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Antimutagenic effect of *Phellinus rimosus* (Berk) Pilat against chemical induced mutations of histidine dependent *Salmonella typhimurium* strains

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ABSTRACT

Mutations are one of the important factors contributing to oncogenesis. Somatic mutations have been detected in oncogenes and tumor suppressor genes in various types of cancers. *In vitro* antimutagenic activity of ethyl acetate extract of macro fungus, *Phellinus rimosus* was evaluated by Ames' mutagenicity assay. The effect was evaluated against the direct acting mutagens (sodium azide, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, doxorubicin and 4-nitro-*o*-phenylenediamine) and mutagen needing activation (2-acetyl aminofluorine, and benzo[*a*]pyrene). The extract was significantly ($p < 0.05$) and dose dependently effective against direct acting mutagens and mutagen needing activation. Among the antimutagenic activity against directly acting mutagens, effect was found to be highest against doxorubicin-induced mutation. The antimutagenic effect of the extract against indirect acting mutagen in the presence of mammalian metabolic activation system was also found to be significant ($p < 0.01$). The background bacterial growth and number of revertant colonies in the extract alone treated plate with or with out metabolic activator was almost same as that of spontaneous revertants. This indicated the non-toxic nature of the extract. The effect was partially ascribed to the antioxidant activity. The results of the study suggest the possible antitumor mechanisms of *P. rimosus*.

1. Introduction

The contemporary view of cancer is that malignant tumor arises and progresses through the accumulation of successive mutations, which involve activation of protooncogenes and inactivation of tumor suppressor genes, leading to uncontrolled proliferation of the progeny cells. Molecular genetic analysis of tumor samples suggests that the accumulation of multiple genetic changes is essential for normal cell to progressively acquire malignant phenotypes. Somatic mutations have been detected in oncogenes and tumor suppressor genes in various types of cancers (Fearon and Vogelstein, 1990). Point mutations of K-*ras*, *p53* and *APC* were most commonly found in human tumors (Tahara, 1990; Wright and Williams, 1993). Mutations of K-*ras* and *APC* can occur in early lesions, while alterations of *p53* and *DCC* often occur in advanced tumors (Mao and Sidransky, 1994). The frequency of *p53* mutations is increased during the progression of colorectal cancer (Calistri et al., 2005). Mutations of *p53* were also involved in the anaplastic thyroid carcinomas (Quiros et al., 2005). Average spontaneous mutation frequencies per base pair in human cells are estimated to be in the range of 10^{-8} to 10^{-10} and are found to be increased by 10- to 1000-folds upon exposure to a mutagen. Chemicals and radiation are known mutagens that are associated with the initiation and progression of human cancers.

Among the Basidiomycetes, medicinal macro fungi and their secondary metabolites such as lectins, alkaloids, terpenes, and antibiotics have an established history of application in medicine (Wasser, 2010). There are many clinical studies proving the cancer inhibitory effects of macro fungi especially medicinal mushrooms (Wasser, 2010). However, the use of such medicinal fungi or their active principles in the modern medicine for the prevention and/or treatment of chronic diseases suffers from lack of scientific evidences and only very few have attracted interest to the scientists. *Phellinus* is a large and widely distributed genus of the family Hymenochetaceae (Donk). Some of the species of *Phellinus* are extensively studied in China, Japan and Korea especially *Phellinus linteus*, which has been considered to be a traditional Chinese medicine. Extracts from *P. linteus* and *Phellinus igniarius* were reported to possess antimutagenic activity (Shon and Nam, 2001). *P. linteus* has also been reported for its inhibitory effect mediated by suppressing the redox-based NF-κB activation in macrophages (Yoon et al., 2007). *Phellinus rimosus* is a wood rotting macro fungus, found growing on jackfruit tree trunks. Investigations on the pharmacological activities of *P. rimosus* are fragmentary. Among the activities demonstrated, ethyl acetate extract of *P. rimosus* showed profound antioxidant, anti-inflammatory and antitumor

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activities (Ajith and Janardhanan, 2002, 2003). Recently, we have reported the ameliorating effect of ethyl acetate extract of *P. rimosus* against DMBA initiated, croton oil promoted skin papilloma formation in mice (Ajith and Janardhanan, in press). Investigations on the mechanism of antitumor effect of *P. rimosus* have not yet been established. Therefore, in this study we evaluate the antimutagenic activity using Ames test which is a standard and simple assay to determine mutagenic/antimutagenic effects of different substances.

2. Material and methods

2.1. Chemicals

Nicotinamide adenine dinucleotide phosphate (disodium) (NADP), glucose-6-phosphate, D-biotin, L-histidine, agar–agar, sodium azide (NaN_3), nicotinamide adenine dinucleotide phosphate tetrasodium reduced (NADPH), were from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. *N*-nitrosodiethylamine (NDEA), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), 4-nitro-*o*-phenylenediamine (NPDA) and benzo[*a*]pyrene (B[*a*]P) were purchased from Sigma, St. Loius, USA. All other chemicals and reagents used were analytical reagent grade.

2.2. Animals

Male Spraque Dawly rat (200 g) selected from inbreed animal colonies of Amala Cancer Research Centre were used for the preparation of microsomal fraction (S9). The experiment was carried out according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) Government of India and approved by the Institutional Animal Ethics Committee, Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India.

2.3. Preparation of the extract

Sporocarps of *P. rimosus* growing on the jackfruit tree trunks were collected from the out skirts of Thrissur, Kerala, India. The specimen was identified and a voucher specimen was deposited in the Herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB MUBL 3171).

Extract was prepared according to the procedure described in Ajith and Janardhanan (2002). Briefly, 200 g of dried, powdered sporocarps were extracted with petroleum ether. The defatted material was extracted with ethyl acetate for 8–10 h using Soxhlet apparatus. The solvents were completely evaporated at 40 °C using a rotary vacuum evaporator. The residue was designated as ethyl acetate (EtOAc) extract (2.5 g) which was pre-solubilised in DMSO for the assay.

2.4. Bacterial strains

Antimutagenic activity was determined using *Salmonella typhymurium* strains TA 98, TA 100, TA 102 and TA 1535. The strains were procured from Professor B.N. Ames, University of California, Berkeley, USA. The strains were initially cultured in nutrient broth for 12 h and stored at −70 °C as frozen permanent in the presence of 9% DMSO. Thawed frozen permanent (40 μl) inoculated in nutrient broth (5 ml), incubated overnight at 37 °C and was used for the antimutagenic assay (Ajith et al., 2005).

2.5. Mutagens

NaN_3, MNNG, doxorubicin (DXN), B[*a*]P, 2-AF, and NPDA were used as the mutagens. NPDA, B[*a*]P, 2-AF and MNNG were dissolved in DMSO. NaN_3 and DXN were dissolved in distilled water.

2.6. Antimutagenic assay using direct acting mutagens

Antimutagenic activity was determined by the method of Maron and Ames (1983). NaN_3 (2 μg/plate), MNNG (1 μg/plate), DXN (10 μg/plate), and NPDA (20 μg/plate) were used as the direct acting mutagens.

Freshly grown overnight culture of *Salmonella* tester strain (TA 98, TA 100, TA 102 and TA 1535) in nutrient broth (0.1 ml, approximately 10^9 colony forming bacterial cells/ml) was mixed with 0.1 ml of various concentrations of extract of *P. rimosus* (0.5, 1 and 2 mg/plate), 0.2 ml of 0.5 mmol/l histidine plus biotin solution and 0.01 ml of mutagen in 2 ml of molten top agar at 45 °C. The mixture was gently poured on minimal glucose agar plate and incubated for 48 h at 37 °C. After the incubation, numbers of revertant colonies per plate were counted against the slight background growth using a colony counter. The percent inhibition was calculated using the formula $\{(1-(R_2-SR)/(R_1-SR)\}\times 100$, where R_1 is the average number of revertant colonies in the presence of mutagen alone (control), R_2 the average number of revertant colonies in the presence of mutagen plus extract and SR (spontaneous revertants) is the average number of revertant colonies in the plate without extract or mutagen. The antimutagenicity of the extract will be evaluated from the increase in percent inhibition of mutations. To evaluate the toxicity of the extract, the revertant colonies as well as the background bacterial growth in the plates treated without mutagens but with extract (2 mg/plate) in a similar manner, as described above, was employed (Ajith and Soja, 2006). All the experiments were repeated twice in triplicate.

2.7. Antimutagenic activity against mutagens needing activation

2.7.1. Preparation of rat liver microsomal fraction (S9)

Male Spraque Dawly rat was treated with sodium phenobarbitone (0.1%) in drinking water for 4 days (Maron and Ames, 1983). After an overnight fasting, animal was killed by decapitation, liver removed and washed several times in 0.15 M chilled KCl. Homogenate was prepared aseptically using 0.15 mol/l KCl (3 ml/g wet liver). The homogenate was centrifuged in a cooling centrifuge at 9000*g* for 10 min at 4 °C. The supernatant was used as the S9 fraction.

2.7.2. Preparation of S9 mix

Five milliliter of the S9 mix was prepared by adding sterile reagents in the following order, 1.675 ml of sterile distilled water; 2.5 ml of 0.2 mol/l sodium phosphate buffer (pH 7.4); 0.2 ml of 0.1 mol/l NADP; 0.025 ml of 1 mol/l glucose-6-phosphate; 0.1 ml of $MgCl_2$–KCl solution (1.65 mol/l KCl + 0.4 mol/l $MgCl_2$); and 0.5 ml of rat liver S9 fraction (Ajith et al., 2005).

The antimutagenic activity test against indirect acting mutagens, B[*a*]P (0.005 mg/plate) and 2-AF (0.025 mg/plate), was carried out by the Ames plate incorporation method (Maron and Ames, 1983). Activation was accomplished by treating the mutagen with the S9 mix before plating on the minimal agar plate. The reaction mixture was prepared by adding 0.5 ml of S9 mix, 0.01 ml of mutagen, 0.1 ml of various concentrations of the extract of *P. rimosus* (0.5, 1 and 2 mg/plate), 0.1 ml of the freshly grown overnight TA 98 *S. typhimurium* culture and 0.2 ml of 0.5 mmol/l histidine plus biotin solution to 2 ml of molten top agar at 45 °C. The mixture was poured on the minimal agar plate. The plates were incubated at 37 °C for 48 h. Number of revertants colonies and percent inhibition of mutation were calculated as described above. All the experiments were repeated in triplicate.

2.8. Preliminary phytochemical analysis

Preliminary phytochemical analysis of the extract was done by thin layer chromatography on silica gel-G using *n*-butanol:acetic acid:water (4:1:5 or 12:3:5), ethyl acetate:methanol:water (100:13.5:10), toluene:ethyl acetate:formic acid (60:30:10) or chloroform:methanol (90:10) as solvent systems (Harbone, 1973). The chromatogram was examined under u.v and also detected using the following reagents: $FeCl_3$:$K_3Fe(CN)_6$ (1:1), 1% alcoholic $FeCl_3$, vanillin-H_2SO_4, acetic anhydride-H_2SO_4 and 10% alcoholic KOH (Wagner et al., 1984).

2.9. Statistical analysis

All experimental data were expressed as mean ± S.D. The data were analyzed by one-way analysis of variance (ANOVA) using MSTAT-C, soft ware package, UK. If found significant, the average number of revertant colonies in the extract treated plate with that of mutagen alone treatment was further analyzed by the post test, Dunnett's-*t* test. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Antimutagenic activity against direct acting mutagens

The ethyl acetate extract of *P. rimosus* showed significant ($p < 0.05$) antimutagenic activity against direct acting mutagens. The extract at 2 mg/plate exhibited 34%, 50%, and 66.5% inhibition of mutagenicity induced by NaN_3 on *Salmonella* strains TA 100, TA 102 and TA 1535, respectively (Table 1). MNNG induced revertants were found to be reduced significantly ($p < 0.01$) in 2 mg ethyl acetate extract incorporated plate (Table 2). The inhibition was 60.2 (TA100) and 92.4% (TA102) compared to the mutagen alone treated plate. We found that the antimutagenic activity of the extract at 2 mg/plate against NPDA-induced revertants was also significant (Table 3). Ethyl acetate extract at 2 mg/plate inhibited NPDA induced mutation of TA 98 (64.4%) and TA 100 (63.3%). Similarly, the extract (2 mg/plate) inhibited the DXN induced mutations of TA 98 (89%) and TA 102 (97.8%) (Table 4), which was found to be the maximum among the antimutagenic activity evaluated using the direct acting mutagens.