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Screening of Basidiomycete mushroom extracts for antigenotoxic and bio-antimutagenic activity

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In this study we screened crude methanol:water extracts of 89 different mushroom species for their antigenotoxic and bio-antimutagenic activity. The screening was performed with the SOS/*umu* test and we monitored the ability of extracts to inhibit UV induced expression of *umu*C gene in *Salmonella typhimurium* TA1535/pSK1002. Seventeen extracts inhibited *umuC* expression by more than 50%. These extracts were further evaluated for the ability to inhibit UV induced mutations in *Escherichia coli* WP2. Five extracts (*Cortinarius evernius, Rozites caperatus, Lactarius vellereus, Russula integra* and *Pleurotus cornucopiae*) inhibited also UV induced mutations. The study showed that certain mushrooms contain substances with bio-antimutagenic potential. Particularly interesting for further investigations are *Pleurotus cornucopiae* (Lentinaceae), which was the most effective and species of Russulaceae and Cortinaceae families, which might contain common family specific bio-antimutagenic substance(s).

1. Introduction

From epidemiological and laboratory studies it has become evident that a variety of dietary and medicinal phytochemicals have substantial cancer preventive properties [1]. Most of the cancer preventive phytochemicals were identified and isolated from plants while mushrooms were since recently neglected as a source of natural cancer preventing agents. *Basidiomycetes* have been used in folk medicine all over the world since ancient times [2]. They show diverse beneficial physiological and therapeutic effects such as immunomodulatory, lipid lowering, antithrombotic, antihypertensive, antiinflamatory and antitumor [3]. It has been known for many years that selected species from higher Basidiomycetes are effective against cancer of the stomach, esophagus, lung etc. [4]. However, the mechanisms of antitumor and cancer chemopreventive effects of Basidiomycetes are poorly understood.

Anticarcinogens may act to inhibit either the initiation, promotion or progression phase of carcinogenic process [5]. It is known that many anticarcinogens are also antimutagens. Based on the mode of action, antimutagens are further classified into two categories: desmutagens and bio-antimutagens [6]. Desmutagens interact with mutagens directly or indirectly before the mutagens attack DNA, while bio-antimutagens suppress the process of mutation fixation after DNA is damaged by mutagens.

In the literature the data concerning antimutagenic activity of mushrooms is limited. Therefore we decided to perform a screening of a range of different species of mushrooms for their antigenotoxic and bio-antimutagenic potential. We used the SOS/*umu* test [7], which is a simple and rapid test for the detection of potential mutagens and is also increasingly used for identifying potential antimutagens [8–14]. The test is based on the ability of physical and chemical genotoxic agents to induce expression of *umu*C gene, one of the SOS genes responsible for error-prone DNA repair, which plays central role in SOS mediated mutagenesis in bacteria [15]. To assay the extracts for potential antimutagenic activity we induced SOS functions with UV irradiation and measured inhibition of UV induced expression of *umu*C by mushroom extracts. Selected active extracts were further evaluated for the ability to inhibit UV-induced mutations in *Escherichia coli* B/r WP2.

2. Investigations and results

In this study we evaluated crude methanol : water extracts of mushrooms for their ability to inhibit UV induced expression of *umu*C in *Salmonella typhimurium* TA1535/pSK1002. In order to preserve the extracted mushroom components as intact as possible no additional concentration, purification and enrichment procedures were applied. Before the screening, the extracts were coded and each extract was tested at a single dose (10% v/v of the extract). The Table indicates that extracts of 96 mushrooms, belonging to 89 different species and 21 different families were included. The results of the initial screening (Table) show that 17 extracts inhibited UV induced expression of *umu*C by more than 50% ($umuC_{inh} < 0.5$), three of them by more than 80% ($umuC_{inh} < 0.2$). Two extracts (*Suillus granalatus* and *Melanoleuca melanoleuca)* enhanced UV induced *umu*C expression ($umuC_{inh} > 1.4$). The extracts that inhibited UV induced *umu*C expression by more than 50% were then re-tested at different dilutions (Fig. A). Eight of them (*Lactarius quietus, Lactarius vellereus, Russula cyanoxantha, Russula ochroleuca, Russula viscida, Cortinarius violaceus, Clitoclybe odora* and *Pleurotus cornucopiae*) inhibited the UV induced expression of *umu*C in a dose dependent manner. The other 9 extracts gave reproducible results at the dilution of 10% (v/v), while at higher dilutions they did not inhibit UV induced expression of *umu*C.

Only the extract of *Lactarius vellereus* inhibited the viability of UV irradiated bacteria (Table). The reduced β-galactosidase activity was thus not due to the toxicity of the extracts, except for the extract of *Lactarius vellereus* at 10% dilution (higher dilutions did not inhibit viability – data not shown).

The active extracts were then tested for the ability to inhibit UV induced mutations in *E. coli* WP2. The results showed that only the extracts of *Cortinarius evernius* and *Pleurotus cornucopiae* reduced the number of UV induced revertants by more than 50% (MIF < 0.5) (Fig. B). Another three extracts (*Russula integra*, *Lactarius vellereus* and *Rozites caperatus*) significantly reduced the number of revertants in a dose dependent manner and can be considered to have moderate effect (Fig. B).

Table: Inhibition of UV (2.5 J/m²) induced *umu*C expression in *Salmonella typhimurium* TA1535/pSK1002 by methanol : water mushroom extracts

Family Species	*umu*C$_{inh}$ [a]	Viability (%) [b]
Control (methanol : water 1 : 1)	1.00	100
Agaricaceae		
Agaricus abruptilus	0.52**	106
Amanitaceae		
Amanita battare (Boud.) Bon	0.93	101
Amanita muscaria (L.: Fr.) Hook	0.52*	139
Amanita pantherina (D.C.: Fr.) Krbh.	0.63	140
Amanita phalloides (Vaill.: Fr.) Link	0.57*	166
Amanita spissa (Fr.) Kumm	0.80*	100
Amanita virosa (Lamarck) Bertill.	0.60	115
Amanita virosa (Lamarck) Bertill.+	1.06	99
Balbitiaceae		
Panaeolus ater (Lge.) K & R. ex Bon	1.01	96
Panaeolus papilionaceus (Bull.: Fr.) Quel.	0.85	114
Pycnoporus cinnabovinus (Jacq.: Fr.) Karst	1.07	122
Banceraceae		
Sarcodon imbricatus (L.: Fr.) Karst.	0.59	129
Bjerkanderaceae		
Climacocystis borealis (Fr.: Fr.) Kotl. & Pouz.	0.98	101
Boletaceae		
Boletus rhodoxanthus (Krbh.) Kall.	0.64*	157
Suillus bovinus (L.: Fr.) Ktze.	0.99	91
Suillus granulatus (L.: Fr.) Roussel	1.65**	100
Suillus gravillei (Klotzsch) Sing.	0.77*	116
Suillus tridentinus (Bres.) Sing.	0.80	105
Suillus variegatus (Sw.: Fr.) Ktze.	0.60*	93
Suillus viscidus (L.) Roussel	0.76*	101
Tylopilus felleus	0.36**	95
Cantharellaceae		
Cantharellus cibarius (Fr.: Fr.) Fr.	0.76	104
Cantharellus lutescens Pers.: Fr.	0.68*	112
Coprinaceae		
Coprinus comatus (Muell.: Fr.) Pers.	0.58*	107
Coriolaceae		
Gleophyllum odoratum (Wulf.: Fr.) Imaz.	0.55**	129
Heterobasidion annosum (Paul.) Gill.	0.58	134
Lenzites betulina (L.: Fr.)	1.30	132
Pycnoporus cinnabovinus (Jacq.: Fr.) Karst	1.07	122
Trametes hirsuta (Pers.: Fr.) Fr. fo. Pil.	0.72	127
Trametes gibbosa (Pers.: Fr.) Fr.	0.73*	107
Trametes versicolor (L.: Fr.) Lloyd	0.94	98
Cortinaceae		
Cortinarius evernius (Fr.: Fr.) Fr.	0.43**	113
Cortinarius paleiferus Svrček	0.60**	112
Cortinarius praestans (Cord.) Gill.	1.39*	117
Cortinarius subtortus (Pers.: Fr.) Fr.	0.66*	97
Cortinarius violaceus (L.: Fr.) Fr.	0.35**	164
Hebeloma sinapizans (Paul.) Gill.	0.53**	113
Inocybe rimosa (Bull.: Fr.) Kumm.	0.64*	116
Inocybe terrigena (Fr.) Kuehn	0.39**	97
Rozites caperatus (Pers.: Fr.) Pers.+	0.82	118
Rozites caperatus (Pers.: Fr.) Pers.	0.39*	161
Fomitopsidaceae		
Fomitopsis pinicola (Sow.: Fr.) Karst.	0.91	105
Gomphidaceae		
Chroogomphus helveticus (Sing.) Mos. ssp. tatrensis Pil.	0.68	154
Gomphidius glutinosus (Schff.: Fr.) Fr.	0.68*	114
Gyrodontaceae		
Boletinus caviceps (Klotzsch: Fr.) Kalchbr.	0.73	100
Hydnaceae		
Hydnum repandum L.: Fr. A	1.03	104
Hygrophoraceae		
Hygrocybe conica (Scop.: Fr.) Kumm.	0.58*	96
Lentinaceae		
Pleurotus cornucopiae (Paul.) Roll.+	0.11**	251
Lycoperdaceae		
Lycoperdon perlatum Pers. : Pers.	1.23	118
Lycoperdon piriforme Schff.: Pers.	0.72*	120
Calvatia excipuliformis (Scop.: Pers.) Perdeck	0.68	147
Polyporaceae		
Grifola umbellata	0.89	103
Ramariaceae		
Ramaria flavobrunnescens (Atk.) Corn.	0.90	108
Ramaria largentii Marr. & Stun.	0.63**	93
Ramaria sanguinea (Pers.) Quel.	0.55**	131
Russulaceae		
Lactarius badiosanguineus K. & R.	0.97	92
Lactarius blennius (Fr.: Fr.) Fr.	0.70	92
Lactarius bresadolanus Sing.	0.38**	114
Lactarius deterrimus Groeg.	1.35	106
Lactarius porninsis Roll.	0.89	88
Lactarius quietus (Fr.: Fr.) Fr.	0.09**	129
Lactarius rufus (Scop.: Fr.) Fr.	0.62	109
Lactarius scrobiculatus (Scop.: Fr.) Fr.	0.71*	105
Lactarius scrobiculatus (Scop.: Fr.) Fr.+	1.28	101
Lactarius torminosus (Schff.: Fr.) Gray	0.66*	116
Lactarius vellereus (Fr.: Fr.) Fr.	0.08**	23
Russula cyanoxantha (Schff.) Fr.	0.17**	172
Russula emetica (Schff.: Fr.) Pers.	0.45**	91
Russula emetica (Schff.: Fr.) Pers. var. silvestris Sing.	0.84	92
Russula integra (L.) Fr.	0.48**	110
Russula ochroleuca (Hall.) Pers.	0.23**	143
Russula viscida Kudr.	0.27**	171
Stereaceae		
Stereum hirsutum (Willd.: Fr.) Fr.	0.91	141
Strophariaceae		
Hypholoma fasciculare (Huds.: Fr.) Kumm.	0.97	124
Hypholoma sublateritium (Schff.: Fr.) Quel.	0.34**	108
Hypholoma sublateritium (Schff.: Fr.) Quel.	0.41**	104
Kuehneromyces mutabilis (Scop.: Fr.) Sing. & Sm.	0.62*	104
Pholiota squarrosa (Muell.: Fr.) Kumm.	0.89	105
Tremellaceae		
Pseudoydnum gelatinosun (Scop.: Fr.) Karst.	0.83*	95
Tricholomataceae		
Armillariella mellea (Vahl.: Fr.) Kumm.	0.82	121
Clitocybe costata K. & R.	0.76*	108
Clitocybe gibba (Pers.: Fr.) Kumm.	0.56*	115
Clitocybe odora (Bull.: Fr.) Kumm.	0.24**	123
Clitocybe odora (Bull.: Fr.) Kumm.+	0.77*	99
Lepista nebularis	0.60**	119
Leucopaxillus giganteus	0.62	121
Lyophyllum connatum	1.00	96
Lyophyllum connatum (Schum.: Fr.) Sing.	0.78	102
Melanoleuca melanoleuca (Pers.: Fr.) Murr.	1.56*	113
Tricholoma columbetta (Fr.: Fr.) Kumm.	0.54	120
Tricholoma pardinum Quel.	0.91	93
Tricholoma pardinum Quel.	0.66*	124
Tricholoma saponaceum (Fr.: Fr.) Kumm.	0.32**	151
Tricholoma sulfureum (Bull.: Fr.) Kumm.	0.60*	142
Tricholoma ustaloides Romagn.	0.66*	116
Tricholoma vaccinium (Pers.: Fr.) Kumm.	0.71	106
Tricholomopsis rutilans	0.56**	71

[a] *umu*C$_{inh}$ = enzyme units of β-galactosidase activity of UV irradiated culture in the presence of the extract/enzyme units of β-galactosidase activity of the UV irradiated control; EU of β-galactosidase activity of solvent controls were 34.5 ± 8.8; enzyme units of β-galactosidase activity of UV irradiated control were 479.9 ± 98.2.

[b] Viability% = OD_{600} of UV irradiated culture in the presence of the extract/OD_{600} of UV irradiated control; the viability after UV irradiation was 32 ± 11%.

* $p < 0.01$; ** $p < 0.001$ (Student t-test);

\+ the extract was prepared from air dried mushroom

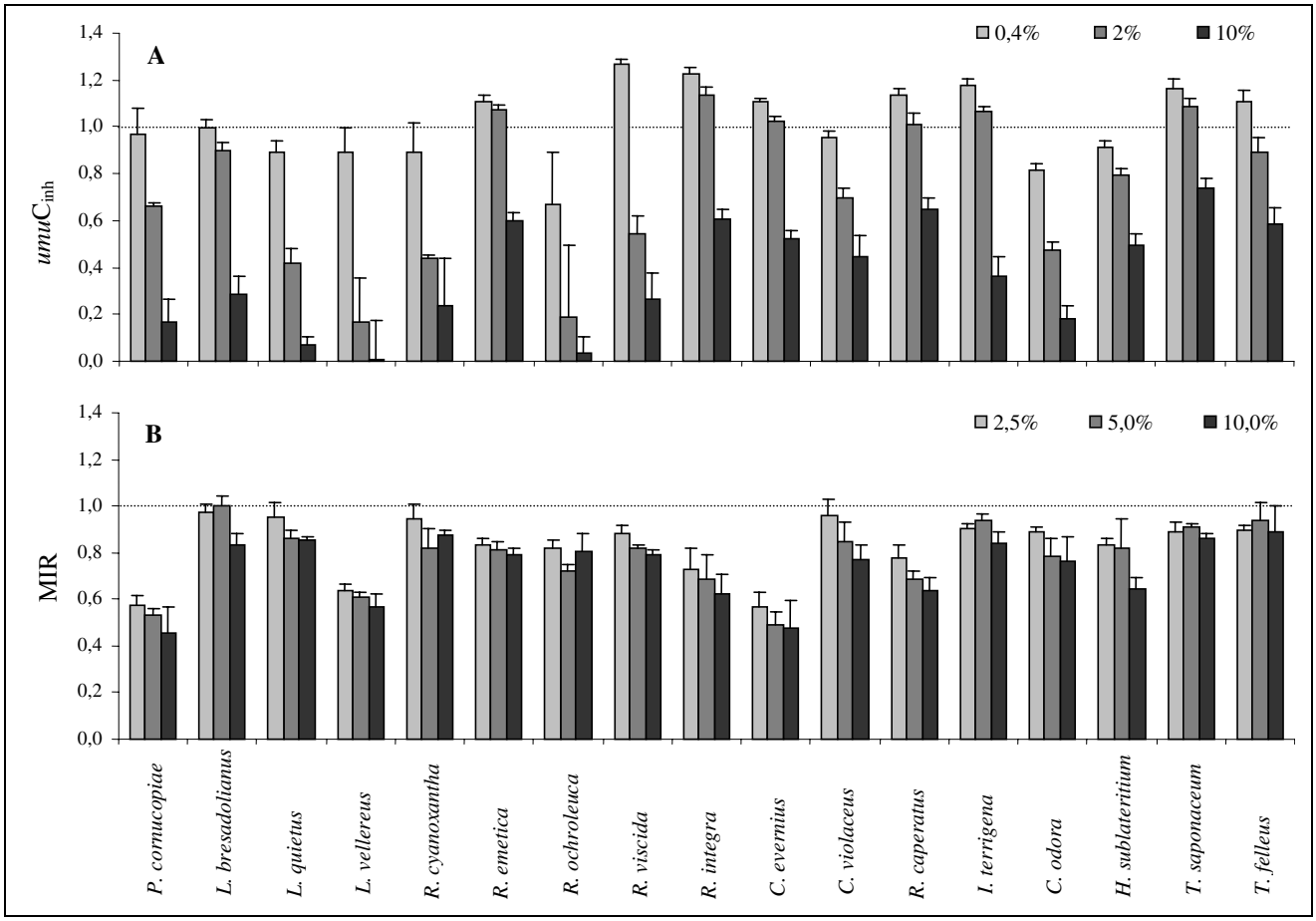

Fig.: Inhibition of UV induced SOS response in *S. typhimurium* TA1535/pSK1002 (A) and mutagenesis in *E. coli* WP2 (B) by selected mushroom methanol : water extracts. *umu*C_{inh} is the ratio between the induced β-galactosidase activity units of the UV irradiated culture in the presence of the test sample and the induced β-galactosidase activity units of the UV irradiated culture in the absence of the test sample. MIR is the ratio between the number of UV induced revertants in the presence of the test sample and the number of UV induced revertants in the absence of the test sample. Data shown are mean values with standard deviation of the mean of three replicates for *umu*C_{inh} and mean values with standard deviations of two independent experiments with three replicates for MIR.

3. Discussion

By screening of methanol : water mushroom extract for antigenotoxic activity with the SOS/*umu* test we found extracts of 17 species out of 89 tested that inhibited UV induced SOS response by more than 50%. The extracts were added to a bacterial culture after UV irradiation thus the observed antigenotoxic effect was due to the inhibition of the expression of *umu*C gene and not due to the protection of DNA against UV induced damage by some other mechanism. As the products of *umu*DC genes are essential for UV induced mutagenesis in bacteria [16, 17], we expected that extracts that efficiently inhibited UV induced expression of *umu*C would also inhibit UV induced mutagenesis. Five extracts (*Cortinarius evernius, Rozites caperatus, Lactarius vellereus, Russula integra* and *Pleurotus cornucopiae*) inhibited also UV induced mutations in *E. coli* WP2. This result indicates that the mechanism of bio-antimutagenic effect of these extracts is suppression of error-prone DNA repair pathway so that less UV induced DNA lesions are fixed as mutations. Several substances were reported to inhibit both UV induced SOS response and mutagenesis. The most interesting of them is curcumin, a phenol isolated from turmeric (*Curcuma longa*), which also proved to have anti-initiation and anti-promotion effect and has been considered as a potential cancer chemopreventive agent [12, 18]. Bio-antimutagenic effects mediated by inhibition of SOS functions were reported also for St. John's Wort extract [19], for natural antimutagens garlicin and cinnamaldehide [20], for protease inhibitor antipain [21] and for pirimidine analogues 5-fluorouracil and 5-fluorodeoxiuridine, which are used as antineoplastic and antiviral agents, respectively [22].

Species from different families and also different species that belong to the same family were included in the screening so we could compare their responses to see if there is any intrafamiliar similarity regarding antigenotoxic activity. Out of 16 different species of the Russulaceae family, extracts of eight species inhibited the UV induced *umu*C expression by more than 50% and two of them inhibited also UV induced mutations. Of nine species of Cortinaceae family four inhibited UV induced *umu*C expression and two of them also UV induced mutations. The findings indicate, that Russulaceae and Cortinaceae might contain some family specific substance(s) with antigenotoxic activity.

None of the mushroom species included in this study was previously reported to have antimutagenic activity. With the *Salmonella*/microsome test the antimutagenic activity against different chemical mutagens was reported for *Agaricus abruptilus, Agaricus bisporus, Cratarellus cornucopioides, Cantharellus cibarius, Lactarius lilacinus, Lyophyllum connatum* and *Xerocomus chrysenteron* [23], *Agaricus blazei* [24], *Phellinus linteus* [25] and *Tiramaria*

pinoyi [26]. Inhibitory effect on chemically induced SOS response was shown for acetone extracts of *Grifola frondosa* [27]. Recently it was reported that *Ganoderma lucidum* extract protects DNA from strand breakage caused by hydroxyl radical and UV irradiation [28].

This study showed, that certain mushrooms contain substances with bio-antimutagenic activity. Particularly interesting mushrooms for further investigations are *Pleurotus cornucopiae* (Lentinaceae), which most effectively inhibited UV induced mutations and species of Russulaceae and Cortinaceae families, which might contain common family specific bio-antimutagenic substance(s).

Lactarius vellereus represents the species of a particular interest. It has been thoroughly researched in order to resolve chemical composition [29–31]. All *Lactarius species* contain sesquiterpenes and some of them are considered as chemotaxonomic markers. In injured *Lactarius vellereus*, the tasteless and inactive sesquiterpene stearoylvelutinal is converted in a few seconds into the two pungent and potent antimicrobial and antifeedant sesquiterpene aldehydes isovelleral and velleral. These compounds were shown to be gradually (within minutes to hours) converted by mushroom enzymes to less toxic and non-pungent compounds [31]. We speculate, that during the transport, freezing and extraction of mushroom isovelleral, which was reported to be mutagenic [31] and velleral were converted into compound(s) with antimutagenic activity. It might be a mechanism that protects the mushroom against its own defence agents. Further experiments are being conducted to confirm this hypothesis and to isolate and identify the antimutagenic compound(s).

4. Experimental

4.1. Preparation of methanol : water extracts

Different species of mushrooms (Table) were collected from their natural habitat in the Northwestern part of Slovenia in autumn 1998. Fresh mushrooms were frozen and kept at −20 °C until extraction. 2 g of frozen fresh mushroom was minced and extracted with 10 ml of methanol : water (1 : 1 v/v) mixture in the ultrasonic bath for 10 min. The extracts were centrifuged two times (3000 rpm, 10 min) to remove insoluble matter, and the supernatants retained. The supernatants were coded and used for antigenotoxicity screening. Voucher specimens are deposited at Department of Pharmaceutical Biology, Faculty of Pharmacy, Ljubljana, Slovenia.

4.2. Media and culture

S. typhimurium TA1535/pSK1002 cells were cultured overnight in TGA medium at 37 °C with shaking. The TGA medium contained: 1% Bacto tryptone; 0.5% NaCl; 0.2% glucose and 50 μg/ml ampicillin. The overnight grown culture was then diluted 5-fold with fresh pre-warmed TGA medium and incubated for additional 2 h at 37 °C to bring the cells into log-phase.

Escherichia coli WP2 cells were grown overnight in nutrient broth (Oxoid No2, 25 g/l) at 37 °C with shaking. For the reverse mutation assay we used semienriched minimal agar medium (SEM). The SEM contained 1 g $(NH_4)_2SO_4$; 10 g KH_2PO_4; 0.1 g $MgSO_4 \cdot 7\,H_2O$; 0.5 g trisodiumcitrate $\cdot\, 2\,H_2O$ (neutralized with KOH), 4 g glucose, 0.16 g Difco nutrient broth and 15 g Difco bacto agar/l. The soft agar medium contained 7 g Difco bacto agar and 6 g NaCl/l and was kept at 45 °C until use.

4.3. UV irradiation

Bacterial cultures were UV irradiated with a germicidal lamp (BSP-30, Elektromedicina Ljubljana) in Petri dishes (90 mm). The suspension of *S. typhimurium* TA1535/pSK1002 in 1/15 M phosphate-buffered saline was irradiated with UV dose of 2.5 J/m^2 and the suspension of *E. coli* WP2 with an UV dose of 15 J/m^2.

4.4. SOS/umu test

The SOS/*umu* test was carried out in microtiter plates as described by Reifferscheid et al. [32] with minor modification. To 270 μl of UV irradiated bacterial culture in TGA medium (absorbance at 600 nm adjusted to 0.2 to 0.3) 30 μl of mushroom extract or 30 μl of methanol : water (1 : 1 v/v) for control were added and incubated at 37 °C on a rotary shaker. After 2 h incubation the bacterial suspension was diluted 10-fold with fresh warm TGA medium and incubated for an additional 2 h. At the end of the incubation, the cell density was measured by absorbance at 600 nm. The expression of *umu*C gene was measured as a degree of β-galactosidase activity according to Miller [33].

The antigenotoxic potential is expressed as *umu*C inhibition ratio ($umuC_{inh}$), which is defined as the ratio between the induced β-galactosidase activity units of the UV irradiated culture in the presence of the test sample and the induced β-galactosidase activity units of the UV irradiated culture in the absence of the test sample.

4.5. Antimutagenicity assay with Escherichia coli WP2

To the UV irradiated culture of *E. coli* WP2 different amounts of mushroom extracts (2.5, 5 and 10% v/v in the reaction mixture) or methanol : water (1 : 1) for control were added and pre-incubated on rotary shaker for 30 min at 37 °C. 100 μl of pre-incubation mixture was then added to 2 ml of soft top agar, poured onto SEM plates and incubated at 37 °C for 48 h. After the incubation the revertant colonies were counted. Mutation inhibition ratio (MIR) was defined as the ratio between the number of UV induced revertants in the presence of the test sample and the number of UV induced revertants in the absence of the test sample.

4.6. Data analysis

All the experiments with the SOS/*umu* test were performed in triplicate and the results are given as a mean of three parallels. For antimutagenicity assay with *E. coli* WP2 at least two independent experiments with three parallels were performed. The results are shown as the mean of two independent experiments. The significance of differences in β-galactosidase units and number of induced revertants, respectively between test and control samples was determined by two-tailed Student t-test for homoscedastic samples at α 0.05 using Microsoft Excell 5.0 statistical analysis tool pack.

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