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COPRINELLUS DISSEMINATUS (PERS.) J.E. LANGE 1938: IN VITRO ANTIOXIDANT AND ANTIPROLIFERATIVE EFFECTS

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ABSTRACT: *Coprinellus disseminatus (Pers.) J.E. Lange 1938* is an edible saprotrophic fungal species with bioactive potential which is not extensively investigated, mostly due to its small size. Accordingly, the aim of this study was to determine chemical and biological properties of crude ethanol (CdEtOH) and water extracts (CdAq). The bioactivity screens included antioxidant and antiproliferative (human breast MCF-7 cancer cell-line; MTT and SRB assays) activity. In addition, CdAq and CdEtOH were primarily characterized by UV-VIS spectrophotometry, with an aim of determining its chemical composition (total phenol and flavonoid contents). The best antioxidant activities were obtained for hydroxyl radicals (OH') and superoxide anion radical (SOA) assays for both crude extracts (IC₅₀ =4.02 μ g/ml for CdAq and IC₅₀ =1.40 μ g/ml for CdEtOH) while total phenolic (TP) and total flavonoid (TF) content was higher in CdEtoH than in CdAq. Both crude extract after 72h in MTT and SRB assay, reaching IC₅₀ at 217.90 and 205.90 μ g/ml, respectively. Based on obtained results, the saprotrophic fungal species *Coprinellus disseminatus (Pers.) J.E. Lange 1938* could be considered as a good potential alternative source of nutraceuticals and biologically active compounds.

Key words: Coprinellus disseminatus, antioxidant activities, antiproliferative activities, total phenolic content, total flavonoid content

INTRODUCTION

Free radicals are classified as highly reactive molecules that have one or more unpaired electrons (Halliwell, 2006). Consequently, they can induce oxidative damage in biomolecules and play an important role in aging process as well as in many diseases, such as cardiovascular and inflammatory diseases, cancer, diabetes, impaired immune system etc. (Valko et al., 2007; Ferreira et al., 2009). Nowadays, we are witnesses of challenges that humanity meets trying to resolve growing incidence of various diseases for which modern medicine still does not have appropriate formulations via standard preparations. Since modern style of living continuously causes disturbance of homeostasis through stress, radiation or increased presence of xenobiotics in the environment (Kalogeropoulos et al., 2013), there is an essential need for enhancement of human antioxidant system in order to prevent oxidative cell damage. The best approach is to enhance the prevention, over curing, by increasing consumption of foods rich in natural antioxidants, such as vegetables, fruits or mushrooms (Barros et al., 2007).

Traditional medicinal use of fungi has been well-known in many Asian countries, such as China, Russia, Japan and Korea, while the western medicine has recently taken an interest in their valuable bioactive properties (Zaidman et al., 2005; Kalogeropoulos et al., 2013; Karaman et al., 2014). Since a growing interest in natural resources with bioactive potentials, including fungi, in the treatment and prevention of many diseases during the recent decades (Espín et al., 2000), edible mushrooms are also analyzed as therapeutic alternatives (Zaidman et al., 2005; Kalogeropoulos et al., 2013; Karaman et al., 2014). Mushrooms represent rich sources of nutraceuticals (Benzie and Strain 1999; Ribeiro et al., 2009) and have demonstrated to have a physiological benefit or provide protection against certain chronic diseases (Zhou et al., 2009). Nowadays, both entire fungal sporophores and extracts are used for cancer treatment (Beattie et al., 2011), while medicinal mushroom preparations together with isolated fractions and specific compounds used as adjuvants to surgery, are radiotherapy or chemotherapy (Lindequist et al., 2005). Numerous bioactive properties of mushrooms, such as antioxidant, antitumor (Wasser 2002; Zaidman, et al., 2005; Ferreira et al., 2010; Vaz et al. 2012), and antimicrobial (Barros et al., 2007; Stojković et al., 2013) are being discovered during the last two decades. Many high molecular compounds (Wasser, 2002; Lindequist et al., 2005; Moradali et al., 2007; Zhang et al., 2007) and low molecular compounds (Zaidman et al., 2005; Paterson, 2006; Barros et al., 2007; Ferreira et al., 2010; Stojković et al., 2013; Karaman et al., 2014) have been proven to possess antioxidant activities. These compounds have already been determined as the main bioactive components in some medicinal mushrooms (e.g. Ganoderma lucidum, Grifola frondosa, Coprinus comatus, Meripilus giganteus) which exhibit both antioxidant and antimicrobial activeties (Barros et al., 2007; Ferreira et al., 2010; Karaman et al., 2014). Therefore,

myco-pharmacological investigations of medicinal mushrooms still play an important role in the development of new biotech-products and bio-pharmaceuticals.

Coprinellus disseminatus (Pers.) J.E. Lange 1938 is a small fungal species known as "fairies bonnets" or "trooping crumble cap" which belongs to the phylum Basidiomycota and family Psathyrellaceae. Unlike other coprinoid species, its sporocarp does not deliquesce (dissolve into black ink) with maturity. It is an edible species that can be found growing in saprotrophic clusters near the bases of stumps, mostly in very high numbers. Only one recently published study reported that methanol extracts of this species possess antioxidant activities, while dichloromethane, methanol and aqueous extracts show antibacterial properties (AI-Fatimi et al., 2013). Data on antiproliferative activity of this species on human tumor cell lines, breast cancer cells, are very modest (Gu and Leonard, 2006).

Since the bioactivity of fungal species is mostly influenced by geographical origin (Puttaraju et al., 2006) or specificity of the single fungal strain, the aim of this study was to evaluate antioxidant and antiproliferative properties of both ethanol and water crude extracts of autochthonous lignicolous species *C. disseminatus* from the area of northern Serbia (Novi Sad).

MATERIAL AND METHODS

Chemicals

Folin-Ciocalteu (FC) reagent, anhydrous sodium carbonate, gallic acid (GA), aluminium trichloride hexahydrate, sodium acetate trihydrate, quercetin hydrate, 2,2diphenyl-1-picrylhydrazyl (DPPH), anhydrous iron(III) chloride, 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), 2-thiobarbituric acid. disodium hydrogen phosphate, thiazolyl blue tetrazolium bromide, phenazine methosulfate (PMS), β-nicotinamide adenine dinucleotide (NADH), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), sulforhodamine B (SRB) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle's Medium with

4.5% of glucose (DMEM) and fetal calf serum (FCS) were obtained from PAA Laboratories (Pasching, Austria).

Mushroom samples

The species C. disseminatus was collected from urban area (street) in Novi Sad during 2012. Identification of the species was based on classical determination based on determinative keys (Phillips, 1983) and a voucher specimen (12-00662) was deposited at the Herbarium BUNS (Faculty of Sciences Department of Biology and Ecology, University in Novi Sad, Serbia). The samples were frozen at -20 °C and freeze-dried during 48 h (Bio alpha, Martin Christ GmbH, Switzerland). Lyophilized samples were ground to a fine powder, and after that packed in plastic tubes and stored in a dark place at room temperature prior to analysis.

Extraction

The whole freeze dried and powdered sporocarps of C. disseminatus (10 g) were extracted with two solvents separately: ethanol (EtOH) and distilled water (Aq) for 24 h on a shaker (Thermofisher Scientific, USA) at 120 rpm at room temperature (25 °C). The extracts were filtered through Whatman No.4 filter paper and the solvents were removed by rotary evaporation in vacuum at 40 °C (Büchi, Switzerland). The obtained ethanolic (CdEtOH) and aqueous (CdAq) extracts were stored at -20 °C prior to analysis. Dry residues were redissolved in DMSO for determination of antioxidant and antiproliferative activities (5 mg/ml, w/v).

Total phenolic content

Total phenol content (TP) of CdEtOH and CdAq respectively was determined according to method by Singleton et al. (1999), adapted for a 96-well plate reader (Multiskan Ascent, Thermo Electron Corporation). Folin-Ciocalteu reagent (125 μ l, 0.1 M) was added to 25 μ l of diluted extracts. After 10 min, 100 μ l of 7.5% w/v sodium carbonate was added and reaction mixture was incubated for 2 h. Absorbance was read at 690 nm. TP was expressed as mg gallic acid equivalents (GAE)/g of dry

weight (d.w.). The experiments were performed in triplicate.

Total flavonoid content

Total flavonoid (TF) content of of CdEtOH and CdAq respectively was measured spectrophotometrically, in a 96-well plate reader, using a modified method by Chang et al. (2002). The relevant sample (30 μ l) was mixed with 90 μ l of methanol, 6 μ l of 0.75 M aluminium-trichloride, 6 μ l of 1 M sodium acetate and 170 μ l of distilled water. Absorbance was measured at 414 nm, after incubation of 30 min. The experiments were performed in triplicate, while results were expressed as mg quercetin equivalents (QE)/g of dry weight (d.w.).

DPPH' scavenging activity

DPPH' scavenging activity was evaluated according to Espin et al. (2000). The reaction mixture consisted of 10 μ l of sample, 60 μ l of DPPH' solution and 180 μ l of methanol. After incubation of 60 min (dark place, at room temperature), absorbance was measured at 540 nm. Each sample was tested at five double diluted concentrations (100-1600 μ g/ml) to obtain IC₅₀, and experiments were performed in triplicate.

Nitric oxide radical scavenging capacity - NO assay

Nitric oxide scavenging capacity was determined according to the procedure of (Green et al. 1982). The reaction mixtures in test tubes consisted of 30 µl extract, 500 µl SNP, and 500 µl of phosphate buffer, (pH=7.4.) The control contained equivalent volume of ethanol, while reagents were replaced with phosphate buffer in the correction. Test tubes were incubated at room temperature for 90 min, under light exposure. After incubation, 1 ml of Griess reagent (0.2% solution of NEDA and 2% solution of SA in 4% phosphoric acid in the ratio 1:1 (v:v)) was added equally to samples, corrections and control. Aliquots of 250 µl were transferred to the plate, and their absorbance was measured using plate reader at 540 nm. Samples were tested at five different concentrations (70-600

 μ g/ml) to obtain IC₅₀, and experiments were performed in triplicate.

Ferric reducing antioxidant power (FRAP)

FRAP assay was performed according to a modified procedure of Benzie and Strain (1999). The FRAP reagent consisted of 300 mM acetate buffer (pH=3.6), 10 mM TPTZ in 40 mM HCl and 20 mM FeCl₃, in the ratio 10:1:1 (v:v:v). The sample (10 μ l), 225 μ l of FRAP reagent and 22.5 μ l of distilled water were added in a 96-well plate. Absorbance was measured after 6 min at 620 nm. The results were ex-pressed as mg ascorbic acid equivalents (AAE)/g of dry weight (d.w.). Each analysis was performed three times.

Superoxide anion radical scavenging capacity – SOA assay

Superoxide anion radical scavenging capacity of extracts was determined by measuring their ability to neutralize superoxide anion radicals generated during aerobic reduction of nitro blue tetrazolium by NADH mediated by PMS (Nishikimi et al., 1972). 100 μ l of 677 μ M NADH, 100 μ l of 60 μ M PMS, 200 μ l of 144 μ M NBT and 1.1 ml of phosphate buffer (pH = 8.3) were mixed with 10 μ l of extract in the test tube.

The control contained ethanol instead of extract, and correction contained 10 μ l of extract and 1.5 ml of phosphate buffer. After 5 min, aliquots of 250 μ l were transferred to the well plate and their absorbance was measured at 540 nm using plate reader. Five different concentrations of each sample (0.4-6.6 μ g/ml) were tested to obtain IC₅₀, and experiments were performed in triplicate.

Hydroxyl radical scavenging capacity - OH assay

The content of OH radicals was determined from the degradation reaction of 2deoxy-D-ribose into fragments (Cheesman et al., 1988), while the malondialdehyde MDA reaction with 2-tiobarbituric acid TBA reagent was determined spectrophotometrically at 532 nm. Each reaction was performed using 10 μ L of fungal extract (1.9–38.38 μ g/ml) to obtain IC₅₀, and experiments were performed in triplicate.

Antiproliferative activity *Cells*

Estrogen dependent MCF-7 cells were grown in DMEM (PAA Laboratories) supplemented with 10% FCS. The cells were seeded in a 96-well microplate (5000 cells per well). After incubation of 24 h, the growth medium was replaced with 100 μ l of medium containing the examined samples (extracts) at four different concentrations (33.3 μ g/ml, 100.0 μ g/ml, 300.0 μ g/ml, and 900.0 μ g/ml). Untreated cells served as the control, while pure DMSO was used as a positive control. The effects of the extracts on the growth of MCF-7 cells were evaluated by standard colorimetric assays – MTT and SRB.

MTT Assay

After 24 h and 72 h respectively, the cell viability was determined by the proliferation MTT assay (Mosmann, 1983). This assay is based on the colour reaction of mitochondrial dehydrogenase in living cells with MTT reagent. After incubation, MTT reagent was added to each well (50 μ g/100 μ l/well; at 37 °C, in 5% CO₂ for 3 h). The crystals of produced formazan were dissolved in 100 µl acidified isopropanol (0.04 M HCl in isopropanol). Absorbance was measured at 540 nm and 690 nm on a 96 well plate reader (Multiskan Ascent, Thermo Electron Corporation, USA). These experiments were performed in triplicate. The results were expressed in IC₅₀ values (sample concentration that inhibited 50% of the net cell growth).

SRB Assay

This assay estimates cell number indirectly, by staining cellular protein with the protein-binding dye SRB (adapted procedure, by Skehan et al. (1990)). After incubation, the cells were fixed adding cold 50% TCA and incubated for 1 h at 4 °C. Wells were washed with deionized water and dried; SRB solution (0.4% in 1% acetic acid) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (pH 10.5), while absorbance was measured at 492 nm and 690 nm, using the microplate reader. DMSO was used as a positive control. The results were expressed in IC_{50} values (sample concentration that inhibited 50% of the net cell growth). These experiments were performed in triplicate.

The absorbance was calculated from the difference of two absorbances: $A=A_{540}$ - A_{690} for MTT assay and $A=A_{492}$ - A_{690} for SRB assay. Percentage of cytotoxicity was calculated as the ratio of treated group absorbance and the control group absorbance, multiplied by 100.

Experiments were performed twice in triplicate, and the obtained results were expressed as IC_{50} values (sample concentration that inhibited 50% of the net cell growth). IC_{50} values were calculated from the cytotoxicity (%) - extract concentration (µg/mL) plot using the Origin v. 6.0 graphing and data analysis software (1999).

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Statistica software system (STATISTICA, 2013). Significant differences between two groups were determined by Student's t-test.

Finally, Pearson correlation coefficients (r^2) were calculated for TP and TF, DPPH, FRAP and IC₅₀ values for antiproliferative activity.

RESULTS AND DISCUSSION

Antioxidant activity and total phenol and flavonoid contents

Radical scavenging capacity (RSC) and total phenolic and flavonoid content of analyzed extracts are presented in Table 1. According to the obtained results, both crude extracts possessed good RSC activity. Based on statistical analyses the water extract exhibited significantly higher activity than ethanol extract in NO[•] assay (thirteen times lower IC₂₅ value), while the ethanol extract showed better activity in SOA assay (IC₅₀ = 1.40 µg/ml).

Moreover, both analyzed extracts showed very good scavenging activity towards DPPH and OH radical although IC_{50} values did not differ significantly. In general, both analysed extracts showed higher activities in DPPH assay than methanol extract of *C. disseminatus* from Yemen with RSC 42.8% at 1000 µg/ml, (Al-Fatimi et al., 2013) as well as from the species *C. comatus* originated from Serbia (Stojković et al., 2013).

These differences between our research results and other published work on this topic, could be assigned to different geographical origin of the fungal species, content of secondary metabolites, different strains as well as different solvents used for extraction.

Table 1.

Antioxidant activity of C. disseminatus extracts and TP and TF content

CdEtOH	CdAq	
397.28 ± 64.17	250.37 ± 15.74	
$1.40 \pm 0.66^{**}$	24.84 ± 2.38 [*]	
$7.37 \pm 1.46^{*}$	$4.02 \pm 0.29^{*}$	
CdEtOH	CdAq	
	21.28 ± 6.08**	
9.74 +0.79	4.02±0.60**	
$59.94 \pm 0^{*}$	30.74 ± 0.36**	
$11.37 \pm 0^{*}$	$0.59 \pm 0.14^{**}$	
	397.28 ± 64.17 1.40 ± 0.66 7.37 ± 1.46 CdEtOH 272.30 ± 21.53 9.74 ± 0.79 59.94 ± 0	

^aResults are expressed as IC_{50} and IC_{25} (µg/ml). ^bFerric reducing antioxidant power (FRAP) is expressed as mg ascorbic acid equivalents/g extract dry weight (mg AAE/g d.w.).^cTotal phenol content (TP) was expressed as mg gallic acid equivalents/g extract dry weight (mg GAE /g d.w.) and total flavonoid content (TF) was expressed in mg quercetine equivalents /g extract dry weight (mg QE/g d.w.). ^{**} Values with different asterisk in superscript within a column are statistically different t-test p<0.001

Furthermore, ethanolic extract showed higher activity than water extract according to reducing power capacity (FRAP) assay which is in accordance with literature data (Vaz et al. 2011), which can be attributed to higher TP and TF content in ethanolic extract compared to water extract (Table 1) according to correlation analysis.

Taking into account both parameters (antioxidant activity and chemical composition), it may be assumed that phenol and flavonoid compounds are not the only components responsible for the observed activities.

Antiproliferative activity

The cytotoxic effect against MCF-7 human breast cancer cell line was evaluated by MTT and SRB assays (Table 2). Under the experimental conditions, both extracts in two different assays showed antiproliferative activity. The ethanol extract demonstrated significantly higher cytotoxic activity (lower IC₅₀) than water extract, except for SRB assay at 72 h.

Differences in IC_{50} values obtained for the same samples by the two assays can probably be attributed to the difference in sensitivity of targets they reflect, since

they measure distinct biological parameters in living cells. While SRB assay does not depend on enzymatic activity but on protein content of the cells, in MTT assay the results reflect the activity of mitochondrial dehydrogenase which is more sensitive parameter, and therefore in many cases with changes detectable at lower concentrations. However, the effects observed for each sample also strongly depend on the specific combination of compounds present in the sample, their interaction and action of their metabolites that induce specific and often unexpected cellular responses. The obtained results were not consistent with previously published data for water extract of Coprinellus sp. that reached IC₅₀ at 120 μ g/ml at 24 h (Gu and Leonard, 2006) because the analyzed extracts showed lower activity (IC₅₀ at 625.26 µg/ml).

In addition, correlation analyses between radical scavenging activity and antiproliferative activities based on IC_{50} values (Table 3) showed very strong correlation for MTT assay with NO, SOA, and FRAP assays at both incubation times, while no significant correlations were observed with DPPH and OH assays.

Table 2.

Cytotoxic activities of *C. disseminatus* extracts on MCF-7 (IC₅₀ µg/ml)

	MTT assay		SRB assay		
Extracts	24h	72h	24h	72h	
CDAq	>900**	718.07±37.36	625.26±26.80	211.01±25.07 [*]	
CDEtOH	249.47±11.53 [*]	217.90±24.79 [*]	511.37±6.46 [*]	205.90±35.98	

All values are represented as mean values and standard deviations obtained from three measurements (mean \pm SD; n=3). ^{***} Values with different asterisk in superscript within a column are statistically different (p<0.05), t-test p<0.001

Table 3.

Correlation (r ²) between ar	ntiproliferative and antioxidant assays and TF, TP content in crude ex	tracts
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IC 50 (µg/ml)	MTT assay		SRB assay	
	24h	72h	24h	72h
DPPH	0.55	0.54	0.6	0.16
FRAP	0.89**	0.87**	0.67*	0.00
NO	0.97***	0.92**	0.82*	0.00
OH	0.54	0.53	0.58	0.26
SOA	0.95***	0.90	0.63	0.00
TF content (mg AAE/100 g)	0.99	0.97***	0.82 [*]	0.00
TP content (mg GAE/100 g)	0.99***	0.95***	0.80 [*]	0.00

Correlations are significant at p<0.05, correlations are significant at p<0.01, correlations are significant at p<0.001

The highest correlation was obtained between antiproliferative effects with TF and TP content especially in MTT assay (Table 3), indicating that these compounds strongly influenced the expressed cytotoxic activities. By comparing two cytotoxic assays, MTT assay showed to be more suitable assay for determination of the antiproliferative activity of mushroom extracts.

CONCLUSIONS

Both crude extracts of C. disseminatus possessed high antiradical and antioxidant properties and antiproliferative activity against MCF-7 human breast cancer cell line. Very high correlation between cytotoxic effects and TP and TF content indicates a significant effect of phenolic compounds on this activity. Based on manifested bioactivities, the saprotrophic fungal species Coprinus disseminatus (Pers.) Gray 1821 could be considered as a good potential alternative source for nutraceuticals and biologically active compounds. Additionally, further biochemical studies regarding the chemical profile of bioactive compounds, as well as possible molecular mechanisms responsible for the demonstrated cytotoxic effects should be conducted in the future.

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Aleksandra Novaković et al., Coprinellus disseminatus (Pers.) J.E. Lange 1938: *in vitro* antioxidant and antiproliferative effects, Food and Feed Research, 43 (2), 93-101, 2016

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COPRINELLUS DISSEMINATUS (PERS.) J.E. LANGE 1938: *IN VITRO* АНТИОКСИДАТИВНИ И АНТИПРОЛИФЕРАТИВНИ ЕФЕКАТ

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Сажетак: *Coprinellus disseminatus* (Pers.) J.E. Lange 1938 је јестива сапротрофна врста гљиве, чији биопотенцијал није много истраживан, углавном због мале величине. Сходно томе, циљ овог истраживања био је да се утврди хемијски и биолошки потенцијал сирових етанолних CdEtOH и водених CdAq екстраката. Скрининг биоактивности обухватио је антиоксидативну и антипролиферативну активност (ћелијска линија хуманог аденокарцинома дојке, MCF-7; тестови MTT и SRB). Поред тога CdEtOH и CdAq хемијски су прелиминарно окарактерисани (садржај укупних фенола и флавоноида) помоћу UV-VIS спектрофотометрије. Највећа антирадикалска активност уочена је за OH and SOA радикал за оба сирова екстракта (IC₅₀ =4,02 µg/ml for CdAq and IC50 =1,40 µg/ml for CdEtOH), док је садржај укупних фенола (TP) и укупних флавоноида био већи код CdEtOH у односу на CdAq. Оба сирова екстракта су показала активност у односу на MCF 7 ћелијску линију, иако је CdEtOH показао бољи ефекат у односу на CdAq екстракт након 72h на MTT и SRB тесту постужући IC₅₀ 217,90 и 205,90 µg/ml. На основу испољене биоактивности сапротрофна врста *C. disseminatus* може се сматрати добрим ресурсом алтернативних извора нутрацеутика и биолошки активних једињења.

Кључне речи: Coprinellus disseminatus, антиоксидативна, антипролиферативна активност, укупни феноли, флавоноиди

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