CHAPTER 4
ANTI INFLAMMATORY AND PLATELETAGGREGATION INHIBITING ACTIVITIES OF PLEUROTUS FLORIDA AND PLEUROTUS SAJOR-CAJU
4.1 INTRODUCTION

Inflammation is a complex reaction in the vascularized connective tissue which can be defined fundamentally a protective response against injury. It has been implicated in pathophysiology of various clinical disorders including cancer. Many different mediators such as prostaglandins, leukotrienes and platelet aggregating factor (PAF) are involved in inflammatory response. By modulating the release of these mediators one can effectively control this process (Cuzzocrea et al., 2001).

It has been demonstrated that metastatic tumour cell lines induce platelet aggregation (Suffness and Pezzuto, 1991) and subsequent to aggregation, platelet release substances that promote tumour cell growth (Cowan and Graham 1982). Thus inhibition of platelet aggregation may be considered an approach for inhibition of tumour dissemination (Ambrus et al., 1982). Thromboxanes and prostaglandins (PGs) appear to be important in the pathogenesis of platelet aggregation and inflammation. Most widely used nonsteroidal anti-inflammatory drugs (NSAID) suffer from severe side effects. Thus the developments of more efficacious agents both potent and selective to inhibit the cyclooxygenase (COX) and lipoxygenase pathways that produce PGs, thromboxanes and leukotrienes are needed.

Mushrooms have a notable place in the folklore throughout the world. They are traditionally used in China and Japan for medicinal and tonic purposes. Oyster mushrooms (Pleurotus species) are known as an efficient blood pressure lowering agent, diuretic, cholesterol reducer, adjuvant and
aphrodisiac (Guzman, 1994) and have also been shown to modulate the immune system (Chang, 1996). Our recent investigations revealed that *P. florida* and *P. sajor-caju* possessed significant antioxidant and antitumour properties. In this chapter we report the anti-inflammatory and antiplatelet aggregating activities of these mushrooms.

**4.2 MATERIALS AND METHODS**

**4.2.1 ANIMALS**

Male Balb/c mice of 4-5 weeks age (20-25g) were employed for studies.

**4.2.2 PREPARATION OF THE EXTRACT**

Methanolic extracts of both *P. florida* and *P. sajor-caju* were prepared as described in the section 2.2.1.

**4.2.3 ANTI-INFLAMMATORY ACTIVITY**

Anti-inflammatory activity was determined by carrageenan induced acute and formalin induced chronic paw edema models in mice.

**4.2.3.1 CARRAGEENAN INDUCED PAW EDEMA IN MICE**

Animals were divided in to six groups comprising six animals in each group. In all groups acute inflammation was produced by sub-plantar injection of 0.02 ml freshly prepared 1% carrageenan in normal saline in the right hind paw of mice. One group injected with carrageenan alone served as positive control. Two groups were administered with methanolic extract of *P. florida* at a concentration of 500 and 1000 mg/Kg body weight intraperitoneally and another two groups with same concentrations of methanolic extract of *P. sajor-caju*, 30 minutes prior to carrageenan injection. One group was administered
with Diclofenac (10 mg/Kg, i.p), as standard reference drug. The paw thickness was measured using vernier calipers before and 3 hour after carrageenan injection (Ajith and Janardhanan, 2001).

Increase in paw thickness was calculated using the formula Pt-Po, where Pt is the thickness of paw at time t (i.e., 3hrs after carrageenan injection) and Po is the paw thickness at 0 time. Percent inhibition was calculated using the formula \( \frac{(C-T)}{C} \times 100 \), where C is the increase in paw thickness of the control and T is that of treatments.

### 4.2.3.2 FORMALIN INDUCED PAW EDEMA IN MICE

Animals were divided into six groups comprising six animals in each group. In all groups chronic inflammation was produced by a single sub plantar injection of 0.02 ml of 2% formalin in the right hind paw of mice (Ajith and Janardhanan, 2001). Two groups of animals were administered intraperitoneally with methanolic extract of *P. florida* (500 and 1000 mg/Kg body weight), and another two groups with same concentrations of methanolic extract of *P.sajor-caju*, one group with standard reference drug Diclofenac (10 mg/Kg body weight) intraperitoneally 30 minutes prior to formalin injection and one group injected with formalin alone served as control. The administration of the extracts (500 and 1000 mg/Kg body weight) and diclofenac was continued once daily for six consecutive days. The paw thickness was measured using vernier calipers before and 6 days after formalin injection.
Increase in paw thickness was calculated using the formula $Pt-Po$, where $Pt$ is the thickness of paw at time $t$ (and 6 days after formalin injection) and $Po$ is the paw thickness at 0 time. Percent inhibition was calculated using the formula $(C-T/C) \times 100$, where $C$ is the increase in paw thickness of the control and $T$ is that of treatments.

4.2.3.3 EFFECT OF METHANOLIC EXTRACTS OF *P. FLORIDA* AND *P. SAJOR-CAJU* ON CROTON OIL INDUCED EDEMA IN MICE

Croton oil was isolated from the seeds of *Croton tiglium* according to the method of Berenblum (1941). Croton oil contains 12-0-tetra decanoyl phorbol -13- acetate (TPA) an inducer of inflammation. The back of 50 mice was shaved using surgical clippers before two days of experiment. Animals with complete hair growth arrest were grouped into 4 groups of 10 animals each and treated as follows. Methanolic extract of *P. florida* (2 and 10 mg in 0.2 ml of acetone) was applied topically to the shaved area of dorsal skin 30 minutes before application of croton oil (0.2 ml 50% croton oil in acetone). After 24 hrs the extract and croton oil application was repeated on the same area. The group treated with 0.2 ml of croton oil in acetone alone was kept as control. One hour after the second treatment of croton oil animals were sacrificed and the skin punches were obtained with 8 mm diameter cork borer. The skin punches were weighed immediately in an analytic balance the percent inhibition was calculated after comparing with the control group (Lakshmi et al., 2002).
Skin punches obtained from the above experiment, after double croton oil treatments were minced in 20 mM Tris HCl buffer (pH 7) and homogenized to obtain 10% homogenate. The homogenate was employed to lipid peroxidation according to the method of Ohkawa et al., (1979) using 1,1,3,3-tetramethoxy propane as the standard and percent inhibition was calculated after comparing with the control. The protein content was determined according to the method of Lowry et al., (1951). Excised skin was fixed in 10% formalin and then embedded in paraffin. Microtome sections were prepared from each skin punch and stained with hematoxylin eosin. The sections were assessed for pathological changes due to inflammation.

The experiment was repeated using methanolic extract of *P. sajor-caju* for determining its anti-inflammatory activity. Treatment schedule and all experimental parameters were repeated as described earlier except *P. sajor-caju* extract was used for experiment.

**4.2.3.3.1 DETERMINATION OF TISSUE LIPID PEROXIDATION**

The level of lipid peroxidation was measured as malondialdehyde (MDA) according to the method of Ohkawa et al., (1979).

**PRINCIPLE**

The tissue malondialdehyde was allowed to react with TBA. The MDA-TBA adduct formed during the reaction in acidic medium was extracted to the organic layer and the absorbance was measured at 532 nm.
PROCEDURE

4 ml of reaction mixture containing 0.4 ml of the tissue homogenate, 1.5 ml of 0.8 % TBA, 1.5 ml of acetic acid (20 %, pH 3.5) and distilled water was kept for 1 h in a boiling water bath at 95°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 1 ml of distilled water. 5 ml of butanol: pyridine mixture (15:1) was added to the reaction tube, mixed thoroughly and centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was measured at 532 nm against butanol:pyridine mixture. The MDA was calculated with the help of a standard graph made by using different concentrations (1-10 nmol) of 1'1'3'3'-tetramethoxypropane in 1 ml distilled water and is expressed as nmol of MDA/mg protein.

4.2.3.3.2 DETERMINATION OF TISSUE PROTEIN

Protein content in the tissue was determined according to the method of Lowry et al. (1951)

PRINCIPLE

The absorbance of blue color developed by the reduction of the phosphomolybdic-phosphotungstic components in the Folin-Ciocalteau reagent by the amino acids tyrosine and tryptophan in the protein and by the biuret reaction of the protein with the alkaline cupric tartrate was measured at 660 nm.

PROCEDURE

0.01 ml of the homogenate was mixed with 0.990 ml of distilled water, 5 ml of alkaline CuSO₄ (0.5 % CuSO₄ in 1 % sodiumpotassium tartrate and 2% Na₂CO₃ in 0.1 N NaOH mixed in the ratio 1:50) was kept for 10 min at room
temperature. 0.5 ml of 1 N Folin phenol reagent was added and absorbance was measured after 20 min at 660 nm against the reagent blank. Protein content was calculated from the standard graph prepared using different concentrations (0.1-0.5 mg/ml) of bovine serum albumin (BSA).

4.2.4 PLATELET AGGREGATION INHIBITING ACTIVITY OF METHANOLIC EXTRACTS OF P. FLORIDA AND P. SAJOR-CAJU

Fresh human blood was collected from healthy donors in anticoagulant solution (2.4% sodium citrate, 1.5% citric acid, and 1.8% dextrose). The ratio of blood to anticoagulant solution was approximately 5:1. The platelet rich plasma (PRP) was separated by centrifugation at 1900 rpm for 7 minutes. PRP thus obtained was centrifuged at 4500 rpm for 18 minutes to sediment the platelets (Chopra et al., 1999). The platelet sediment was dispersed in washing buffer which was composed of (mM) 113 NaCl, 4.3 K$_2$HPO$_4$, 4.3 Na$_2$HPO$_4$, 24.44 NaH$_2$PO$_4$ and 5.5 dextrose, pH 6.5 (Baeziger and Majerus, 1974) and the platelets were collected after centrifugation at 4000 rpm for 10 min. Then platelets were suspended in a buffer composed of (mM) 113 NaCl, 4.3 K$_2$HPO$_4$, 16 Na$_2$HPO$_4$, 8.3 NaH$_2$PO$_4$ and 5.5 dextrose, pH 7.5 (Baeziger and Majerus, 1974). The suspension was adjusted to give a final optical density of approximately 0.5/ml at 600 nm (0.8 X108 cells/ml) (Subramoniam and Satyanarayana, 1989).

The methanolic extract of P. florida (500 µg) was incubated with washed human platelets (1ml) in siliconised tubes for 5, 10 and 20 min at 37°C.
At the end of the incubation period 20 µl of 1mM ADP was added and the O.D at 600 nm was measured at 1 min intervals up to 5min. The reaction mixture devoid of extract was taken as control.

For determining the antiplatelet aggregating activity of P.sajor-caju, fresh human blood was collected from healthy donors in anticoagulant solution and platelets were separated from blood as above. The methanolic extract of P.
sajor-caju (500µg) was incubated with washed human platelets (1ml) in siliconised tubes for 5,10 and 20 min at 37°C. At the end of the incubation period 20 µl of 1mM ADP was added and the O.D at 600 nm was measured at 1 min intervals up to 5min. The reaction mixture devoid of extract was taken as control.

4.3 RESULTS
4.3.1 CARRAGEEANAN INDUCED PAW EDEMA IN MICE
The methanolic extract of both the mushrooms significantly reduced the carrageenan induced paw edema. The reduction of edema by both the mushroom extracts were in a dose dependent manner. P florida extract at concentrations 500 and 1000 mg/Kg bodyweight inhibited the inflammation by 40 and 60 percent respectively and P.sajor-caju inhibited the inflammation by 43.4 and 63 percent at the same concentrations. The inhibition of inflammation at 1000 mg/kg body weight was greater than the standard reference drug diclofenac (Table 4.1).

4.3.2 FORMALIN INDUCED PAW EDEMA
The methanolic extract of both the mushrooms significantly reduced formalin induced paw edema. The reduction of edema by both the mushroom
extracts were in a dose dependent manner. *P. florida* at concentrations 500 and 1000 mg/Kg bodyweight inhibited the inflammation by 43.1 and 64.4 percent respectively and *P.sajor-caju* inhibited the inflammation by 46.6 and 63.1 percent at same concentrations. The inhibition at 1000 mg/kg body weight was greater than the standard reference drug diclofenac (Table 4.2)

4.3.3 CROTON OIL INDUCED SKIN EDEMA

Topical application of the extract prior to croton oil, could markedly inhibit the skin inflammation in mouse. In the 2 and 10 mg treated group, the skin punch weight was significantly reduced compared to the control group (Figs 4.1 & 4.2).

The methanolic extractS of *P.florida* and *P.sajor-caju* were also effective in inhibiting the croton oil induced lipid peroxidation in mouse skin (Fig 4.3 & 4.4). Topical application of the extract 30 minutes prior to croton oil could significantly inhibit the lipid peroxidation compared to control group. The malondialdehyde level as an indicator of lipid peroxidation was elevated in the control group compared to treatments. The extracts at a concentration of 10mg showed the maximum effect.

Histopathological examination of the skin of mice showed marked epidermal thickening, severe lymphocytes infiltration, marked edema and mast cells in the sub epidermis. The pathological manifestations were markedly reduced by the treatment with extracts (Fig 4.7 & 4.8).
4.3.4 PLATELET AGGREGATION INHIBITING ACTIVITY

The addition of ADP to washed human platelets caused a marked decrease in O.D at 600 nm, indicating aggregation of platelets. Preincubation of the platelets with methanolic extract of *P.florida* (500 µg/ml-1) and methanolic extract of *P.sajor caju* (500 µg/ml-1) caused an increase in the O.D indicating the inhibition of platelet aggregation induced by ADP. The inhibitory effect increased with increase in incubation time (Fig 4.5 & 4.6).
Table 4.1. Effect of methanolic extract of *P. florida* and *P. sajor-caju* on carrageenan induced acute inflammation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/Kg)</th>
<th>Increase in paw Thickness after 3hrs</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.120 ± 0.028 a</td>
<td></td>
</tr>
<tr>
<td><em>P. florida</em> extract</td>
<td>500</td>
<td>0.072 ± 0.021 b</td>
<td>40.0</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.048 ± 0.018 d</td>
<td>60.0</td>
</tr>
<tr>
<td><em>P. sajor-caju</em> extract</td>
<td>500</td>
<td>0.069 ± 0.020 b</td>
<td>43.4</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.045 ± 0.016 d</td>
<td>63.0</td>
</tr>
<tr>
<td>Standard (Diclofenac)</td>
<td>10</td>
<td>0.064 ± 0.023 c</td>
<td>47.5</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6 animals
Any two values having a common letter are not significantly different at 5% level, lsd=0.0085

Table 4.2: Effect of methanolic extract of *P. florida* and *P. sajor-caju* on formalin induced chronic inflammation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/Kg)</th>
<th>Increase in paw Thickness after 3hrs</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.225 ± 0.037 a</td>
<td></td>
</tr>
<tr>
<td><em>P. florida</em> Extract</td>
<td>500</td>
<td>0.128 ± 0.029 b</td>
<td>43.1</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.080 ± 0.025 c</td>
<td>64.4</td>
</tr>
<tr>
<td><em>P. sajor-caju</em> extract</td>
<td>500</td>
<td>0.120 ± 0.026 b</td>
<td>46.6</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0.083 ± 0.029 c</td>
<td>63.1</td>
</tr>
<tr>
<td>Standard (Diclofenac)</td>
<td>10</td>
<td>0.131 ± 0.026 b</td>
<td>41.7</td>
</tr>
</tbody>
</table>

Values are mean ± S.D, n=6 animals
Any two values having a common letter are not significantly different at 5% level, lsd=0.01703
Fig 4.1: Effect of methanolic extract of *P. florida* on croton oil induced mouse skin edema

A: Normal, B: Control, C: Methanolic extract of *P. florida* (2mg)
D: Methanolic extract of *P. florida* (10mg)

Values are mean ± S.D, n=6 animals

Any two values having a common letter are not significantly different at 5% level. lsd=3.678
Fig 4.2: Effect of methanolic extract of *P.sajor-caju* on croton oil induced mouse skin edema

A: Normal, B: Control, C: Methanolic extract of *P. sajor-caju* (2mg)
D: Methanolic extract of *P. sajor-caju* (10mg)

Values are mean ± S.D, n=6 animals

Any two values having a common letter are not significantly different at 5% level. lsd= 4.062
Fig 4.3: Effect of methanolic extract of *P. florida* on lipid peroxidation (MDA) inhibition in croton oil induced inflammation in mice

A: Normal, B: Control, C: Methanolic extract of *P. florida* (2mg)
D: Methanolic extract of *P. florida* (10mg)

Values are mean ± S.D, n=6 animals

Any two values having a common letter are not significantly different at 5% level. lsd= 0.1319
Fig 4.4: Effect of methanolic extract of *P.sajor-caju* on lipid peroxidation (MDA) inhibition in croton oil induced inflammation in mice
A: Normal, B: Control, C: Methanolic extract of *P.sajor-caju* (2mg)
D: Methanolic extract of *P.sajor-caju* (10mg)
Values are mean ± S.D, n=6 animals
Any two values having a common letter are not significantly different at 5% level. lsd=0.1204
Fig 4.5: Platelet aggregation inhibiting activity of *P. florida* (500μg/ml)

Fig 4.6: Platelet aggregation inhibiting activity of *P. sajor-caju* (500μg/ml)
Fig 4.7 Antiinflammatory activity of methanolic extract of *P. florida* against croton oil induced inflammation on mice skin. Skin sections stained with H&E. a) Normal b) Croton oil c) Methanolic extract (10mg) + Croton oil
Fig 4.8 Antiinflammatory activity of methanolic extract of *P. sajor-caju* against croton oil induced inflammation on mice skin. Skin sections stained with H&E. a) Normal b) Croton oil c) Methanolic extract (10mg) + Croton oil
4.4 DISCUSSION

The results of the present investigations reveal that methanolic extracts of *P. florida* and *P. sajor-caju* possess significant anti-inflammatory activity against carrageenan induced acute and formalin induced chronic inflammatory models in mice in a dose dependent manner. Several inflammatory mediators such as kinins, PGs and serotonin account for the edema formation caused by sub-plantar formalin or carrageenan injection. This increased synthesis of PGs is due to increased release of arachidonic acid from the membrane phospholipids and the up regulation of cyclooxygenase-2 (Subbaramaiah et al., 1997) The methanolic extract of *P. florida* also showed marked inhibitory effect on platelet aggregation mediated by ADP. Preincubation of the extract (500 µg) with washed human platelets could inhibit ADP induced aggregation. The ADP mediated platelet aggregation pathway consists of an intermediary mechanism where mobilisation of arachidonic acid from membrane phospholipids and its metabolism through COX pathway to the potent proaggregation agent thromboxane A2 has been demonstrated (Miller, 1996). The exact mechanism of anti-inflammatory and antiplatelet aggregating activities by the extracts is unknown.

Inflammation, a fundamental protective response; may be harmful in conditions such as life threatening hypersensitive reactions, insect bites, drugs, toxins and in certain chronic disease such as rheumatic arthritis, atherosclerosis, and lung fibrosis (Cortan et al., 1999). Application of TPA to skin results in rapid accumulation of inflammatory cells such as neutrophils and macrophages (Lewis et al., 1987). The anti-inflammatory activity of *P. florida* and *P. sajor-caju* suggests its potential ability to modulate inflammation, which is increasingly implicated in coronary artery diseases and cancer. Chemical substances, which counter platelet aggregation, have a protective role in thromboembolic disorders. In conclusion, the anti-inflammatory and antiplatelet aggregating activities of *P. florida* and *P. sajor-caju* suggest the potential therapeutic use of these mushroom extracts in human ailments, especially vascular disorders and cancers.