Comparative evaluation of antimutagenic and antimitotic effects of *Morchella esculenta* extracts and protocatechuic acid

Dejan S. Stojković[a], Slobodan Davidović[b], Jelena Živković[c], Jasmina Glamočlija[a], Ana Ćirić[a], Isabel C.F.R. Ferreira[d,*], Marina Soković[a,*]

[a]*Institute for Biological Research "Siniša Stanković", Department of Plant Physiology, University of Belgrade, Bulevar Despota Stefana 142, 11000 Belgrade, Serbia.* dejanbio@yahoo.com; jasna@ibiss.bg.ac.rs; rancic@ibiss.bg.ac.rs; mris@ibiss.bg.ac.rs

[b]*Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, 11010 Belgrade, Serbia.* davidcigla@yahoo.com

[c]*Institute for Medicinal Plant Research "Dr. Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia.* jelenazivkovic1@yahoo.com

[d]*Mountain Research Centre, School of Agriculture, Polytechnic Institute of Bragança, Campus de Santa Apolónia, apartado 1172, 5301-855 Bragança, Portugal.* iferreira@ipb.pt

* Authors to whom correspondence should be addressed (Isabel C.F.R. Ferreira; e-mail: iferreira@ipb.pt; telephone +351-273-303219; fax +351-273-325405 and Marina D. Soković; e-mail: mris@ibiss.bg.ac.rs; telephone +381-11-2078419; fax +381-11-2761433).

Abstract

Morchella esculenta (L.) Pers. methanolic extracts, obtained from fruiting bodies growing wild in Serbia and Portugal, were screened for their antimutagenic properties and compared to protocatechuic acid, previously identified in both species. *Salmonella typhimurium* TA100 reversion assay was used for the antimutagenic properties. Methanolic extracts expressed important antimutagenic potency towards *S. typhimurium*, which was documented by index of antimutagenicity (I). Sample from Serbia expressed slightly higher antimutagenic properties with inhibition rate of 58.7%. Sample from Portugal gave inhibition rate of 51.7%. Protocatechuic acid had inhibition rate I of his^+ revertants of 72.4%. Cell viability in the presence of extracts was also documented. *M. esculenta* samples from Serbia and Portugal possessed novel biological potential for the studied species, as well as its phenolic compound – protocatechuic acid, identified in both samples. Genotoxic effect, regarding mitotic index and chromosomal aberration score, was also assessed by using *Allium cepa* L. assay. Protocatechuic acid showed the most significant decrease in mitotic index, as well as decrease in chromosomal aberration score.

Keywords: *Allium cepa* assay, Antimitotic, Antimutagenic, Ames test, *Morchella esculenta*, Protocatechuic acid.

1. Introduction

The contemporary view of cancer is that malignant tumor arises and progresses through the accumulation of successive mutations, which involve activation of proto-oncogenes and inactivation of tumor suppressor genes, leading to uncontrolled proliferation of the progeny cells (Ajith and Janardhanan, 2011). The accumulation of mutations relates to the development of most cancers and various degenerative disorders, as well as aging and genetic defects in offspring (Migliore and Coppede, 2002). Mutations are important early steps in carcinogenesis; therefore, a short term genetic test, such as the Salmonella/reversion assay, has been successfully used for the detection of mutagens/carcinogens, as well as of antimutagens/anticarcinogens (Rhouma et al., 2012). *Allium cepa* root chromosomal aberration assay has been frequently used to determine the cytotoxic, mutagenic and genotoxic effects of several substances (Fernandes et al., 2007). Identification of antigenotoxic factors is expected to lead to the development of cancer preventing agents (Yoneda et al., 2012).

Edible mushrooms are widely appreciated throughout the world for their nutritional properties (Kalač, 2009), but also for their pharmacological value (Ferreira et al., 2009 and 2010; Alves et al., 2012). *Morchella esculenta* (L.) Pers. (morel) is one of the most widely appreciated wild edible mushrooms. Since commercial cultivation of this mushroom has not been successful till now, its cultured mycelium is extensively used as a flavoring agent. Recently, it has been proven that morel possess anti-inflammatory, antitumor, antioxidant and antimicrobial activities, as well as demelanizing properties on microfungi (Mau et al., 2004; Nitha et al., 2007 and 2013; Nitha and Janardhanan, 2008; Alves et al., 2012; Heleno et al., 2013).

Phenolic compounds are reported to have multiple biological effects, including antioxidant activity, antitumor, antimutagenic and antibacterial properties (Halliwell, 2011 and 2012). Over

the past two decades, researchers, as well as food manufacturers and consumers, have increasingly been attracted by phenolic compounds due to their antioxidant properties, and also due to their role in the prevention of various diseases, such as cancer and cardiovascular diseases (Carocho and Ferreira, 2013).

The present study was designed to evaluate antimutagenic properties of *M. esculenta* extracts obtained from two different countries, Portugal and Serbia. As previously reported by our research group, protocatechuic acid was identified in both *M. esculenta* samples (Heleno et al., 2013), so it was chosen for further elucidation of its antimutagenic properties towards *Salmonella typhimurium* TA 100. Genotoxic effect, regarding mitotic index and chromosomal aberration score, was also assessed by using *Allium cepa* assay.

2. Material and methods

2.1. Samples

Specimens of *Morchella esculenta* (L.) Pers. were collected in Bragança (Northeast Portugal) and Jabučki rid (Northern Serbia) during November of 2011 and April 2012, respectively. The authentications were done by Dr. Anabela Martins (Polytechnic Institute of Bragança) and Dr. Jasmina Glamočlija (Institute for Biological Research, Belgrade). Voucher specimens were deposited at herbarium of School of Agriculture of Polytechnic Institute of Bragança, Portugal, and at Fungal Collection Unit of the Mycological Laboratory, Department for Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia, respectively.

The specimens were immediately lyophilized (FreeZone 4.5, Labconco, Kansas, USA and LH Leybold, Lyovac GT2, Frenkendorf, Switzerland, respectively), reduced to a fine powder (20

mesh), mixed to obtain an homogenate sample and kept at -20 ºC until further analysis. Protocatechuic acid (**Figure 1**) was purchased from Extrasynthese (Genay, France).

2.2. Samples preparation

Mushroom samples (~5 g) were extracted by stirring with 150 mL of methanol (25ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland) to dryness. The extracts and protocatechuic acid were redissolved in water containing Tween 80 (0.02%) for further assays.

2.3. Evaluation of antimutagenic properties

A variation of the Ames test was used (Abdillahi et al., 2012) to screen for antimutagenic activity of *M. esculenta* methanolic extracts and protocatechuic acid. For this activity, *Salmonella typhimurium* TA100 without S9 metabolic activation was used. It is also known that this strain is capable of identifying up to 90% of the mutagens (Mortelmans and Zeiger, 2000). Briefly, 0.1 M phosphate buffer (500 µl) was added to the sample (50 µl – 200 µg/ml, 500 µg/ml and 1000 µg/ml of extracts and 10 µg/ml, 20 µg/ml and 50 µg/ml of protocatechuic acid) in a test-tube. 4-Nitroquinoline-N-oxide (4NQO) (50 µl, 20 µg/ml in DMSO) was added to the mixture and then pre-incubated for 3 min before the addition of overnight *S. typhimurium* TA100 culture (100 µl). After incubation for 48 h at 37 °C, the number of viable cells (cultured on Mueller–Hinton agar) and revertant colonies were determined (on minimal glucose agar), and the percentage of

inhibition (Index of antimutagenicity) was calculated using the following formula (Ong et al., 1986):

$$Percent\ of\ inhibition\ (\%) = \left(1 - \frac{T}{M}\right)\ x\ 100$$

where T is the number of revertants per plate in the presence of the mutagen 4NQO and extracts/compound, and M is the number of revertants per plate in the positive control (4NQO). The antimutagenicity was classified negative, weak, moderate or strong on the basis of the percentage inhibition: 25-40% inhibition was defined as moderate antimutagenicity; 40% or more inhibition as strong antimutagenicity; and up to 25% inhibition as no antimutagenicity (Abdillahi et al., 2012). The extracts and protocatechuic acid were tested in triplicate and were repeated twice.

2.4. Allium cepa genotoxicity assay

A modified *A. cepa* genotoxicity assay was used as previously described (Lateef et al., 2007). For acute tests, seeds of *Allium cepa* were primarily germinated in Petri plates in ultra-pure water until the roots reached 1 cm in length. The germinated onion seeds were then incubated with 20, 50 and 100 µg/mL of *M. esculenta* extracts and 10, 20 and 50 µg/mL of protocatechuic acid. For the negative control (NC), a 19:1 mixture of water containing Tween 80 (0.02%) was used and for the positive control (PC), the carcinogenic agent methyl-methane-sulfonate (MMS) was used at a concentration of $4x10^{-4}$ mol/L. After 24h and 48h of incubation, the roots were fixed in Carnoy solution (3:1 (v/v) ethanol: glacial acetic acid) for 24 h, carefully squashed and hydrolyzed with 1 N HCl at 60 °C for 8 min, washed with distilled water and Schiff stained for 2h in the dark. After this process, five slides were prepared per sample for each treatment. The

slides were examined under an optical microscope (Mikroskop DMLS Typ 020 518 500. Leica, Wetzlar. Neubauer Zählkammer. Eppendorf, Hamburg, Germany). 500 cells were counted for each slide, and the mitotic index (MI) and the chromosomal aberration score (CAS) were determined according to the following equations:

$$MI = \frac{\text{the number of dividing cells}}{\text{the total number of cells}}$$

$$CAS = \frac{\text{the number of damaged cells}}{\text{the number of cells in division}}$$

3. Results

Antimutagenic studies are important tools to find anticarcinogens. In this study, *S. typhimurium* strains carrying mutations in histidine operon were used. In the antimutagenicity assay performed with *S. typhimurium* TA 100, the studied *M. esculenta* extracts and protocatechuic acid showed dose dependent inhibitory effect against direct-acting mutagen 4NQO (**Tables 1, 2** and **3**), and the effect could be partially ascribed to the antioxidant activity previously reported (Heleno et al., 2013). The highest inhibition rate was 72.4% for protocatechuic acid at 500 µg/ml. Our research group previously has determined lethal doses of protocatechuic acid to various bacteria at 3 mg/ml (Stojković et al., 2013), therefore the maximum used doses for antimutagenic properties was considered as non-deleterious to bacterial cells. Sample of morel from Serbia expressed slightly higher antimutagenic properties with inhibition rate of 58.7%, while sample from Portugal gave inhibition rate of 51.7% at 1000 µg/ml.

Cell survival in medium with the samples and not treated with 4-NQO, as well as cell survival treated with 4-NQO in medium with extracts and protocatechuic acid was recorded (**Tables 1, 2**

and **3**). Cell survival, treated with 4-NQO and extracts, slightly decreased with higher concentrations. Viability of cells was higher when extract and 4-NQO were applied simultaneously, but the number of cells decreased in correlation with control samples. The methanolic extracts at a concentration of 1000 µg/ml had no significant stimulatory effect on viable cells. Also, control samples and samples treated with sterilized distilled water containing Tween 80 had no effects on Index of antimutagenicity (I).

Antimitotic index and chromosomal aberration scores of *M. esculenta* from Portugal and Serbia, as well as of protocatechuic acid, are presented in the **Table 4**. It is evident from the table that in comparison to positive control mitotic index decreased with increasing concentrations of extracts. Chromosomal aberration score was higher when extracts were applied, but lower when protocatechuic acid was used, in comparison to both negative and positive controls. Mushroom extracts reduced the mitotic index in onion root meristems, indicating the presence of bioactive substances (protocatechuic acid) with the potential for pharmacological use in chemotherapy. The reduction of the mitotic index might be explained as being due to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or the slowing of the rate of cell progression through mitosis (Yuet-Ping et al., 2012).

4. Discussion

Recently our biological study on the methanolic extracts of *M. esculenta* from Serbia and Portugal reported their relevant antioxidant activity (Heleno et al., 2013). Since many mutations in cells are potentially results of oxidative and nitrosative damages made by Reactive Oxygen Species (ROS) and Nitrogen Reactive Species (RNS), the obtained results encouraged us to extent our evaluation using the Ames Salmonella test and *Allium cepa* genotoxicity assay.

Nowadays, cancer is one of the mortality factors in the world that occurs as a result of different causes, such as mutagenic and carcinogenic chemicals in the environment (Cavalcanti et al., 2012). Prevention of cancer and related diseases is an old but important concept regarding human health. Dietary factors are known to influence cancer development (Tanaka et al., 2011). The concept of chemoprevention is the use of natural or synthetic compounds to block, reverse, or prevent the development of cancers and it has great appeal. Main mechanisms for cancer chemoprevention are anti-mutagenesis and anti-proliferation/anti-progression (Nieva-Moreno et al., 2005).

The Ames *Salmonella* test is a popular test model for studying the antimutagenic effects of substances. As cancer is often linked to DNA damage, the test serves as a quick assay to estimate the anticarcinogenic potential of a compound or extract (Hong and Lyu, 2011). The addition of mutagens results in bacteria reverting back to histidine-independent, forming colonies in histidine-deficient medium. 4-NQO is a well-known genotoxic agent which is able to induce DNA damage (Liu et al., 2012). Its carcinogenic mechanism of action is through generation of ROS and RNS, such as superoxide radicals, hydrogen peroxide and nitric oxide, which induce intracellular oxidative and nitrosative stress (Nunashiba and Demple, 1993). Both tested extracts from *M. esculenta*, as well as protocatechuic acid inhibited 4-NQO-induced mutagenicity. Mechanism of action of tested extracts seems to be due to potent antioxidant activity and subsequent scavenging of ROS by the polyphenols present in the extract. Recently, we showed that protocatechuic acid is one of two phenolic acids detected in both samples (240 and 60 μg/100 g dw for *M. esculenta* from Portugal and Serbia, respectively) (Heleno et al., 2013). Protocatechuic acid was previously reported to have antigenotoxic potential towards human cells and cytotoxic activity on leukemia cell lines (Anter et al., 2011). Also, extracts rich in

protocatechuic acid inhibited mutagenicity of 1-nitropyrene (1-NP) in a dose-response manner (Olvera-Garcia et al., 2008). On the other hand, Stagos et al. (2006) showed no effect for protocatechuic acid on mutagenicity induced by bleomycin and hydrogen peroxide.

Higher plants such *Allium cepa* are accepted as admirable genetic models to evaluate genotoxic effects such as chromosome aberrations and disturbances in the mitotic cycle. *A. cepa* assay enabled the assessment of different genetic endpoints, which are mitotic index and chromosome aberration. Mitotic index is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the mitotic phase of the cell cycle. Hence, the decrease in the mitotic index of *A. cepa* meristematic cells could be interpreted as cellular death (Yuet-Ping et al., 2012).

Overall, protocatechuic acid had the highest antimutagenic potential in comparison to methanolic extracts of *M. esculenta*, obtained from Serbia and Portugal. *M. esculeta* from Portugal gave higher level of protocatechuic acid, while *M. esculenta* from Serbia showed higher anti-mutagenic potential. Demonstrated activity thus may be related to other classes of secondary metabolites, or may be due to synergistic action between present phenolics.

Further studies are needed to elucidate the mode of antimutagenic action of extracts, as well as of protocatechuic acid. As far as we know, this study is the first one to report antimutagenic potential of *M. esculenta* on *S. typhimurium* his^+ mutants and antimitotic activity in *Allium cepa* assay.

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Table 1. Antimutagenic properties of methanolic extracts of *Morchela esculenta* from Serbia, on *Salmonella typhimurium* TA 100, expressed as Index of antimutagenicity (I, mean ± standard deviation).

Concentration	**Viable cells/plate**			**his$^+$ revertants/plate**		
			V %			**I %**
(µg/ml)	-4NQO *****	+4NQO *****		-4NQO *****	+4NQO *****	
control	280	195	69	13	58	
A	237	172	73	14	41	29.3±1.7
B	245	168	69	15	32	44.8±1.7
C	260	185	71	13	24	58.7±1.8

V – Viability of cells, (+4NQO /-4NQO) × 100; A, B and C - concentration of extract used, A – 200 µg/ml, B – 500 µg/ml, C – 1000 µg/ml. * Dilution 10^6

Table 2. Antimutagenic properties of methanolic extract of *Morchela esculenta* from Portugal, on *Salmonella typhimurium* TA 100, expressed as Index of antimutagenicity (I, mean ± standard deviation).

Concentration	**Viable cells/plate**			**his$^+$ revertants/plate**		
			V %			**I %**
(µg/ml)	-4NQO *****	+4NQO *****		-4NQO *****	+4NQO *****	
control	280	195	69	13	58	
A	241	169	70	12	43	25.9±1.8
B	242	174	72	14	34	41.4±1.8
C	258	179	69	15	28	51.7±1.7

V – Viability of cells, (+4NQO /-4NQO) × 100; A, B and C - concentration of extract used, A – 20 µg/ml, B – 50 µg/ml, C – 100 µg/ml.* Dilution 10^6

Table 3. Antimutagenic properties of protocatechuic acid, on *Salmonella typhimurium* TA 100, expressed as Index of antimutagenicity (I, mean ± standard deviation).

Concentration	**Viable cells/plate**			**his$^+$ revertants/plate**		
			V %			**I %**
(µg/ml)	-4NQO *****	+4NQO *****		**-**4NQO *****	+4NQO *****	
control	280	195	69	13	58	
A	257	182	71	12	31	46.5±1.8
B	265	177	67	15	25	56.8±1.8
C	267	181	68	14	16	72.4±1.8

V – Viability of cells, (+4NQO /-4NQO) × 100; A, B and C - concentration of extract used, A – 10 µg/ml, B – 20 µg/ml, C – 50 µg/ml. * Dilution 10^6

Table 4. Mitotic index and chromosomal aberration score of *Morchella esculenta* from Serbia (MES) and Portugal (MEP) and of protocatechuic acid (PA).

Concentration		After 24h		After 48h	
		MI	**CAS**	**MI**	**CAS**
	Negative control	0.031	0.008	0.032	0.010
	Positive control	0.036	0.012	0.041	0.014
20 µg/ml	**MES**	0.027	0.009	0.030	0.011
	MEP	0.029	0.008	0.031	0.009
10 µg/ml	**PA**	0.011	0.001	0.010	0.001
50 µg/ml	**MES**	0.023	0.011	0.024	0.015
	MEP	0.022	0.010	0.025	0.012
20 µg/ml	**PA**	0.010	0.000	0.008	0.001
100 µg/ml	**MES**	0.017	0.014	0.023	0.017
	MEP	0.019	0.013	0.021	0.015
50 µg/ml	**PA**	0.005	0.000	0.006	0.002

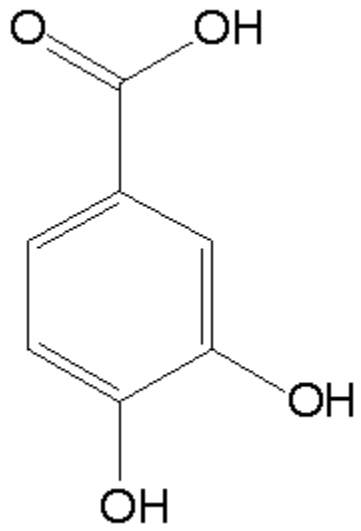

Figure 1. Chemical structure of protocatechuic acid.