Anticancer activity of P. rimosus

Chapter 9

Anticarcinogenic activity of
Phellinus rimosus
Section I

9.1 Introduction

Chemoprevention is an area of cancer research that is considered to be of greatest potential for reducing mortality in particular, gastrointestinal cancers, skin, breast, lung and colon cancers (Lee et al., 1992 and Costa 1993). This intervention strategy appears helpful particularly in subjects at high risk for cancer development. The approach depends on the ability of certain chemical agents to block initiation and promotion events that occur during the process of neoplastic development. Compounds that prevent the initiational (mutational) events of neoplastic development can be expected to be chemopreventive agents.

The role of polycyclic aromatic hydrocarbons (PAH) are clearly implicated in the process of carcinogenesis especially 7,12-dimethylbenz[a]anthracene (DMBA) which is one of the most potent skin carcinogens known. Most of the metabolically activated PAHs are mutagenic to DNA (Miller, 1978). 12-O-tetradecanoylphorbol-13-acetate (TPA) is a skin tumor promoter isolated from seed oil of Croton tiglium and has been extensively studied in DMBA induced mouse skin tumor model. Inflammation and free radicals have been associated with cancer in various tissues including skin, bladder, stomach and colon. The experimental evidences strongly suggested the role of free radical mediated tumor promotion in phorbol ester promoted papilloma on mouse skin (Lewis and Adams, 1987, Cerutti, 1985). The applications of croton oil have been shown to reduce antioxidant enzymes in both epidermal and inflammatory cells (Solanki et al., 1981). Inhibition of ROI generation can serve as an important system for the identification of agents that can inhibit oxidative DNA damage as well as tumor promotion.

Ethyl acetate extract of P. rimosus showed higher antioxidant, anti-inflammatory and antimutagenic activities, was selected for the antipromotional activity. The inhibition of tumor promoting activity was determined using the classic two-stage carcinogenesis model in mouse skin.

9.1.2 Materials and methods

9.1.2.1 Preparation of extract of P. rimosus

Ethyl acetate extract of P. rimosus was prepared as described in the section 3.2.1.

9.1.2.2 Animals

Female Balb/c mice (25 ± 2 g) were used for the study.
9.1.2.3 Determination of antipromotional activity using two-stage carcinogenesis

Female Balb/c mice were shaved on their back using surgical clippers 2 days before the experiment. Animals with complete hair growth arrest were grouped into 3 groups of eight animals each. The skin tumor was initiated with a single topical application of 390 nmol of 7,12-dimethyl benz[a]anthracene (DMBA) in 200 µl acetone (Mimura et al., 1994). One week after tumor initiation, the promotion was induced by topical application of 200 µl of freshly isolated croton oil (section 4.2.4) (10 % in acetone, v/v) twice weekly for 8 weeks to the same area (Verma and Boutwell, 1980 and Divan et al., 1985). The ethyl acetate extract of *P. rimosus* (1 mg and 5 mg in 200 µl acetone/mouse) was applied topically 40 minutes before each croton oil application. The group treated with croton oil alone served as positive control. Skin papilloma formation was recorded weekly in each experimental group. Average number of papilloma per mouse, percent of animals with papilloma and tumor latency period were recorded.

9.1.3 Results

9.1.3.1 Determination of antipromotional activity of ethyl acetate extract

Topical application of ethyl acetate extract inhibited skin papilloma initiated by DMBA and promoted by croton oil on mouse skin (Fig. 9.1.4). Group of animals applied with croton oil and DMBA showed 87.5 % tumor incidence at 15 weeks after DMBA treatment. Application of ethyl acetate extract of *P. rimosus* prior to croton oil reduced the percent of incidence. Topical application of extract at a dose of 1 mg showed 62.5 % incidence at 15 weeks and at a dose of 5 mg showed 37.5 % incidence at 15 weeks (Fig. 9.1.1). The percent animals with tumor in the control group of animals attained maximum at 7 week after tumor promotion by croton oil. The average number of tumor (1mm diameter) per animal in the control group was 5 nos at 7 weeks after the croton oil application, while the average number of tumor per animal in the 1 and 5 mg extract treated group of animals was 2 and 1.33 respectively (Fig. 9.1.2). The tumor latency period in the control, extract 1 and 5 mg treated groups was 39, 49 and 56 days respectively (Fig. 9.1.3).
Figure 9.1.1. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on croton oil induced tumor promotion in mouse skin.

Figure 9.1.2. Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on croton oil induced tumor promotion in mouse skin.
Figure 9.1.4- Effect of ethyl acetate extract (EtOAc) of *P. rimosus* on DMBA induced and croton oil promoted skin papilloma on mice skin. a) and b) DMBA + croton oil; c) DMBA + croton oil + EtOAc (1 mg/skin) and d) DMBA + croton oil + EtOAc (5 mg/skin). (Arrowmark indicated papilloma)
9.1.4 Discussion

Polycyclic aromatic hydrocarbon (PAH) must be metabolically activated to electrophilic intermediates, which can bind to DNA and exert their carcinogenic effects. Studies on the mutagenicity, tumorigenicity of the PAHs have indicated that bay region diol-epoxides are the ultimate carcinogenic species and the binding of these diol-epoxides to DNA can explain their carcinogenicity (Jerina and Daly, 1977; Jerina et al., 1979). Current evidence indicates that the metabolic activation of DMBA occurs primarily through the formation of a 3,4-diol-1,2-epoxide (Tang et al., 2000). ROS production by double or multiple TPA treatments is closely associated with the metabolic activation of proximate carcinogens and the increased levels of oxidized DNA bases. Persistent oxidative stress in cancer may also constantly activate transcription factors, such as NF-κB, through the intracellular signal transduction.
system and induce expression of proto-oncogenes such as c-fos, c-jun and c-myc (Toyokuni et al., 1995). Oxidative stress induces DNA damage such as modified base products and strand breaks that may lead to further mutation and chromosomal aberration, in the single mutated clones.

Experimental results indicate that applications of ethyl acetate extract of P. rimosus before each application of croton oil directly scavenged the free radical or inhibited the generation of free radicals. This was evident from decreased skin lipid peroxidation induced by croton oil when pretreated with the extract (section 4.3.3). The extract also inhibited the croton oil mediated skin inflammation. Hence the antipromotional activity of extract is probably mediated through the radical scavenging activity of the extract (section 5.3.4). The tumor latency period in animals that were treated with 5 mg is extended significantly compared to the control as well as the 1 mg treated group. The average number of tumor/animal is also decreased in the extract treated groups compared to the control group. The results are indicating the efficacy of the extract in delaying the skin tumor incidence in animals.
9.2.1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with limited effective therapeutic options available. Chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and dietary aflatoxin B1 (AFB₁) contamination are considered as important etiological factors (Sheu et al., 1992). The absence of effective systemic chemotherapeutic agents and the mortality associated with intrahepatic, rather than metastatic growth of HCC have led many investigators to focus on developing better methods of local tumor control. Among the several group of chemical carcinogens implicated in human hepatocellular carcinogenesis, nitrosocompounds form the largest group (Preussmann et al., 1984). The exposure to N-nitrosamines can be either from environment or from the diet. Majority of human cancers are currently thought to be caused by environmental factors (Doll and Peto, 1981) with food being one of the most important modifying agent (Waynder and Gobi, 1977). Cooking methods like roasting, grilling, baking and deep-frying in open furnaces, of foods, seem to increase the formation of N-nitrosamines. Similarly tumorigenic agents attributed to the initiation and promotion of tumor in the gas phase of cigarette smoke has also been identified as nitrosamines such as dimethylnitrosamine, diethylnitrosamine and nitropyrrolidine (Block, 1992). Heterocyclic amines in food and cigarette smoke are important exogenous source of free radical.

An alternative approach to control cancer is chemoprevention which refers to the administration of chemical agents to prevent the initiation (mutational) and promotional events that occur during the process of neoplastic development (Boone et al., 1990). Hence a chemopreventive agent exhibiting activities such as anti-inflammation, inhibition of carcinogen induced mutagenesis, inhibition of phase I enzyme activity and scavenging of free radical could play a decisive role in the inhibition of chemical carcinogenesis either at the initiation or promotion stage. Medicinal mushrooms useful against cancer are known in many countries. The anticancer activity of aqueous extract of P. rimosus was studied and the results are presented in this chapter.
9.2.2 Material and Methods

9.2.2.1 Preparation of extract of P. rimosus

The yield of aqueous extract was high and selected for the long-term carcinogenesis study using NDEA. The extract was prepared as described in the section 3.2.1.

9.2.2.2 Animals

Male Wistar rat (150 ± 30 g) were used for the study.

9.2.2.3 Effect of P. rimosus extract on NDEA induced hepatocellular carcinoma

Male Wistar rats were used for the experiment. HCC was induced according to the method of Jose et al., (1999) with some modifications. Animals were divided into 4 groups of 6 animals in each group. The group 1 treated with vehicle (distilled water) was maintained as normal. Group 2 treated with NDEA (4 mg/kg body wt, p.o.) for 5 days/week for 20 weeks were kept as untreated control. Group 3 and 4 were administered orally with 25 and 50 mg/kg body wt respectively with aqueous extract of P. rimosus 1 h prior to each NDEA administration. One week after the last dose of NDEA administration, animals were kept fasting overnight and then sacrificed. Coagulated and noncoagulated (heparin) blood were collected by heart puncture for serum and plasma respectively. Serum was used for the determination of glutamate oxaloacetate transaminase (GOT) (section 3.2.12), glutamate pyruvate transaminase (GPT) (section 3.2.13), alkaline phosphatase (ALP) (section 3.2.14), gamma glutamyl transpeptidase (GGT) (section 3.2.15), total protein (section 3.2.18), albumin (section 3.2.19), and lipid peroxidation (malondialdehyde) (section 3.2.21). Plasma was analyzed for fibrinogen (section 3.2.20).

Liver was removed and washed thoroughly in ice-cold saline and homogenate (10 %) was prepared in PBS (50 mM, pH 7). The homogenate was used for the estimation of reduced glutathione (GSH) (section 3.2.3), glutathione peroxidase (GPx) (section 3.2.6) glutathione S-transferase (GST) (section 3.2.7), and protein by the method as described in the section 3.2.10.

9.2.2.4 Histopathological examination

A portion of the liver was fixed in 10 % formalin and then embedded in paraffin. 5 μm microtome sections were prepared from each liver and stained with hematoxilin-eosin.
9.2.3 Results

9.2.3.1 Effect of extract on NDEA induced hepatocellular carcinoma

Aqueous extract of *P. rimosus* inhibited the NDEA induced hepatocellular carcinoma in a dose dependent manner (Fig. 9.2.2). Treatment of NDEA 5 days/week for 20 weeks induced hepatocellular carcinoma in all the control group animals. The number of tumors and percent of incidence was reduced significantly in animals administered with the 50 mg/kg body wt extract. The activity of the SGOT (345 ± 10 IU/l), SGPT (798.1 ± 25.2 IU/l) and ALP (429.1 ± 20 IU/l) was elevated significantly (*P*<0.01) in the NDEA alone treated animals compared to the normal group of animals. Marked decline (*P*<0.01) was observed in the activities of these enzymes in group treated with the extract plus NDEA (Table 9.2.1).

The NDEA alone treated animal group also showed hyperfibrinogenemia (167.2 ± 14.5 mg/dl) compared to the extract plus NDEA treated animals 8.46 ± 0.92 g/dl (Table 9.2.2). The control animal group showed elevation of total protein (Table 9.2.2), hence the albumin/globulin ratio (Table 9.2.3) was altered (*P*<0.01) compared to the normal animal group. Treatment of the extract prevented the alteration of the A:G ratio. The activity of the serum GGT was found reduced significantly (*P*<0.01) in the extract plus NDEA treated animal group compared to the control group of animals (50 mg/kg body wt) (Table 9.2.4).

The index of lipid peroxidation, MDA was elevated (*P*<0.01) in the serum of the NDEA alone treated animal group (3.4 ± 0.2 nmol/ml) compared to the normal and *P. rimosus* treated groups (Table 9.2.4). The activities of GST (Fig. 9.2.1), GPx and GSH (Table 9.2.5) level in the liver homogenate of the extract (50 mg/kg) plus NDEA treated animals showed a significant decrease (*P*<0.01) compared to the NDEA treated group.

Histopathological analysis indicated that the NDEA alone treated liver cells were arranged mostly in solid and trabecular pattern, with cellular polymorphism, fatty infiltration, varying mitotic figures and focal necrotic changes. All these changes clearly indicated the hepatocellular carcinoma. These pathological manifestations were decreased high to moderate level respectively in the 50 mg/kg and 25 mg/kg body wt extract treated group of animals (Fig.9.2.2).
Table 9.2.1. Effect of aqueous extract (AQ) of *P. rimosus* on serum GPT, GOT and ALP activities in rats with HCC induced by NDEA.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments (mg/kg)</th>
<th>SGPT (IU/l)</th>
<th>SGOT (IU/l)</th>
<th>ALP (IU/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>Vehicle</td>
<td>125.8 ± 10.6</td>
<td>71.1 ± 3.1</td>
<td>146.3 ± 11.5</td>
</tr>
<tr>
<td>(NDEA)</td>
<td>--</td>
<td>798.1 ± 25.2</td>
<td>345.0 ± 10.0</td>
<td>429.1 ± 20.0</td>
</tr>
<tr>
<td>AQ + NDEA</td>
<td>25</td>
<td>442.8 ± 16.2</td>
<td>210.3 ± 14.1</td>
<td>247.6 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>311.8 ± 14.7</td>
<td>142.3 ± 9.1</td>
<td>202.1 ± 14.7</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6

*P<0.01 (lsd) significantly different from normal.

*P<0.01(Dunnett’s t-test) significantly different from control.

Table 9.2.2. Effect of aqueous extract (AQ) of *P. rimosus* on serum fibrinogen and total protein levels in rats with HCC induced by NDEA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments (mg/kg)</th>
<th>Fibrinogen (mg/dl)</th>
<th>Total protein (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal Control</td>
<td>Vehicle</td>
<td>96.6 ± 5.6</td>
<td>5.93 ± 0.08</td>
</tr>
<tr>
<td>(NDEA)</td>
<td>--</td>
<td>167.2 ± 14.5</td>
<td>8.46 ± 0.92</td>
</tr>
<tr>
<td>AQ + NDEA</td>
<td>25</td>
<td>123.0 ± 6.8</td>
<td>7.19 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>110.6 ± 1.9</td>
<td>6.17 ± 0.22</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6

*P<0.01 (lsd) significantly different from normal.

*P<0.01(Dunnett’s t-test) significantly different from control.
### Table 9.2.3. Effect of aqueous extract (AQ) of *P. rimosus* on serum albumin, globulin and albumin/globulin (A/G) ratio in rats with HCC induced by NDEA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments (mg/kg)</th>
<th>Albumin (mg/dl)</th>
<th>Globulin (mg/dl)</th>
<th>A/G (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Vehicle</td>
<td>3.16 ± 0.07</td>
<td>2.84 ± 0.24</td>
<td>1.11 ± 0.08</td>
</tr>
<tr>
<td>Control (NDEA)</td>
<td>--</td>
<td>2.78 ± 0.05*</td>
<td>5.67 ± 0.87*</td>
<td>0.49 ± 0.07*</td>
</tr>
<tr>
<td>AQ + NDEA</td>
<td>25</td>
<td>3.06 ± 0.05a</td>
<td>4.41 ± 0.76a</td>
<td>0.70 ± 0.10a</td>
</tr>
<tr>
<td>, ,</td>
<td>50</td>
<td>3.21 ± 0.08a</td>
<td>2.95 ± 0.23a</td>
<td>1.09 ± 0.09a</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6

*P<0.01 (lsd) significantly different from normal.

aP<0.01(Dunnett’s t-test) significantly different from control.

### Table 9.2.4. Effect of aqueous extract (AQ) of *P. rimosus* on serum GGT and MDA activity in rats with HCC induced by NDEA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments (mg/kg)</th>
<th>GGT (U/l) at 25°C</th>
<th>MDA (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Vehicle</td>
<td>21.5 ± 6.09</td>
<td>1.46 ± 0.08</td>
</tr>
<tr>
<td>Control (NDEA)</td>
<td>--</td>
<td>70.5 ± 13.1*</td>
<td>3.40 ± 0.21*</td>
</tr>
<tr>
<td>AQ + NDEA</td>
<td>25</td>
<td>42.5 ± 3.67a</td>
<td>2.75 ± 0.18a</td>
</tr>
<tr>
<td>, ,</td>
<td>50</td>
<td>31.1 ± 3.25a</td>
<td>1.90 ± 0.05a</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6

*P<0.01 (lsd) significantly different from normal.

aP<0.01(Dunnett’s t-test) significantly different from control.
Table 9.2.5. Effect of aqueous extract (AQ) of _P. rimosus_ on hepatic GPx and GSH activity in rats with HCC induced by NDEA

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments (mg/kg)</th>
<th>GPx (U/l)</th>
<th>GSH (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Vehicle</td>
<td>22.5 ± 1.8</td>
<td>8.40 ± 0.32</td>
</tr>
<tr>
<td>Control (NDEA)</td>
<td>--</td>
<td>36.8 ± 4.0*</td>
<td>11.10 ± 1.00*</td>
</tr>
<tr>
<td>AQ + NDEA</td>
<td>25</td>
<td>32.8 ± 2.6b</td>
<td>9.30 ± 0.47a</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>24.7 ± 0.8a</td>
<td>8.55 ± 0.20a</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6
*P<0.01 (lsd) significantly different from normal.

^aP<0.01 and ^bP<0.05 (Dunnett's t-test) significantly different from control.

Figure 9.2.1. Effect of aqueous extract (AQ) of _P. rimosus_ on hepatic GST activity in rats with HCC induced by NDEA. Values are mean ± S.D, n=6. *P<0.01 (lsd) significantly different from normal. ^aP<0.01 (Dunnett's t-test) significantly different from control.
Figure 9.2.2- Histology of hepatocellular carcinoma induced by NDEA in rat. (a) Normal, (b) NDEA; (c) NDEA + aqueous extract (25 mg/kg body wt.) and (d) NDEA + aqueous extract (50 mg/kg body wt.). Magnification x 20. Stain H&E. (Arrowmark indicated necrotic changes).

Morphology of hepatocellular carcinoma induced by NDEA in rat. e) NDEA applied liver and f) NDEA and aqueous extract (50 mg/kg body wt.) treated liver. (Arrowmark indicated liver tumor).
9.2.4 Discussion

Results of the present investigations indicate that the aqueous extract of *P. rimosus* is an effective chemopreventive agent against the NDEA induced hepatocarcinogenesis. This conclusion is supported by various biological properties of the extract. Treatment of the extract prior to the NDEA administration significantly reduced the tumor incidence compared to the control group of animals. The serum GGT activity was significantly elevated in the NDEA alone treated group of animals indicating the induction of hepatocellular carcinoma. However, treatment of the extract prior to NDEA showed a significant reduction of the tumor marker in a dose dependent manner. This is in agreement with elevated hepatic GST activity in the NDEA treated animal. Various hepatomas exhibited high levels of GST-P protein, as usually observed in pre-neoplastic and neoplastic lesions after chemical hepatocarcinogenesis (Satosh et al., 1991). The low level of the hepatic GST in the extract plus NDEA treated animal supports its ability to inhibit tumor progression. Further, the hepatocellular carcinoma is associated with hyperfibrinogenemia (Amemiya et al., 1997). This was due to synthesis of this protein by the carcinoma cells. The plasma fibrinogen level decreased in the extract treated group. The elevated serum GOT, GPT, ALP and altered A:G ratios are indicative of hepatic damage in the NDEA treated animals compared to animals administered with extract prior to NDEA treatment. The elevated hepatic GGT activity is responsible for the increased GSH level in the control group, which is found to be decreased in the extract treated group. In addition to elevated GST, increased expressions of both γGT and GPx have been implicated in drug resistance (Tew, 1994). Decreased hepatic GPx, GST and serum γGT activity in the extract treated animal group compared to control support the efficacy of the treatment.

NDEA has been shown to metabolized by the microsomal mixed function oxidase (MFO) system to its active ethyl radical metabolites CH$_3$CH$_2^+$. This reactive radical interact with DNA producing mutation and oncogenesis. Studies in the hepatoma indicate disequilibria of the delicate oxidant versus antioxidant balance, which is tilted towards an oxidant side (Boittier et al., 1995). This oxidative stress might be the reason for the elevated MDA level in the serum of NDEA treated animals. Lipid peroxidation can result in the formation of several toxic bye-products such as 4-hydroxynonenal and malondialdehyde that can attack the cellular targets including DNA, inducing mutagenicity and carcinogenesity (Park and Floid, 1992 and Ramal et
The treatment of the extract prior to the NDEA administration significantly reduces the level of lipid peroxidation. Inhibition of lipid peroxidation may largely because of scavenging lipid peroxyl radicals. The reducing activity of the aqueous extract also partially explains the antiperoxidation activity. The observation that dietary ascorbate inhibits the carcinogenic action of several nitroso-compounds fed to animals can be attributed to its ability to reduce them to inactive forms (Halliwell and Gutteridge, 1999). The histopathological observations support the above findings. The liver of animals treated with the extract and NDEA shows a significant reduction of mitotic level and hyperplasia compared to the liver of NDEA alone treated animals.

Reactive oxygen species such as $O_2^-$, $^\cdot OH$, $H_2O_2$ and $NO^-$ participate in the initiation or promotion of cancer through their ability to cause point mutations, DNA cross-links and DNA strand breaks (Park and Fleid, 1992 and Nguyen et al., 1992). Oxidants have the capacity to induce the transcription of growth competence related protooncogene C-fos and C-jun (Burdon, 1995). Induction of these immediate genes represents a prerequisite for the stimulation of the cell proliferation. A marked increase in the expression of cellular onogenes such as C-ras, C-fos, C-myc and N-myc, involved in neoplastic transformation, has been detected in the rat hepatomas as early as the first month after diethylnitrosamine treatment (Boitier et al., 1995).

The in vitro radical scavenging activity of the extract partially explains its mechanism in the prevention of hepatocarcinogenesis. Reducing the nitric oxide generation in the digestive tract was found to be effective in preventing the reaction of nitrites with amines and amides to form carcinogenic nitrosamines and nitrosamides (Boone et al., 1990). The NO$^-$ scavenging activity of P. rimosus extract could also support the preventive role against NDEA induced hepatocellular carcinoma. Recently, neutrophil-mediated nitrosamine formation has been showed to be a possible endogenous carcinogen, which may promote neoplasia (Grishman et al., 1992). The results of the earlier study reveal that aqueous extract of P. rimosus possesses antimitogenic and anti-inflammatory activities that may also contribute to the exhibited anticancer activity.

Phytochemical analyses of the extract show the presence of polysaccharides, protein bound polysaccharides and polyphenols. A number of polysaccharides and protein bound polysaccharides isolated from mushrooms are clinically used for the treatment of cancer. Cancer preventive properties possessed by the aqueous extract of P. rimosus might be mediated through these active ingredients.