate *in vitro* pe limfocite din sânge periferic. Printre compușii polifenolici determinați s-au regăsit predominant acid ferulic și quercetină. Extractul a demonstrat o activitate mai puternică de neutralizarea a radicalilor liberi. MIC a fluctuat în intervalul 0,009-10 mg/mL. Activitatea citotoxică (pe baza IC50) a variat de la 116,5 până la 158,33 µg/mL. Cele mai mari concentrații testate ale extractului au prezentat o activitate genotoxică semnificativă, în timp ce extractul de mitomicină C a prezentat o activitate protectoare. Rezultatele au arătat că extractul acetonic al *H. repandum* conține metaboliți secundari care au prezentat activități biologice antioxidante, antimicrobiene, citotoxice, genotoxice și protectoare împotriva chimioterapicelor, indicând faptul că includerea lor în nutriție ar putea avea o mare importanță în prevenirea și tratamentul diferitelor afecțiuni patologice.

Keywords: *Hydnum repandum*, antioxidant activity, antimicrobial activity, cytotoxic activity, genotoxic, antimutagenic activity

Introduction

Ever since the time of ancient civilizations, such as China, Japan, Europe, Mesoamerica and Africa, the medicinal properties of mushrooms have been used for the benefit of humans as food and medicine [1]. Moreover, in the recent years, the interest in the consumption of mushrooms has increased because of their nutritional composition. In addition to that, mushrooms are a significant source of natural biologically-active substances [2].

Mushrooms contain different secondary metabolites such as polyphenols, peptides, polysaccharides, vitamins, iron, zinc, sodium, minerals, terpenes, etc., with various biological activities [3]. Polyphenols are the widest group of bioactive substances, composed of phenolic acids, flavonoids, hydroxybenzoic acids, lignans, tannins, stilbenes and oxidized polyphenols, obtained from fruit bodies and the mycelium [4].

Hydnum repandum L. (popularly known as sweet tooth or wood hedgehog) is an edible and medicinal mushroom belonging to the Hydnaceae family [5]. Studies have confirmed that *H. repandum* possesses antioxidant [6] and antimicrobial [7] activities.

Repandiol isolated from *H. repandum* showed potent cytotoxic activity against various cancer cells [8].

Available literature contains very few data about the biological activities of this species. Thus, the aim of this study was to determine the polyphenolic compounds, the antioxidant, antimicrobial, cytotoxic, genotoxic and antimutagenic activities of the mushroom *H. repandum* acetone extract.

Materials and Methods

Collection and identification of the fungal sample

The fungal sample of *H. repandum* was collected in Kragujevac, Serbia, in September in 2015. The demonstration sample is preserved in the facilities of the Department of Biology and Ecology of Kragujevac, Faculty of Science. The identification of the mushroom was conducted using standard methods [5].

Preparation of the extract

Finely dry ground thalli of the examined mushrooms were extracted with acetone in a Soxhlet extractor. The extract was filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extract was stored at –18°C until it was used in the tests. The extract was dissolved in 5% dimethyl sulphoxide (DMSO) for the experiments. DMSO was dissolved in sterile distilled water to the desired concentration. The final concentration of DMSO solvent never exceeded 0.5% [9].

High performance liquid chromatography (HPLC)

Chromatographic analysis was performed using a Shimadzu HPLC system (Shimadzu, Duisburg, Germany), consisting of a G1312A binary pump, a 7725i manual injector and a G1379A degasser. Separations of the analyses were achieved on a Luna C18 reversed - phase column (250 mm × 4.6 mm I.D., 5 µm, Phenomenex) connected with an Analytical KJ0-4282 C18 guard cartridge system for HPLC (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile (A) and 0.1% formic acid aqueous solution (B) using the following gradient elution program for separation: 0–5 min, 5% (A); 5–30 min, 5–60% (A); 30–32 min, 60–90% (A); 32–35 min, 90% (A), 35–36 min, 90–5% (A); 36–40 min, 5% (A). The column temperature was maintained at 30°C, the flow rate was 1.0 mL/min and the injection volume was 20 µL. The developed HPLC-DAD method was subsequently applied for the analysis of polyphenolic compounds in a sample of mushroom.

Determining the antioxidant activity

DPPH radical scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazil) radical scavenging capacity of the extract was evaluated according to a method previously used by some other authors [10], but it was modified in its details. Two milliliters of methanolic solution of DPPH radical in the concentration of 0.05 mg/mL and 1 mL of the test sample (62.5, 125, 250, 500 and 1000 µg/mL) were placed in cuvettes. The mixture was shaken and allowed to stand at room temperature for 30 min. DPPH solution is initially violet in color which fades when antioxidants donate hydrogen. The change in color is monitored spectrophotometrically (Bibby Scientific Limited, Stone, UK) at 517 nm against methanol as blank. Ascorbic acid was used as a positive control. The DPPH radical concentration was calculated using the following equation: DPPH scavenging ability *(%) = [(A0 - A1) / A0] × 100*, where A0 is the absorbance of the negative control (2 mL of methanol solution of DPPH radical + 1 mL of 5% DMSO) and A1 is the absorbance of reaction mixture or standard. For both extract and ascorbic acid, the IC_{50} values (the concentration required for 50% inhibition of DPPH radical) were determined.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of the sample was detected according to the method of Nishimiki *et al*. [11] Briefly, 0.1 mL of test sample (62.5, 125, 250, 500 and 1000 µg/mL) was mixed with 1 mL nitroblue tetrazolium solution (156 µM in 0.1 M phosphate buffer, pH 7.4) and 1 mL nicotinamide adenine dinucleotide (NADH) solution (468 µM in 0.1 M phosphate buffer, pH 7.4). The reaction started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM in 0.1 M phosphate buffer, pH 7.4). The mixture was incubated at room temperature for 5 min, and the absorbance was measured spectrophotometrically at 560 nm (Jenway, UK) against the blank sample (phosphate buffer). Ascorbic acid was used as positive control. The inhibition percentage of superoxide anion generation was calculated using the following formula: Superoxide anion scavenging ability *(%) = [(A0 - A1) / A0] × 100*, where A0 is the absorbance of the negative control (consisting of all the reaction agents except the extract) and A1 is the absorbance of reaction mixture or standard. For both the extract and ascorbic acid, IC_{50} values were determined.

Reducing power

The reducing power of the samples was determined according to the method described by Oyaizu [12]. One milliliter of the test samples (62.5, 125, 250, 500 and 1000 µg/mL) was mixed with 2.5 mL of phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixtures were incubated at 50°C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged (HermLe, Wehingen, Germany). Finally, the upper layer was mixed with distilled water (2.5 mL) and ferric chloride (0.5 mL of 0.1%). The absorbance of the solution was measured spectrophotometrically at 700 nm (Bibby Scientific Limited, Stone, UK). The blank was prepared with all the reaction agents without the extract. Higher absorbance of the reaction mixture

indicated that the reducing power was increased. Ascorbic acid was used as a positive control.

Determining the antimicrobial activity

Microorganisms and media

The following bacteria were used as test organisms: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Bacillus cereus* (ATCC 10987), *Escherichia coli* (ATCC 25922) and *Proteus mirabilis* (ATCC 12453). The bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). The fungi used as test organisms were: *Aspergillus niger* (ATCC 16888), *Candida albicans (*ATCC 10259), *Penicillium italicum* (ATCC 10454), *Mucor mucedo* (ATCC 20094), *Trichoderma viride* (ATCC 13233). All the bacteria and fungi used were obtained from the American Type Culture Collection (ATCC). The fungal cultures were maintained on potato dextrose (PD) agar, except for *Candida albicans* which was maintained on Sabourad dextrose agar (Torlak, Belgrade). All of the cultures were stored at 4°C and subcultured every 15 days.

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37ºC on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/mL. The suspensions of fungal spores were prepared from fresh mature (3- to 7-day-old) cultures growing at 30ºC on a PD agar substrate. Spores were rinsed with sterile distilled water, used to determine the turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/mL according to the procedure recommended by NCCLS [13].

Minimum inhibitory concentration (MIC)

The MIC value was determined using 96-well micro-titer plates (Spektar, Čačak, Serbia) by the broth microdilution method [14]. A series of extract dilutions with concentrations ranging from 40 to 0.004 mg/mL were used in the experiment against every microorganism tested. The starting solution of test samples was obtained by measuring off a certain quantity of sample and dissolving it in DMSO. Two-fold dilutions of test sample were prepared in Müller-Hinton broth for bacterial cultures and broth for fungal cultures. The MIC was determined with resazurin. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. It is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The boundary dilution without any changing color of resazurin was defined as the MIC for the tested microorganism at the given concentration. Streptomycin (for bacteria) and ketoconazole (for fungi) were used as a positive control. The solvent control test was performed to study an effect of 5% DMSO on the growth of microorganisms. All experiments were performed in triplicate.

Determining the cytotoxic activity

Cancer cell lines

Human epithelial carcinoma HeLa cells, human lung carcinoma A549 cells and human colon carcinoma LS174 cells were obtained from ATCC (Manassas, VA, USA). All cancer cell lines were cultured as a monolayer in the RPMI 1640 nutrient medium, with 10% FBS (inactivated at 56ºC), 3 mM of L-glutamine and antibiotics, at 37ºC in humidified air atmosphere with 5% CO_2. IC_{50} values are expressed as the mean ± SD (standard deviation) determined from the results of 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT) assay in two independent experiments.

In vitro cytotoxic assay

In vitro cytotoxic assay of the investigated extract was performed when the cells reached 70–80% confluence. A stock solution (50 mg/mL) of the extract was dissolved in the corresponding medium to the required working concentrations. Neoplastic HeLa, A549 and LS174 cells (5000 cells *per* well) were seeded into 96 - well microtiter plates, and 24 h later, after cell adherence, 5 different, double diluted concentrations of investigated extract were added to the wells. Final concentrations of the extract were 12.5, 25, 50, 100, 200 µg/mL except for the control wells, where only nutrient medium was added. The cultures were incubated for the next 72 h. The activity on cancer cell survival was determined 72 h after the addition of the extract, by the MTT test [15]. Briefly, 20 µL of MTT solution (5 mg/mL PBS) was added to each well and incubated for a further 4 h at 37°C in 5% CO_2 and humidified air. Subsequently, 100 µL of 10% sodium dodecyl sulphate was added to solubilize the formazan crystals formed from MTT after the conversion by mitochondrial dehydrogenases of viable cells. Absorbances proportional to the number of viable cells were measured using a microplate reader (Multiskan EX, Thermo Scientific, Finland) at 570 nm. Each experiment was performed in triplicate and independently repeated at least four times.

Determining the genotoxic and antimutagenic activities

In vitro cytokinesis-block micronucleus (MN) assay

Peripheral blood lymphocytes (PBLs) of healthy donors were used in determining the *in vitro* genotoxic and antimutagenic activities of five different concentrations of mushroom extract (12.5, 25, 50, 100 and 200 µg/mL). The experiments were in accordance with the guidelines of the World Medical Assembly (Declaration of Helsinki) and informed consent was obtained from all donors.

Whole heparinized blood (0.5 mL) was cultured in 5 mL of PB-MAX Karyotyping (Gibco by Life Technologies, USA), the complete medium for lymphocyte cultivation. Cell cultures were incubated at 37ºC for 72 h. The extract in five different concentrations and small volume (0.1

mL), separately or in combination with known mutagen mitomycin C (MMC, final concentration of 0.5 µg/mL), were added to cell cultures 24 h after the beginning of incubation. After forty-four hours of incubation cytochalasin B (Sigma, St. Louis, MO, USA) was added to cultures, in the final concentration of 4 µg/mL. Further, the cells were centrifuged and treated with cold (4ºC) hypotonic solution (0.56% KCl) and fixed with methanol: glacial acetic acid = 3:1, three times. The slides were lamp dried and stained with 2% Giemsa (Alfapanon, Novi Sad, Serbia). The MN frequency was determined in 1000 binucleated (BN) cells from each donor (3000 BN cells *per* concentration). To calculate the nuclear division index (NDI), 500 cells from each donor were scored. NDI were calculated using the formula NDI = ([1 x M1] + [2 x M2] + [3 x M3] + [4 x M4]) / N, where M1–M4 represented the number of cells with 1 to 4 nuclei and N was the total number of the cells scored [16].

Statistical analysis

Statistical analysis was performed using the EXCEL (version 2010) and SPSS (version 20) software packages. The IC_{50} values were calculated from the dose curves by software (CalcuSyn). To determine the statistical significance of the antioxidant and genotoxic activities Student's *t*-test was used. The relationship between the tested concentrations of the extract and both MN and NDI was determined by Pearson correlation coefficient. All values are expressed as mean ± SD. In all cases $p < 0.05$ was considered statistically significant.

Results and Discussion

HPLC analysis

The content of the polyphenolic compounds in the acetone extract of *H. repandum* and retention times (tR) determined by HPLC analysis are shown in Table I.

Table I

The polyphenolic compounds of *Hydnum repandum* acetone extract

Polyphenolic compounds	tR (min)	*H. repandum* (µg/g)
Phenolic acids		
gallic acid	8.252	1.30 ± 0.10
p-coumaric acid	13.249	8.30 ± 0.70
chlorogenic acid	16.277	1.81 ± 0.03
caffeic acid	17.329	n.d.
syringic acid	17.656	2.15 ± 0.09
ferulic acid	20.601	12.10 ± 0.20
Flavonoids		
catechin	15.855	4.95 ± 0.06
rutin	18.471	3.38 ± 0.02
quercetin	24.901	22.50 ± 0.10

Five phenolic acids (gallic, *p*-coumaric, chlorogenic, syringic and ferulic acid) and three flavonoids (catechin, rutin and quercetin) were found in the extract. Caffeic acid was under the limit of detection in the extract.

The chromatograms for standards and acetone extract are represented in Figure 1 and 2.

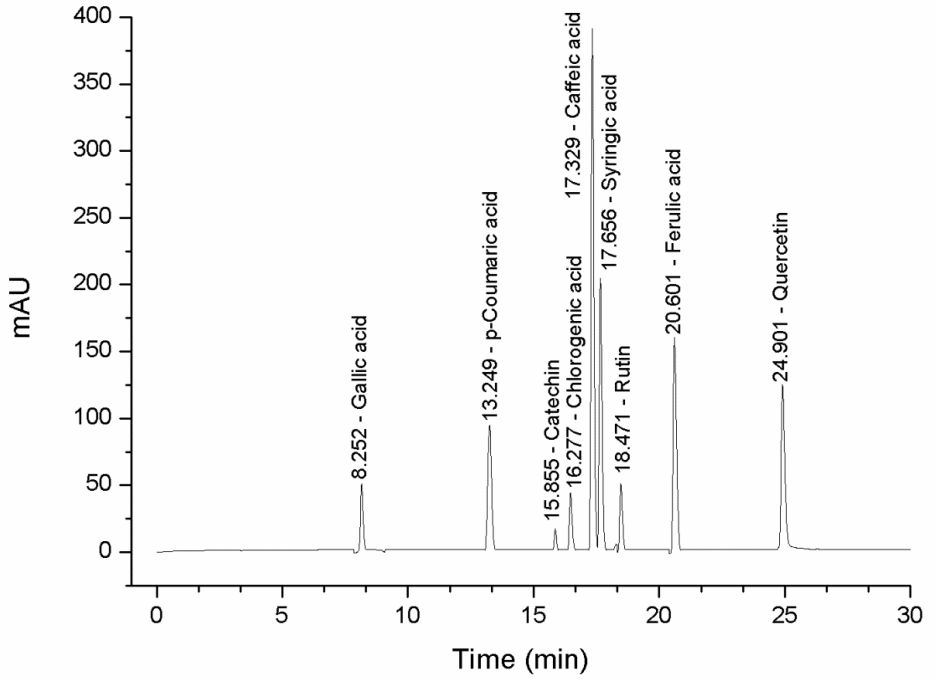

Figure 1.
HPLC chromatogram for mushroom standards of selected polyphenolic compounds

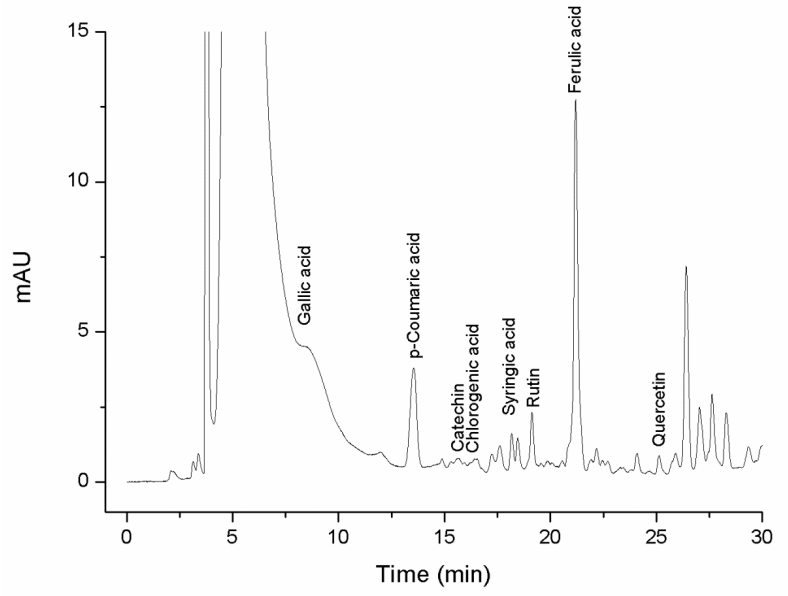

Figure 2.
HPLC chromatogram of the acetone extract of *Hydnum repandum*

Antioxidant activity

Free radical scavenging and reducing power of the studied extract are presented in Tables 2 and 3. The IC_{50} values for DPPH radicals and superoxide anion radicals scavenging activity were 35.41 and 19.28 µg/mL, respectively (Table 2). As shown in Table 3, the reducing power of the extract was concentration dependent. The measured values of absorbance for reducing power varied from 0.032 to 0.081. In various antioxidant activities there was a statistically significant difference between the extract and control samples ($p < 0.05$).

Table II

Free radical scavenging activity of acetone extract obtained from *Hydnum repandum*

Samples	DPPH radical scavenging IC_{50} (µg/mL)	Superoxide anion scavenging IC_{50} (µg/mL)
H. repandum	35.41 ± 1.05	19.28 ± 1.01
Positive control (ascorbic acid)	6.42 ± 0.18	115.61 ± 1.16

Table III

Reducing power of acetone extract obtained from *Hydnum repandum*

Samples	2000 µg/mL	1000 µg/mL	500 µg/mL
H. repandum	0.081 ± 0.031	0.044 ± 0.025	0.032 ± 0.008
Positive control (ascorbic acid)	3.862 ± 0.992	2.113 ± 0.032	1.654 ± 0.021

Antimicrobial activity

The antimicrobial activity of the tested extract against the test microorganisms is shown in Table 4. The extract from *H. repandum* acted on all the tested microorganisms. The most sensitive among the microorganisms was *B. cereus* (MIC = 0.009 mg/mL). The antimicrobial activity was compared with the standard antibiotics, streptomycin for bacteria or ketoconazole for fungi. The results showed that streptomycin had similar antibacterial activity, while ketoconazole was more active than the tested mushroom.

Table IV

Minimum inhibitory concentration (MIC) of acetone extract obtained from *Hydnum repandum*

Microorganisms	*H. repandum* (mg/mL)	S (mg/mL)	K (mg/mL)
Bacteria			
Bacillus cereus	0.019	0.031	-
Bacillus subtilis	0.009	0.016	-
Escherichia coli	0.039	0.016	-
Proteus mirabilis	0.078	0.062	-
Staphylococcus aureus	0.039	0.062	-

Microorganisms	*H. repandum* (mg/mL)	S (mg/mL)	K (mg/mL)
Fungi			
Aspergillus niger	10	-	0.078
Candida albicans	5	-	0.039
Mucor mucedo	10	-	0.156
Penicillium italicum	10	-	0.156
Trichoderma viride	5	-	0.078

Antibiotics: K – ketoconazole, S – streptomycin

Cytotoxic activity

The results obtained for the anticancer activity of the extract are shown in Table 5. The IC_{50} against HeLa, A549 and LS174 cancer cell lines was 116.42, 150.74 and 158.33 μg/mL, respectively. In all tested cell lines, the extract had weaker inhibiting activity than cis-dichlorodiammineplatinum (cis-DDP), as a positive control.

Table V

Growth inhibitory effect of acetone extract obtained from *Hydnum repandum* on HeLa, A549, LS174 and MRC5 cell survival

Cell lines	*H. repandum* extract (mg/mL)	IC_{50} (μg/mL)
	Cis-DDP	
HeLa	116.42 ± 2.53	0.86 ± 0.33
A549	150.74 ± 1.64	4.91 ± 0.42
LS174	158.33 ± 0.81	3.18 ± 0.29
MRC5	> 200	13.21 ± 0.37

CIS-DDP = cis-dichlorodiammineplatinum

Genotoxic and antimutagenic activities

The activities of different concentrations of the mushroom extract on MN frequencies, NDI values and their distributions in both separate and against MMC treatments are shown in Table 6. The extract increased the MN frequency, but significantly only at higher concentrations ($p < 0.05$). Pearson correlation coefficient showed that the extract increased MN frequency in a dose dependent manner ($r = 0.837$, $p < 0.01$). NDI values decreased with the increase of extract concentration, but significantly only at the highest concentration, in comparison to untreated PBLs. In combined treatment, the extract of the MMC-increased MN frequencies slowly decreased, but significantly only at highest concentration. Correlations between the extract concentrations and MN frequencies was negative and significant ($r = -0.730$, $p < 0.001$). All tested concentrations of the extract decreased the MMC-induced NDI values dose dependently ($r = -0.522$, $p < 0.05$) and with statistical significance only at the highest tested concentration ($p < 0.05$). The analysis of MN distribution *per* 3000 BN cells *per* treatment revealed that in both separate and against MMC treatments the most BN cells were with 1 MN, while cells with 2 or more MN were considerably less present.

Over the last few decades, the biological activities of the various mushrooms have been investigated for their protective activity on human health. It is well known that solvents play an important role in the extraction of important bioactive substances. Acetone proved to be the most efficient solvent for the extraction of bioactive secondary metabolites [17]. Therefore, in this study acetone was chosen as a solvent for extracting the bioactive substances from *H. repandum*.

The chemical composition of the extract determines its biological activity which is reflected in stronger or weaker antioxidant, antimicrobial, genotoxic, cytotoxic or antiproliferative activity [18]. The content of polyphenolic compounds was positively correlated with the biological activities of mushroom extracts. However, the total phenolic acids and flavonoids contents are not as well as important for the exhibited activity, as the concentration of individual polyphenolic compounds [19].

We analyzed the presence of nine secondary metabolites. The main phenolic acid was ferulic, and beside that, the extract contained *p*-coumaric, chlorogenic, gallic and syringic acids. Quercetin was the major flavonoid, but both catechin and rutin were also present in high quantity too. Similarly, Sułkowska-Ziaja *et al*. [6] reported the presence of gallic and syringic acids in *H. repandum* mushroom.

It is known that ferulic acid can modulate molecular mechanisms involved in a multitude of biological activities. Previously published results showed that ferulic acid has cytotoxic activity against human prostate cancer cell lines PC-3 and LNCaP [20], and TT medullary thyroid cancer cell line [21].

Table VI

The micronuclei frequency (MN) and nuclear division index (NDI) in peripheral blood lymphocytes of healthy donors in the treatments with *Hydnum repandum* extract *in vitro*

Treatments	*H. repandum* Concentrations	Total BN cells analyzed	MN/1000BN cells (X ± SD)	BN with MN (%)	Distribution of MN				NDI
					1MN (%)	2MN (%)	3MN (%)	≥ 4MN (%)	
Negative control cells	0	3000	7.0 ± 1.73	20 (0.67)	19 (0.64)	1 (0.03)			1.58 ± 0.03
Positive control cells	0 + MMC	3000	64.67 ± 9.74	165 (5.50)	142 (4.73)	17 (0.56)	6 (0.20)		1.48 ± 0.04
Separate treatment	12.5 µg/ml	3000	10.33 ± 1.76	30 (1.00)	29 (0.96)	1 (0.03)			1.45 ± 0.07
	25 µg/ml	3000	10.33 ± 0.88	31 (1.03)	31 (1.03)				1.50 ± 0.02
	50 µg/ml	3000	12.33 ± 1.45	37 (1.23)	37 (1.23)				1.50 ± 0.04
	100 µg/ml	3000	15.00 ± 1.53*	44 (1.47)	43 (1.44)	1 (0.03)			1.52 ± 0.02
	200 µg/ml	3000	18.00 ± 1.53*	50 (1.67)	46 (1.54)	4 (0.13)			1.44 ± 0.03*
Combined treatment	12.5 µg/ml + MMC	3000	53.67 ± 4.33	136 (4.53)	120 (4.00)	10 (0.33)	4 (0.14)	2 (0.07)	1.37 ± 0.07
	25 µg/ml + MMC	3000	50.00 ± 5.29	134 (4.47)	121 (4.03)	11 (0.38)	1 (0.03)	1 (0.03)	1.35 ± 0.06
	50 µg/ml + MMC	3000	48.33 ± 3.84	138 (4.60)	131 (4.37)	7 (0.23)			1.38 ± 0.07
	100 µg/ml + MMC	3000	41.67 ± 6.89	121 (4.03)	117 (3.90)	4 (0.13)			1.35 ± 0.07
	200 µg/ml + MMC	3000	33.00 ± 4.73**	91 (3.03)	83 (2.67)	8 (0.27)			1.28 ± 0.04**

* $p < 0.05$ compared to untreated control cells; ** $p < 0.05$ compared to positive control cells; BN = binucleated, MN = micronuclei frequency, NDI = nuclear division index

Similarly, Jaganathan *et al*. [22], studying the effect of *p*-coumaric acid concluded that this acid has a good inhibitory activity against two colon cancer cells (HCT 15 and HT 29).

Srivastava *et al*. [23] came to the same conclusion, studying the inhibitory activities of flavonoids, especially quercetin against leukaemia and breast cancer.

Our results on the antioxidant activity against various oxidative systems *in vitro* indicate that the extract showed very strong free radical scavenging activity, while the reducing power was less emphasized. This activity of the extract was based on its ability to form the non-radical form of DPPH-H as well as on its ability to destroy the superoxide radical produced from the PMS/NADH reaction which is a weak oxidative agent but induces the formation of highly reactive free radicals.

The antioxidant activity of *H. repandum* extract was also examined by Sułkowska-Ziaja *et al*. [6], but these authors used different solvent agents. Different extraction solvents, according to their polarity, may extract various compounds involved in the antioxidant activity. This means that synergistic activities may occur between these constituents leading to the pronounced antioxidant activity of extract [24].

The intensity of the antimicrobial activity of *H. repandum* acetone extract in our study depended on the used concentration of the extract and the tested microorganisms. We observed slightly higher activity against Gram-positive than Gram-negative bacteria strains, while the tested mushroom extract, in the same concentrations, showed a stronger antibacterial than antifungal activity. These results are comparable with previous results regarding the antimicrobial and antifungal activity of mushrooms. For example, Kosanić *et al.* [9] proved that bacteria are more sensitive to herbal extracts compared to the fungi. The reason for different sensitivity between the fungi and bacteria can be found in different transparencies of the cell wall. Similar to our results, Ozen *et al.* [7] demonstrated that the highest antimicrobial activity of *H. repandum* methanolic extract was expressed against *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (W3110), followed by *Staphylococcus aureus* (ATCC 6535), *Bacillus cereus* (ATCC 7064) and only one fungal species of *Candida albicans* (ATCC 10231). The probable mechanisms of the antimicrobial action of the tested mushroom are the inhibition of cell wall synthesis, protein synthesis, or nucleid acid synthesis, but less effective compared to the classical antibiotics. *P*-coumaric and ferulic acids extracted from mushrooms exhibited antimicrobial activity against *Escherichia coli* and *Proteus mirabilis* [25].

The tested extract expressed moderate cytotoxic activity on the used cancer cells, among which HeLa were the most sensitive. There has been no available information about the cytotoxic potential of *H. repandum* species, but some researchers examined the cytotoxic potential of other mushrooms. For example, Kosanić *et al*. [9] showed that methanolic extracts from *Lactarius deliciosus* and *Macrolepiota procera* exhibited significant cytotoxic activities. Also, edible mushrooms, *Agaricus bisporus*, *Lentinula edodes* and *Pleurotus ostreatus,* extracted by different polar and non-polar solvents, inhibited the proliferation of human liver carcinoma (HepG2), the human colon epithelial carcinoma (HCT116) and the human cervical cancer cells (HeLa) [26].

Edible mushrooms are regarded as non-toxic and safe, but recent investigations reported that some of them cause DNA damages [27]. Numerous studies showed that the CBMN assay is a valid test for detection of genomic damage caused both *in vitro* [28, 29] and *in vivo* [30, 31].

Our results of CBMN assay showed that the extract was genotoxic in higher tested concentrations. In treatment against MMC all tested concentrations of the extract showed protective activity. Namely, the acetone extract lowered the MMC-induced MN frequencies and NDI values dose dependently, but significantly only in the highest tested concentration. The analysis of MN distribution showed that the percentage of cells with 1MN and 2 MN increased for about two and four times (0.64 *vs*. 1.54% and 0.03 *vs*. 0.13%) in the treatment with the highest tested concentration of the extract compared to the negative control (without treatment). In treatment with MMC the percentage of cells with 2 MN decreased for about two times after the highest tested concentration of the extract compared to the positive control (0.56 *vs*. 0.27%), while cells with 3 and 4 MN were only seen in the positive control (MMC alone) and in the treatment with lower concentrations of the extract (12.5 and 25µg/mL).

To our knowledge, there are no available results about the genotoxic activity of *H. repandum,* but some authors have investigated this activity on other mushrooms. Thus, it has been shown that the aqueous extract of *Lentinula edodes* has moderate genotoxic activity on the epidermal cells of human larynx carcinoma (HEp-2), and that the highest concentration of the extract shows antigenotoxic activity against methyl methanesulfonate [32]. Also, Knežević *et al.* [33] and Živković *et al*. [34] showed that the ethanolic extract of *Agaricus brasiliensis* had high antigenotoxic activity against H_2O_2-induced DNA damage in human PBLs applying

comet test. They concluded that the antigenotoxic activity of mushrooms could be attributed to their scavenging properties and antioxidant mechanisms.

Based on our results in the treatment with MMC, we can conclude that the extract did not accomplish the antimutagenic activity by inhibition of the effect of MMC, but that the cells which collected a large amount of genetic damage were transferred into natural processes of apoptosis. This is indicated by the reduction of both MN frequency and NDI values in all tested concentrations of the extract, especially in the highest. The proapoptotic effect of the extract can be attributed to the rich polyphenolic composition of mushroom, especially ferulic acid and quercetin. Recently, Srivastava *et al*. [23] came to the similar conclusions.

Conclusion

This study showed that the acetone extract from *H. repandum* showed different biological activities such as strong antioxidant activity after DPPH solution, antimicrobial activity depending on the concentration of the extract and tested microorganism, moderate cytotoxic activity on HeLa, A549 and LS174 cancer lines, among which HeLa cells were the most sensitive ones, genotoxic activity in cultured human healthy PBLs depending on the concentration of the extract, and protective activity against MMC. These activities are in correlation with the rich composition of polyphenols, especially ferulic acid and quercetin which were the most common in the extract. The extraction of polyphenolic compounds and use in a diet can be of great importance for the prevention and treatment of various pathological conditions.

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