CHAPTER 4

Anti-carcinogenic activity of Phyllanthus amarus extract
4.1 INTRODUCTION

Prevention of carcinogenesis is one of major strategies for cancer control. Carcinogenesis is a multistage process that encompasses prolonged accumulation of injuries at several different biological level and producing both biochemical and genetic changes in cells. At each level, there is an opportunity for intervention, a chance to prevent, slow or even halt the gradual change of healthy cells towards malignancy (Peter., 1996). In spite of the immense efforts to improve treatment and find cure for advanced disease, overall mortality rates for most forms of epithelial cancer have not declined in the past 25 years (Hong and Sporn., 1997). Efforts to prevent the disease would be a more desirable as well as practical approach for cancer control.

Stomach cancer is the third commonest malignancy in South India. Exposure to environmental nitrite and nitrosation of smoked foods has been associated with an increased risk of stomach cancer. Although the key nitrosating agent is nitrite, the situation with regard to the formation of carcinogenic nitrosocompounds is greatly complicated by the presence of other chemicals in the environment.

In our center several phytochemicals have been isolated and identified and have been demonstrated to block or suppress the different stages of carcinogenesis (Sukumaran et al., 1994, Kumar and Kuttan., 2001, Jose et al., 1997, Joy et al., 2000, Jose et al., 1999).

Simultaneous administration of *P. amarus* extract along with carcinogen has been reported to inhibit the hepatocellular carcinoma development induced by NDEA (Joy and Kuttan., 1998). *P. amarus*
extract could inhibit the hepatocarcinogenesis and increased life span in tumour bearing animals (Kumar and Kuttan., 2000). *P. amarus* extract administration increased the life span of ascites tumour harboring mice and inhibited the sarcoma development induced by 20MC (Kumar et al., 2002).

The present study was designed to assess its anti-carcinogenic activity of *P. amarus* against DMBA induced papilloma formation in BALB/c mice as well as its effect on MNNG induced gastric cancer in Wistar Rats.

4.2 Methods

4.2.1 Determination of the effect of *P. amarus* treatment on papilloma formation initiated by DMBA and croton oil

The concept of two stage carcinogenesis consisting of initiation and promotion was first proposed by Berenblum., 1941. The former stage is an irreversible process while the latter is associated with reversible and irreversible changes, 12-0-Tetradecanoyl phorbol 13-acetate (TPA), present in croton oil is a typical tumour promoter having various biological and biochemical effects on susceptible tissues (Harris., 1991).

Methanolic extract of *P. amarus* was prepared as mentioned earlier. Dried concentrations of *P. amarus* was dissolved in 200μl methanol.

Male BALB/c mice were used for these studies. They were kept as groups of 10 animals / group. Aggressive males were removed and kept separately. The dorsal region (2 cm diameter) of mice was shaved
with a razor at least two days before treatment with DMBA. Mice, which did not show signs of hair regrowth, were used for the experiments. Animals were divided into different groups;

Group I- DMBA + croton oil, twice weekly for 8 weeks (Positive control).
Group II- DMBA alone (Initiation only).
Group III- DMBA + methanol (200µl/ mouse, topical) 30 minutes before croton oil application, twice weekly for 8 weeks (Vehicle control).
Group IV- *P. amarus* (1mg/ mouse, topical), 10 continuous days prior to the application of DMBA followed by croton oil, twice weekly for 8 weeks (Prior to initiation).
Group V- *P. amarus* (5mg / mouse, topical), 10 continuous days prior to the application of DMBA followed by croton oil application, twice weekly for 8 weeks (Prior to initiation).
Group VI- DMBA + *P. amarus* (1mg/ mouse, topical) 30 minutes before each croton oil application, twice weekly for 8 weeks (Drug promotion).
Group VII- DMBA + *P. amarus* (5mg/ mouse, topical) 30 minutes before each croton oil application, twice weekly for 8 weeks (Drug promotion).

Single dose of DMBA (470nmol/ mouse in 200µl acetone) was used in this study (George & Kuttan, 1997). Two weeks after DMBA application animals in Group I, III, IV, V, VI, and VII were applied with 10% croton oil (in 200µl acetone). *P. amarus* was administered topically dissolved in methanol. The animals in all groups were watched for food intake as well as any apparent symptoms like weight loss or mortality.
during the entire period of study. Skin tumour formation was recorded weekly, and the tumours greater than 1mm in diameter were included in the cumulative total if they persisted for 2 weeks or more. Delays in the onset of tumours in various groups were recorded.

4.2.2 Determination of the effect of P. amarus on N-Methyl-N' nitro-N nitrosoguanidine (MNNG) induced stomach cancer

Treating the animals with MNNG results in the formation of preneoplastic cells. Further treatment of MNNG causes conversion of preneoplastic cells to neoplastic cells. Anti-neoplastic activity of extract is evaluated by administering P. amarus extract with MNNG, which may prevent the development of neoplastic cells.

Male Wistar rats (10 animals / group) were used for the study. They were divided into four groups:

Group I : Normal, untreated
Group II : MNNG treated
Group III : MNNG + P. amarus 150mg/kg. b. wt
Group IV : MNNG + P. amarus 750 mg/ kg. b. wt.

MNNG was given orally as a solution in water at a concentration of 1mg/ ml for the first 28 weeks. P. amarus was given orally starting from the day of MNNG administration and continued for 20 weeks. The control group (Group II) was given distilled water. All the rats were killed at the 44th week of the experiment. The esophagus, forestomach, glandular stomach and duodenum were removed. The stomach was opened along the greater curvature and the gastric mucosa was examined grossly. The location and size of tumours were recorded.
The following biochemical parameters were done in the homogenized stomach mucosa to assess the anti-carcinogenic activity of *P. amarus*, γ-glutamyl trans peptidase (Tate and Meister., 1974), cytosolic glutathione-S-transferase (Habig et al., 1974), tissue glutathione (Moron et al., 1979) and cytosolic glutathione reductase activity (Racker., 1955).

Tumour and normal appearing stomach sections were stained with haematoxylin and eosin and were examined under microscope (10x). Nucleolar organizer region associated proteins (AgNORS) were studied in paraffin sections of stomach mucosa (Method given in chapter II).

4.3 RESULT

4.3.1 Effect of *P. amarus* extract on papilloma formation

*P. amarus* administration prior to DMBA application (Group IV & V) showed an inhibition in papilloma development in mice, indicating that *P. amarus* had an effect on the tumour initiation process. These inhibitory effects were also dependent on the concentration of *P. amarus*. Topical application of *P. amarus* was found to be effective in delaying tumour appearance. In the control animals the first tumour appeared at 4 weeks after DMBA application where as in *P. amarus* treated groups the first tumour appeared at 8 weeks (Fig 4.1). *P. amarus* also lowered the percentage of tumour bearing mice (Fig 4.2). All the control animals developed tumours by 20 weeks while only 56% of the 5mg *P. amarus* treated group-developed tumours at the 20th week. The average number of tumours per mouse at the 20th week were
6.2± 2.6 in the control group, and in 1 and 5 mg *P. amarus* treated groups were 3.4± 1.5 and 2.6±1.8 respectively (Table 4.1).

Topical application of *P. amarus* prior to croton oil administration in DMBA initiated mice also resulted in a significant protection against skin tumour promotion in a dose dependent manner. *P. amarus* administration substantially lowered the percent of mice with tumours and decreased the total number of tumours per mice (Fig 4.3)

Application of DMBA (470nmol/mouse) alone (group II) did not produce any tumours, suggesting that this concentration level was ineffective to elicit carcinogenic potential without further promotion. Moreover animals topically treated with DMBA, croton oil and methanol (Group III) was found to produce tumours like control group suggesting that methanol itself could not inhibit papilloma formation.

### 4.3.2 Effect of *P. amarus* extract on MNNG induced carcinogenesis

*P. amarus* extract administration inhibited the stomach cancer induced by MNNG in a dose dependent way. All the untreated rats in MNNG group developed tumours. Administration of *P. amarus* inhibited tumour development in the stomach of rats as seen in the 44th week of the experiment. Only 66% animals developed tumours in the 150mg/kg *P. amarus* treated group and only 44% in the animals treated with 750mg/kg body weight of *P. amarus* extract.

A typical morphological picture of stomach of rats treated with MNNG as well as MNNG and *P. amarus* is given in Fig 4.4. All the untreated animals had significant number of tumourus in the non-glandular region of stomach. Number of tumours developed in *P.
amarus treated group was found to be significantly less.

In MNNG treated group, the stomach weight was increased to 1.06±0.10g/100g b.wt as compared to normal rat stomach weight 0.53± 0.05g/100g b.wt. The rats which were treated with 150 and 750mg/ kg of P. amarus extract showed 0.89± 0.08 and 0.71± 0.06g/ 100g b.wt of stomach weight (Table 4.2).

The treatment with P. amarus extract effectively lowered the elevated γ-GT, a marker of neoplasm (Hanigan and Pitot., 1985) from 20.3± 6.7 to 2.8± 0.9μmol/min/mg protein (Table 4.3). This result indicated that P. amarus could reduce the proliferation of tumour cells. MNNG administration increased mucosal GST to 1317.6±211 n mol/min/mg protein as compared to normal value, which was 344.9± 22 n mol/min/mg protein. Administration of P. amarus 150 and 750mg/ kg b.wt significantly reduced these elevated levels to 779.8±144 and 494.8±76 n mol/min/mg protein respectively. Similarly stomach mucosal GR was increased to 368±66 nmol/min/mg protein as compared to normal value of 129±24 nmol/min/mg protein, which was significantly reduced by 150 and 750mg of P. amarus administration to 286± 41 and 192±45 n mol/min/mg protein respectively. MNNG administration decreased GSH from the normal value of 9.8±1.2 n mol/min/mg protein to 4.6±0.9 n mol/min/mg protein. Administration of 150 and 750 mg/ kg b. wt of P. amarus increased these levels to 5.5±1.3 and 8.5±1.4 n mol/min/mg protein respectively.

During the experiment, the rats in the control, MNNG treated
group and *P. amarus* treated group did not show any significant difference in the body weight (Fig 4.5).

### 4.3.2.1 Effect of *P. amarus* on AgNOR counts

The normal rats showed very low AgNOR values. This is to be expected since in the normal cells, hypertranscriptional activity of rDNA genes is not necessary. AgNOR dots and clusters of MNNG administered rats were increased (Fig 4.6) as compared to normal rats. AgNOR dots and clusters of normal rats were found to be 1.5±0.29 and 0.4±0.11, which were raised to 3.9±0.98 and 1.5±0.45 respectively by MNNG administration. AgNOR dots and clusters were reduced by *P. amarus* treatment. *P. amarus* treatment 150 and 750mg/ kg reduced dot value to 2.9±0.30 and 1.8±0.51 and cluster value to 1.0±0.47 and 0.7±0.37 respectively (Table 4.4).

### 4.3.2.2 Histopathology

Histopathology of rat stomach treated with MNNG with or without *P. amarus* is shown in Fig 4.7.

Fig 4.7. a, Normal stomach. There was no evidence of malignancy or any other specific lesions in the sections.

Fig 4.7. b, MNNG treated rat stomach

Sections show stomach with non-secretary area showing squamous epithelial lining which in some areas show hyperplasia, papillary formation, loss of polarity and increased mitosis. Some areas show hyperkeratinisation. Section from the secretary area show a few mucosal glands, most areas showing squamous metaplasia, the squamous cells having features of malignancy as evidenced by
pleomorphism, loss of polarity and increased mitosis. Some areas show normal columnar epithelial lined mucosal glands.

Fig 4.7.c, MNNG and *P. amarus* 150mg/ kg treated rat stomach

Sections from the non-secretory area of the stomach show squamous lining in many areas with hyperkertosis of outer layers. The epithelial cells in some areas had hyperplasia, pleomorphism, loss of polarity and increased mitosis. The muscle layer appears normal but some areas showed tumour necrosis. Sections from the secretary portions of the stomach were having squamous metaplasia in some areas. The columnar cells of the gastric glands showed pleomorphism, loss of polarity, and increased mitosis. The muscle layer is normal. There are areas of tumour cell necrosis.

Fig 4.7.d, MNNG and *P. amarus* 750mg/ kg

Sections show stomach with the non-secretory area being lined by squamous epithelium. Squamous cells show hyperplasia, some areas showing pleomorphism and loss of polarity. Gastric wall in the non-secretary area appear normal. Tumour cell necrosis are seen in some places. Sections from the secretary area show necrosis of the glands in some places, where the outline of the cells could not be made properly. The other places the cells show pleomorphism and loss of polarity. The muscle layer is normal. Necrosis tumour cells are seen in some places.

In summary histopathology of rat stomach treated with MNNG with or without *P. amarus* indicated that there was marked necrosis of the stomach tumour in animals treated with *P. amarus* and tumour burden was much less compared to untreated animals.
4.4 DISCUSSION

In this chapter we have shown the anti-carcinogenic activity of *P. amarus* extract. *P. amarus* administration was found to inhibit papilloma formation by the two stage carcinogenesis induced by DMBA as initiator and croton oil as promoter in mice and MNNG induced stomach cancer in rats. Administration of *P. amarus* was found effective indicating a direct role of *P. amarus* in modulating carcinogenesis. *P. amarus* was found to be having anti-oxidant activity and was shown to scavenge the oxygen free radicals generated *in vitro*. Cancer promotion involves increased oxygen radical production that produces DNA strand breaks. This may be the reason for increased effectiveness when the drug administration was continued during promotion period.

The involvement of oxidative stress and lipid peroxide formation have been found in the process of DMBA carcinogenesis (Baoxu et al., 1998). Sasaki et al., 1995 reported that transversion of adenine to thymine occurs in H- ras oncogene in mouse treated with DMBA/TPA. The free radical hypothesis is also supported by the fact that the anti-oxidants can inhibit the DMBA/TPA carcinogenesis (Slaga et al., 1995).

Several anti-oxidant and anti-inflammatory compounds isolated from plants such as resveratol, quercetin, kaemphenol, rutin, curcumin etc effectively inhibit the DMBA/croton oil induced carcinogenesis in experimental animals (Meishiang et al., 1997, Kato et al., 1993, Soudamini and Kuttan., 1989).

*P. amarus* extract was also found to have significant activity against MNNG induced stomach cancer. γ-GT, GST and GR, which
were elevated very high in MNNG treated animals, were almost normalized by co-treatment with *P. amarus* extract. MNNG treatment was found to suppress the level of GSH and this was found to be significantly increased by *P. amarus* treatment.

Nucleolar organizer regions (NORs) are loops of DNA located on acrocentric chromosomes in the nuclei of normal and abnormal cells (Sirsat and Khanolkar., 1962, Massimo et al., 1990). They encode for ribosomal RNA (rRNA) and the associated proteins are argyrophilic (hence the term AgNOR). Since RNA are the sites of protein synthesis, the number of AgNORS per nucleus has a direct relationship to the cellular activity (Newbold et al., 1988). It has been shown recently that the increase in the number of AgNORS in a proliferating cell, is due to a wider dispersal of otherwise compact clusters of NOR associated proteins (Crocker et al., 1988).

AgNOR staining has now been recommended as a prognostic (Simha et al., 1996) and diagnostic tool in human (Anon., 1987) and canine (Bostock et al., 1989) histopathology. Statistical significance of AgNORs, showed that tumours having less than 4 AgNOR counts and less proliferative index will be of a benign lesion, while more counts than that will indicate tumours of a malignant nature (Mehrotra and Chandra., 1998). *P. amarus* extract also produced significant decrease in AgNOR counts indicating that the extract has anti-carcinogenic activity.
Histological studies of MNNG induced gastric tumourigenesis indicated that *P. amarus* prevents gastric mucosal development or stops its progression.

The novel hydrolysable tannin, named amariin and geraniin, corilagin, 1, 6- digalloylucopyranoside, rutin and quercetin -3-0-glucopyranoside, were isolated from the polar fraction of the methanolic extract of aerial parts of *P. amarus* (Foo., 1993). Some of the hydrolysable tannins isolated from *P. amarus* were found to be potent inhibitors of wheat embryo Ca\(^{2+}\) dependent protein kinase (CDPK), rat brain Ca\(^{2+}\) protein kinase and phospholipid dependent protein kinase (PKC) and Ca\(^{2+}\)- calmodulin dependent myosin light chain kinase (Polya et al., 1995). How this activity is related with the anti- carcinogenic activity of the extract is not known at present. The extract was also found to inhibit P450 enzymes, which are needed in the activation of carcinogen (Kumar et al., 2002). Inhibition of cell cycle regulation, topoisomerase II (Kumar et al., 2002), P450 enzymes as well as anti-oxidant activity may tribute to the overall activity of the extract against carcinogenesis induced in animals.
Table 4.1 Effect of *P. amarus* extract on papilloma induction initiated by DMBA and promoted by Croton oil

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal status</th>
<th>Number of mice developed papillomas by 20 weeks</th>
<th>Number of papillomas per tumour bearing mice</th>
<th>% reduction in papillomas per tumour bearing mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DMBA+ Croton oil</td>
<td>9/9</td>
<td>6.2±2.6</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>DMBA alone</td>
<td>0/7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>III</td>
<td>DMBA+ Croton oil+ Methanol</td>
<td>8/8</td>
<td>6.1±2.5</td>
<td>-</td>
</tr>
<tr>
<td>IV</td>
<td>DMBA+ Croton oil+ <em>P. amarus</em> (1mg/mouse, Prior treatment, Topical)</td>
<td>6/9</td>
<td>3.4±1.5*</td>
<td>45.2</td>
</tr>
<tr>
<td>V</td>
<td>DMBA+ Croton oil+ <em>P. amarus</em> (5mg/mouse, Prior Topical)</td>
<td>5/9</td>
<td>2.6±1.8**</td>
<td>58.1</td>
</tr>
<tr>
<td>VI</td>
<td>DMBA+ Croton oil+ <em>P. amarus</em> (1mg/mouse, Topical)</td>
<td>7/9</td>
<td>3.8±1.9*</td>
<td>38.7</td>
</tr>
<tr>
<td>VII</td>
<td>DMBA+ Croton oil+ <em>P. amarus</em> (5mg/mouse, Topical)</td>
<td>5/9</td>
<td>3.2±2.2**</td>
<td>48.4</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01 as compared with group I
Table 4.2 Effect of *P. amarus* in incidence of tumours and stomach weight in MNNG induced rat gastric carcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (Dose/kg b. wt)</th>
<th>Number of tumour bearing rats</th>
<th>Relative stomach Weight (g/100g/body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>Nil</td>
<td>0.53±0.05</td>
</tr>
<tr>
<td>II</td>
<td>Control (MNNG)</td>
<td>9/9</td>
<td>1.06±0.10</td>
</tr>
<tr>
<td>III</td>
<td><em>P. amarus</em> 150mg</td>
<td>6/9</td>
<td>0.89±0.08***</td>
</tr>
<tr>
<td>IV</td>
<td><em>P. amarus</em> 750mg</td>
<td>4/9</td>
<td>0.71±0.06***</td>
</tr>
</tbody>
</table>

***P< 0.001 as compared with group II
Table 4.3 Effect of *P. amarus* on γ-GT, GST, GSH and glutathione reductase levels in the stomach mucosa of rats treated with MNNG

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (Dose/kg b. wt)</th>
<th>γ-GT (umol/min/mg protein)</th>
<th>GST (nmol/min/mg protein)</th>
<th>GSH (nmol/min/mg protein)</th>
<th>GR (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>1.6± 4.6</td>
<td>344.9± 22</td>
<td>9.8± 1.2</td>
<td>129± 24</td>
</tr>
<tr>
<td>II</td>
<td>Control (MNNG)</td>
<td>20.3± 6.7</td>
<td>1317.6± 211</td>
<td>4.6± 0.9</td>
<td>368± 66</td>
</tr>
<tr>
<td>III</td>
<td><em>P. amarus</em> 150mg</td>
<td>10.5± 1.4**</td>
<td>779.8± 144**</td>
<td>5.5± 1.3</td>
<td>286± 41**</td>
</tr>
<tr>
<td>IV</td>
<td><em>P. amarus</em> 750mg</td>
<td>2.8± 0.9***</td>
<td>494.8± 76***</td>
<td>8.5± 1.4**</td>
<td>192± 45***</td>
</tr>
</tbody>
</table>

* P< 0.05; **P< 0.01; ***P< 0.001 as compared with group II
Table 4.4 Mean frequency of AgNOR dots and clusters with different doses of *P. amarus*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment (Dose/kg b.wt)</th>
<th>AgNOR dots</th>
<th>AgNOR clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Normal</td>
<td>1.5± 0.29</td>
<td>0.4± 0.11</td>
</tr>
<tr>
<td>II</td>
<td>Control (MNNG)</td>
<td>3.9± 0.98</td>
<td>1.5± 0.45</td>
</tr>
<tr>
<td>III</td>
<td><em>P. amarus</em> 150mg</td>
<td>2.9± 0.30*</td>
<td>1.0± 0.47</td>
</tr>
<tr>
<td>IV</td>
<td><em>P. amarus</em> 750mg</td>
<td>1.8± 0.51**</td>
<td>0.7± 0.37*</td>
</tr>
</tbody>
</table>

*P< 0.05; **P< 0.01 as compared with group II
Fig 4.1 Inhibitory effect of P. amarus extract on the number of papilloma/mice developed by DMBA/Croton
Fig 4.2 Effect of *P. amarus* extract on DMBA/Croton oil induced papilloma
Fig 4.3

**Photograph of Balb/c mice**

a, Mouse showing papillomas induced by DMBA and croton oil

b, Mouse treated DMBA and croton oil along with *P. amarus* 5mg/ dose shows reduction in the number of papillomas
Fig 4.4

Morphology of stomach in rats

a, Morphology of normal rat stomach

b, Stomach of rats administered with MNNG showing numerous tumours with hairs

c, Stomach morphology of MNNG administered animals treated with 150mg/ kg of *P. amarus* showing reduction in the number of tumours

d, Stomach morphology of MNNG administered animals treated with *P. amarus* 750mg/ kg showing similarity to normal rats
Fig 4.5 Effect of *P. amarus* on body weight of rats treated with MNNG
Fig 4.6

Histology of rat stomach

a, Ag NOR staining of MNNG administered rat stomach showing increased AgNOR dots and clusters (40x)

b, *P. amarus* 750mg/kg treated rats stomach showing AgNOR dots and clusters similar to that of normal stomach (40X)
Fig 4.7

Histopathology of rat stomach

a, Histology of stomach of normal rats showing normal mucosa, sub mucosa and muscle layer (H& E 10x)
b, Non secretary area of stomach of MNNG administered animal showing hyperplasia, papillary formation, loss of polarity, increased mitosis. Squamous cells in secretary area showing pleomorphism, loss of polarity and increased mitosis (H& E 10x)
c, Stomach of MNNG administered animals treated with P. amarus 150mg/ kg body weight showing tumour cell necrosis (H& E 10x)
d, Histology of rat stomach treated with P. amarus 750mg/ kg showing normal gastric wall in the non secretary area, muscle layer in the secretary area is normal (H& E 10x)
Figure 4.7