

Supplementary data for the article:

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SUPPLEMENTARY MATERIAL

***Trametes versicolor* ethanol extract, a promising candidate for health promoting food supplement**

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Abstract

This study aimed to estimate antiradical, antioxidant (AO) and cytotoxic activities of the fungus *Trametes versicolor* ethanol fruiting body extract. The extract was found to effectively scavenge both $O_2^{\cdot-}$ and NO^{\cdot} (29.62 and 52.48 $\mu\text{g/mL}$, respectively). It also showed a good AO activity in the polarographic HPMC assay (950 $\mu\text{g/mL}$). *p*-Hydroxybenzoic acid may be one of the responsible compounds for the aforementioned activities. The same extract also exhibited a concentration-dependent cytotoxicity against MCF-7 and HepG2 tumour cell lines reaching IC_{50} values of 123.51 and 134.29 $\mu\text{g/mL}$ respectively with no any cytotoxic activity against normal MRC-5 cells. Gentisic, syringic and protocatechuic acids may be among the bioactive principles for the observed cytotoxicity. Taken all together, *T. versicolor* ethanol extract can be considered as a promising candidate for development of health promoting food supplement.

Keywords: *Trametes versicolor*, ethanol extract, antiradical activity, antioxidant activity, cytotoxic activity, hydroxybenzoic acids

3. Experimental

Chemicals

Herein the following reagents were used: dimethyl sulfoxide and ethanol (Baker Analyzed™ reagent, J.T. Baker, Netherlands); β-nicotinamide adenine dinucleotide, quercetin, pyrogallol, Folin-Ciocalteu phenol reagent and gallic acid were (Sigma Aldrich, Germany); hydrogen peroxide (Merck Darmstadt, Germany); sodium nitroprusside was (Reanal, Hungary); nitro-blue tetrazolium chloride (Alfa Aesar, Thermo Fisher Scientific, UK); phenazine methosulfate (Fluka). Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Media (MEM), RPMI-1640 medium and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Aldrich (Germany), while fetal bovine serum was obtained from Gibco, Thermo Fisher Scientific, USA.

Extract

Trametes versicolor basidiocarps were collected in January 2014 on the locality of Iriški Venac (Fruška Gora Mountain, Serbia). The ethanol extract was prepared as previously described (Janjušević et al. 2017).

Anti-O₂^{•-} radical activity

Anti-O₂^{•-} radical activity was determined according to the procedure of Nishikimi et al. (1972). The working extract concentrations ranged from 0.08 to 132.45 mg/mL. The reaction mixture contained 200 μL of NBT, 10 μL of the extract, 100 μL of NADH, 100 μL PMS and 1.1 mL of phosphate buffer (pH 8.3). Pirogallol (PG) used as a standard compound (1 mg/mL). The absorbance was measured at 560 nm after 5 min of incubation. The results are expressed as a mean value of three measurements (IC₅₀ ± SD, μg/mL).

Anti- NO[•] radical activity

Anti-NO[•] radical activity was estimated as previously described (Green et al. 1982). The working concentrations of the extract ranged from 2.16 to 138.00 μg/mL. The reaction mixture contained 500 μL of SNP, 30 μL of the extract and 500 μL of buffer (pH 7.4). After incubation period of

90 min at 25 °C under continuous light, 1 mL of Griess's reagent was added and absorbance was read at 546 nm.

Antioxidant activity

HydroxoPerhydroxoMercury(II) Complex (HPMC) assay – the assay based on decrease of anodic limiting current originating from HydroxoPerhydroxoMercury(II) Complex formation in alkaline solutions of hydrogen peroxide at potential of mercury dissolution, observed upon gradual addition of antioxidants – was performed according to Sužnjević et al. (2011). The polarographic current-potential (i-E) curves were recorded using the polarographic analyser PAR (Princeton Applied Research), model 174A, equipped with X-Y recorder (Houston Omnigraphic 2000), as previously described. A dropping mercury electrode (DME) with a programmed dropping time of 1 s as a working electrode, saturated calomel electrode (SCE) as a reference, and a Pt-foil as auxiliary electrode, were used in the electrolytic cell. All i-E curves were recorded from 0.1 V towards negative potentials with a scan rate of 10 mV/s. Diluted ethanol extract (1:9) were gradually added in aliquots of 20 µL into 20 mL of starting 5 mM hydrogen peroxide in Clark & Lubs (CL) buffer (pH 9.8). Dependence of decrease of anodic limiting current of HPMC on volume of gradually added extract was followed. The slope of the starting linear part of plot of that dependence is used as a measure of AO activity. That is expressed as percentage of peak height decrease per volume of the extract added (%/mL). In the case of a pure substance (*p*-hydroxybenzoic acid, PHBA), the same value is expressed as percentage of peak height decrease per amount of the substance screened (%/µmol).

Determination of Total Phenol Content

Folin-Ciocalteu method was used to determine the Total Phenol Content (TPhC) (Singleton and Orthofer, 1999). This method is based on spectrophotometric detection of phenols that form a coloured complex with a FC reagent. The absorbance was read at 760 nm (MultiscanGO, ThermoScientific, USA). For the preparation of a calibration curve, gallic acid (GA) was used in the range from 0.1 to 1000 µmol/L. The content of TPhC is expressed as mg eq. gallic acid (GAE)/g d.w. of the extract.

Determination of Total Flavonoid Content

Determination of Total Flavonoid Content (TFC) in the extract was determined spectrophotometrically (Chang et al. 2002). Standard solution of quercetin (QUE) was used for preparing a calibration curve. The result is expressed as the mean of three measurements (mg eq. QUE/g d.w. of the extract).

Cytotoxicity

Estrogen-dependent human breast adenocarcinoma (MCF-7 – HTB22), human hepatocellular carcinoma (HepG2 – HB-8065) and human lung fibroblasts (MRC-5 – CCL-171) cell lines were used for measuring cytotoxicity of *T. versicolor* ethanol fruiting body extract. MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), HepG2 cells were cultured in Minimum Essential Media (MEM), while MRC-5 cells were grown in RPMI-1640 medium. The media were supplemented with 10 % fetal bovine serum, L-glutamine and penicillin (streptomycin).

The cells were maintained in a humidified 5% CO₂ atmosphere at 37 °C (Series 8000 WJ CO₂ Incubator, Thermo Scientific, USA). Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The following extract concentrations were tested: 33.3, 100, 300 and 900 µg/mL. The final concentration of solvent (dimethyl sulfoxide, DMSO) never exceeded 0.5 %, i.e. a non-toxic concentration to the cells.

For MTT assay, 100 µL of MTT solution (5 mg/mL) was added to each well of a 96-well plate and incubated for 3 h. The supernatant was then removed, while the obtained formazan crystals were dissolved in 100 µL of 0.04 M HCl in izopropanol. Light absorption was measured after 10 min using spectrophotometer, at 540 nm, with a reference wavelength of 690 nm.

Statistical analysis

All measurements were performed in triplicate. The data were recorded as mean ± SD. IC₅₀ values were determined by the linear regression analysis of RSC (Microsoft Excel programme for Windows, v. 2007 and Origin 8) and analysed using STATISTICA (StatSoft, Inc. (2012), version 10.0, www.statsoft.com). One-way analysis of variance (ANOVA) was a method of choice. Finally, significant differences between means were determined by Tukey's post hoc test.

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