CHAPTER 6

Anti-inflammatory activity and inhibition of gastric lesion by Phyllanthus amarus extract
6.1 INTRODUCTION

The role of oxygen free radicals in the inflammatory process is well established (Halliwell and Gutteridge, 1985). Oxygen radicals and products derived from the action of lipoxygenase and cyclooxygenase enzymes play an important role in the process of inflammation. As the inflammation is mainly produced by the oxidative burst of the macrophages, many antioxidants may be effective to reduce the inflammation. Most widely used non-steroidal anti-inflammatory drugs (NSAID) suffer from severe side effects such as gastrointestinal disturbances, irritations of the stomach mucosa and renal injury. Thus the development of more efficacious agents both potent and selective to inhibit cyclooxygenase (COX) (Hrish et al., 1981) and lipoxygenase (Feuerstein and Hallenbeck, 1987) pathways that produce prostaglandins, thromboxanes and leukotriones are needed.

Ulcerative lesions of the gastrointestinal tract are one of the major side effects associated with the use of NSAIDS (Pihan et al., 1987), alcohol (Mizui et al., 1987) stress (Cochran et al., 1982) and ischemic reperfusion (Itoh and Guth, 1984). Although the mechanism of NSAIDS, ethanol and other drug induced gastric lesion is unclear, accumulating neutrophils (Perry et al., 1986), oxygen free radicals (Istav and Kay, 1988), inhibition of prostaglandin synthesis (Glavin and Szabo, 1992) play a crucial role. Several herbal drugs and Ayurvedic preparations (Satayavati et al., 1987) inducing saponin glycosides have been shown to be protective against drug induced gastric mucosal
injury (Vanisree et al., 1996, Manonmani et al., 1995). Several natural
drugs have been reported to possess anti-ulcerogenic activity by virtue
of their predominant effect on mucosal defensive factors (Sairam et al.,
2001). Infusion of young shoots of *P. amarus* has been recommended to
lessen ooedematous swelling and ulcers (Mhaskar et al., 2000).

Based on these evidences, we investigated anti-inflammatory
activity of *P. amarus* using experimental paw oedema produced by
carrageenan administration. We have also looked the protection of
gastric lesion by *P. amarus* extract.

**6.2 METHODS**

**6.2.1 Determination of anti-inflammatory activity of methanolic
and aqueous extracts of *P. amarus***.

Anti-inflammatory activity was determined by carrageenan
inbred strains of BALB/c mice weighing 25-28g (6-7 weeks old) were
used for the experiment. They were divided into 4 groups of four
animals each.

- **Group I** : Control, carrageenan alone
- **Group II** : Carrageenan + 100mg/ kg body wt. of *P. amarus* extract
- **Group III** : Carrageenan + 250mg/ kg body wt. of *P. amarus* extract
- **Group IV** : Carrageenan + 500mg/ kg body wt. of *P. amarus* extract

*P. amarus* extract (methanolic and aqueous extracts) were given
orally as a single dose 1 h prior to the experiment. Paw oedema was
induced by injecting carrageenan (200μg/20μl) into the sub-plantar
region of the left paw. The thickness of paw oedema was measured by vernier calipers before treatment and after injection with carrageenan. Measurement was continued at 60 minutes intervals up to 8h and further at 24th h. The inhibition of paw oedema was calculated by comparing the difference in paw thickness of the control and treated group. Experiment was repeated twice and average values were taken.

6.2.2 Effect of P. amarus on induction of ethanol induced gastric lesion

Ethanol induced peptic ulcer is occurring as a result of disturbance of the natural balance between aggressive acid / pepsin and mucosal defence/ mucosal turnover. Administration of ethanol produces ulcerative lesions and increases lipid peroxidation in the gastric mucosa, which plays a significant part in the pathogenesis of the mucosal lesions. Free radicals created by ethanol injury attack protein in gastric mucosa proteins which leads to a reduction in protein levels.

Adult male Wistar rats weighing 120 g were used for the experiment. They were grouped into four groups of five animals each. All the animals were fasted for 16h and deprived of water for 12h prior to the experiments.

Group I : Normal, untreated
Group II : Alcohol alone
Group III : Alcohol + 50 mg P. amarus extract/kg body wt
Group IV : Alcohol + 200 mg P. amarus extract/kg body wt
Group V : Alcohol + 1000 mg P. amarus extract/kg body wt
P. amarus extract was given as a single dose 30 minutes prior to the experiment. Acute gastric lesion was induced by absolute ethanol (Robert et al., 1979). Briefly, absolute ethanol (8 ml /kg body wt) was administered intragastrically to control and drug treated animals. Each animal was sacrificed by ether overdose 1h after administration of ethanol. Latter on the stomach was excised, opened along the greater curvature and washed gently with ice cold solution. The stomach weight and intraluminal bleeding were recorded.

The extent of erosion of stomach mucosa was assessed from a scoring system designed by Merazzi- Uberti Turba as follows. 0: no erosions; 1: 1-3 small erosions (4 mm or smaller); 2: more than three small erosions or one large erosion; 3: Two large erosions; 4: 3-4 large erosions and 5: more than 4 large erosions or lesion proliferation (Giordano et al., 1990). The results were expressed in terms of an ulcer index, which is the average severity of erosions of rat, per each group on the scale from 0-5.

The mucosa of glandular stomach was removed by scraping with a blunt knife and 10% homogenate was prepared. Reduced glutathione (GSH) in the gastric mucosa was determined (Moron et al., 1979). The protein content of the gastric mucosa was quantified by the Lowry method (Lowry et al., 1951). The details are given in Chapter II.

A portion of the stomach from each group was stained with hematoxylin and eosine and evaluated by light microscopy. Details are given in Chapter II.
6.3 RESULTS

6.3.1 Effect of *P. amarus* extract on inflammation

Development of paw oedema was observed in both control and treated group after carrageenan injection. Thickness of the paw was found to be increased initially upon injection of carrageenan due to volume effect. Difference in the thickness of mice paw oedema was further increased during the time interval of 60 to 180 min in control group. Water extract of *P. amarus* (100, 250 and 500 mg/kg) produced an inhibition of 26%, 33% and 39% respectively at 3h (Fig. 6.1). While the methanol extract of *P. amarus* (100, 250 and 500 mg/kg) produced an inhibition of 29%, 37% and 42% respectively at 3h (Fig. 6.2) and significant inhibition of paw oedema was observed throughout the course of the experiment up to 8 hours.

6.3.2 Effect of *P. amarus* extract on gastric lesion

The present investigation indicated that rat mucosal gastric injury induced by ethanol was significantly and dose dependently reduced by methanolic extract of *P. amarus*. Administration of absolute ethanol to fasted rats resulted in severe gastric damage visible from the outside of the stomach as thick reddish-black lines. After opening, the gastric lesions were found in the mucosa and consisted of elongated bands, 1-10 mm long, usually parallel to the long axis of the stomach. They were located mostly in the corpus (the portion of the stomach secreting acid and pepsin). No gross lesions developed in the fore stomach (the non-secretary part of the stomach) (Fig 6.3).
Intragastric administration of the absolute ethanol to rats resulted in 50% mortality due to acute reaction of the alcohol and its metabolites (Table 6.1). Increased mortality in the controls was found to be aggravated due to the fasting (16h) and deprivation of water (12h). The rats, which died, had perforated lesions and severe intraluminal bleeding. Stomach weight in alcohol treated rats was increased to 1.02±0.13 g/100 g body weight as compared to normal rat stomach weight 0.68±0.05 g/100 g body weight possibly due to inflammation (Table 6.2). In the treated animals because of scavenging of the oxygen radicals generated by ethanol, the mortality rate and increase in stomach weight induced by ethanol was found to be significantly less. All animals treated with absolute ethanol caused intraluminal bleeding in the glandular portion of the stomach, while all animals in the P. amarus (200 and 1000 mg/kg) pre treated group were found to be significantly protected from intraluminal bleeding (Table 6.2).

Ethanol administration to rats produced gastric damage with an ulcer index of 4.75±0.5 (Table 6.3). P. amarus pre treatment (50, 200 and 1000 mg/kg) significantly reduced the ulcer index to 3.5±0.6, 2.0±0.5 and 0.6±0.5, respectively. Doses of 50, 200 and 1000mg/kg of P. amarus extract inhibited ulceration by 26.3, 57.9 and 87.4% respectively.

Animals treated with absolute ethanol produced 48.8% reduction in gastric mucosal GSH (Table 6.4). 50, 200, 1000mg/kg of P. amarus extract reduced the depletion of GSH to 36, 16.5 and 5.5 % respectively.
Histological analysis of ethanol treated rat stomach revealed the presence of necrotic debris in the lamina propria of the mucosa which are infiltrated with polymorphonuclear leucocytes (Fig 6.4). The depth of the lesion extended up to the muscularis mucosae with red blood corpuscles extravasation. The submucosa of the corpus was markedly thickened by oedema but devoid of polymorphonuclear leucocytes. Histologically, the stomach of the *P. amarus* pretreated groups (250 and 1000 mg/kg) showed superficial erosion in the mucosa and moderate degree of sub-mucosal oedema with neutrophilic infiltration.

### 6.4 DISCUSSION

Narcotizing agents such as ethanol, when given intragastrically to rats produce sever gastric hemorrhagic erosions. Oxygen free radicals are implicated in the pathogenesis of ethanol induced gastric mucosal injury (Istav and Brune., 1988, Hiraishi et al., 1999) apart from other mechanisms such as mucosal leukotriene release (Peskar et al., 1986), submucosal venular constriction (Oates and Hakkineu., 1988). Ethanol induced gastric injury is associated with the significant production of free radicals (Istav and Brune., 1988) leading to increased lipid peroxidation which cause damage to cell and cell membranes (Fridovich., 1978). Accumulation of activated neutrophils in the gastric mucosa may be a source for free radicals (Tepperman and Soper., 1990). The ethanol induced gastric mucosal damage was shown to be associated with the significant reduction in the non-protein sulphhydryl concentration in cultured rat gastric mucosa cells (Szabo et al., 1981).
Ethanol induced gastric lesion formation may be due to stasis in gastric blood flow which contributes to the development of the haemorrhage and necrotic aspects of the tissue injury.

The present study indicating that *P. amarus* extract has significant effect in reducing inflammation and ulcer is again reflective of its activity oxygen radical scavenger. Reactive oxygen species has been shown to have significant effect on the cellular system, damaging its structure and inducing alteration especially in its high molecular weight components. Large doses of ethanol have been shown specifically affecting the inner lining of the stomach producing erosion. In liver, ethanol is converted to ethanal (aldehyde) which is more toxic. Ethanal has been shown to reduce GSH levels in liver tissues. Fall in GSH increases lipid peroxidation. Pre treatment with the *P. amarus* extract may reduce oxygen radical production and there by reduces its effect on the liver. Similar mechanism could also be postulated for its anti-inflammatory activity of *P. amarus*. Inflammation produced by carageenan is mainly due to macrophage activation and thereby producing oxygen radicals. *P. amarus* extract cold inhibit the oxygen radicals and there by reduces the inflammation.

Another possible mechanism for the activity of the extract to inhibit gastric lesion produced by alcohol may be due to the formation of a protective layer of the polyphenolic compounds present in the extract with the protein of the stomach lining by hydrophobic interaction. Moreover the extract may inhibit the prostaglandin
synthesis as in the case of nonsteroidal anti-inflammatory drugs. It has been shown that *P. amarus* extract could inhibit the onset of diarrhea induced by castor oil and reduced frequency of defecation and also reduced gut meal travel distance significantly (Odetola and Akojenu., 2000). This effect has been attributed to the inhibition of prostaglandin synthesis. Extract may also inhibit macrophage migration which can reduce the inflammatory response produced by carrageenan.

A drug that possess both anti-inflammatory and anti-ulcer activity is of great therapeutic importance as most of the anti-inflammatory drugs used in the modern day medicine are ulcerogenic.
Table 6.1 Effect of *P. amarus* administration on mortality of rats treated with absolute ethanol

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>P. amarus</em> extract</th>
<th>Mortality</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td>Number</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Normal rats</td>
<td>Nil</td>
<td>0/5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>Nil</td>
<td>5/10</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>50</td>
<td>1/5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>200</td>
<td>0/5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>1000</td>
<td>0/5</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table 6.2 Effect of *P. amarus* administration on stomach weight and intraluminal bleeding of rats treated with absolute ethanol

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>P. amarus</em> extract mg/kg</th>
<th>Stomach weight g/100g b. wt</th>
<th>Intraluminal bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>Nil</td>
<td>0.68±0.05</td>
<td>0/5 0%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Nil</td>
<td>1.02±0.13</td>
<td>5/5 100%</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>50</td>
<td>0.85±0.10*</td>
<td>2/4 50%</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>200</td>
<td>0.79±0.05**</td>
<td>0/5 0%</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>1000</td>
<td>0.70±0.08**</td>
<td>0/5 0%</td>
</tr>
</tbody>
</table>

* P< 0.05; **P< 0.01 as compared with group II
Table 6.3 Effect of *P. amarus* administration on the ulcer index of rats treated with absolute alcohol

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>P. amarus</em> extract mg/kg</th>
<th>Ulcer index ±SD</th>
<th>Inhibition % in ulcer index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>Nil</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Nil</td>
<td>4.75±0.5</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>50</td>
<td>3.5±0.6***</td>
<td>26.3</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>200</td>
<td>2.0±0.5***</td>
<td>57.9</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>1000</td>
<td>0.6±0.5***</td>
<td>87.4</td>
</tr>
</tbody>
</table>

***P < 0.001 as compared with group II
Table 6.4 Effect of *P. amarus* administration on the glutathione (GSH) content of the mucosa of rats treated with absolute alcohol

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>P. amarus</em> extract mg/kg</th>
<th>GSH (nmol/mg protein)</th>
<th>% reduction in GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>Nil</td>
<td>12.7±0.8</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Nil</td>
<td>6.5±0.6</td>
<td>48.8</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>50</td>
<td>8.1±1.1*</td>
<td>36</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>200</td>
<td>10.6±1.1***</td>
<td>16.5</td>
</tr>
<tr>
<td>Ethanol+<em>P. amarus</em></td>
<td>1000</td>
<td>12.0±0.5***</td>
<td>5.5</td>
</tr>
</tbody>
</table>

* P< 0.05; ***P< 0.001 as compared with group II
Fig 6.1 Anti-inflammatory activity of aqueous extract of *P. amarus*
Fig 6.2 Anti-inflammatory activity of methanolic extract of *P. amarus*
Gross morphology of rat stomach

a, Morphology of normal rat stomach

b, Stomach of rat administered with absolute ethanol showing intraluminal bleeding and inflammation

c, Stomach morphology of ethanol administered animals treated with *P. amarus* 200mg/kg

d, Stomach morphology of ethanol administered animals treated with *P. amarus* 1000mg/ kg showing normal size and texture
Figure 6.3
Fig 6.4

Histopathology of rat stomach

a, Histology of normal rat stomach (H& E 10x)
b, Gastric mucosa of ethanol administered rats showing severe lesion that extents to muscularis mucosa (H& E 10x)
b,Stomach lesions of ethanol administered animals treated with *P. amarus* 200mg/ kg showing lesion in the gastric mucosa (H& E 10x)
d, Gastric mucosa of ethanol administered animals treated with *P. amarus* 1000mg/ kg showing pattern similar to that of normal gastric mucosa (H& E 10x)