Chapter 3

Allylpyrocatechol and its screening for anti-inflammatory activity
Introduction

The inflammatory response protects the host against tissue injury and microbial invasion but, if the response is not short lived, it results in the pathogenesis of many immune mediated diseases like chronic inflammation, cancer, septic shock, rheumatoid arthritis, Crohn's disease, etc (Pulendran et al., 2001, Kern, 2007, Hesslinger et al., 2009, Kanwar et al., 2009). Therapeutic interventions are required to maintain a balance between suppressing an inflammatory process and maintaining normal functions of essential immune responses. Several plant derived compounds have shown anti-inflammatory properties, these include *Piper betle*, a commonly available plant (Ganguly et al., 2007). In the present study, we have purified and investigated the component responsible for the anti-inflammatory activity of the leaves of *Piper betle*.

Piper betle: the green gold

*Piper betle* is a perennial climber, belonging to the 'Piperaceae' family. It is popularly known as 'paan' in India and is consumed commonly with betel nut and lime paste (Figure 1). The leaves are large, heart shaped, bright green and shiny on both sides. It is cultivated in the hotter parts of India and Ceylon (Kirtikar & Basu, 1975). *Piper betle* has its ethno medicinal properties traced back to Sanskrit literature as early as 3000 B.C. Its medicinal properties as a carminative, aromatic, digestive and stimulant are described in the Susruta Samhita, a medico-scientific treatise on the indigenous Ayurvedic system of medicine (Chatterjee & Pakrashi, 1994). It is still in use as a folklore medicine and the essential oil obtained from the leaves have been successfully used in the treatment of catarrhal disorders and an antiseptic (Kirtikar & Basu, 1975). The act of chewing the betel leaf constituents show enhanced salivary peroxidase activity which plays a crucial role in oral hygiene (Kumar & Tripathi, 1999).

The radioprotective role of the *Piper betle* ethanolic extract has been established by its ability to prevent gamma-ray induced lipid peroxidation and DNA strand breaks (Bhattacharya et al., 2005). It also possesses antimicrobial properties as the crude aqueous extract caused cell membrane damage, coagulation of nucleoid and reduced acid production in *Streptococcus mutans* (Nalina & Rahim, 2007). The phenolic compounds present in the leaf extract exhibited antimicrobial activity against bacteria present in the mouth e.g. *Staphylococcus aureus*, *S. viridans* etc. (Panda & Kar, 1996). Hematological and enzymatic studies (transaminases and phosphatases) with an alcoholic extract of leaf stalk in rats and mice indicated that the plant extract was devoid
of toxicity (Sarkar et al., 2000). Its cytoprotective, antifertility (Sarkar et al., 2000) and anti-fungal (Rahman et al., 2005) activities have also been well documented.

Figure 1: Leaves of *Piper betle*

**Anti-Inflammatory properties of *Piper betle***

The anti-inflammatory activity of *Piper betle* was demonstrated by its ability to inhibit dextran mediated acute and Freund's adjuvant mediated chronic inflammation; it also reduced nitric oxide production which was due to its ability to downregulate mRNA expression of inducible nitric oxide synthase (Ganguly et al., 2007). However, the key phytoconstituents contributing to these pharmacological activities remain to be identified. Purification of the ethanolic extract of leaves has revealed that it mainly consists of phenols, allylpyrocatechol, chevibetol and their respective glycosides (Rathee et al., 2006, Ganguly et al., 2007). Phenols are generally reported to be anti-oxidant in nature but to date, no studies pinpointing the phytoconstituent(s) in the ethanolic extract of *Piper betle*, responsible for the anti-inflammatory activity, have been established. In the present study, the phytoconstituents of the *Piper betle* leaf extract were isolated and initial screening of the components revealed that allylpyrocatechol was responsible for the anti-inflammatory activity of *Piper betle*.

**Mechanism of acute inflammation**

Inflammation may be acute which occurs over seconds, minutes, hours, and days and chronic, which occurs over longer periods. Acute inflammation is a short-term process which begins within seconds to minutes following the injury of tissues. It occurs as long as the injurious stimulus is present and ceases once the stimulus has been removed (Gryglewski, 1981). The damage may be purely physical, or it may involve activation of
an immune response. Increased blood flow as well as accumulation of fluid in the interstitial spaces causes the area to swell causing oedema. The increased permeability of capillaries occurs because endothelial cells are separated from one another at their edges. Neutrophils (also macrophages) migrate from the blood to the tissues with the help of cell adhesion molecules. Initially, adhesion molecules called selectins tether the neutrophils loosely to the endothelium, so that it begins rolling along the surface (Springer, 1990). Later, a much tighter binding occurs through the interaction of ICAMs on the endothelial cells with integrins on neutrophils (Harlan & Lin, 1992). Once bound to the endothelium, neutrophils squeeze through gaps between adjacent endothelial cells into the interstitial fluid, a process called diapedesis. Once outside the blood vessel, a neutrophil is guided towards an infection by various diffusing chemotactic factors. The other cells involved in acute inflammation are eosinophils (when inflammation is caused due to parasitic worms), mast cells (release histamine and other inflammatory paracines) and macrophages (important source of inflammatory cytokines such as IL-1β and TNF-α). The arachidonic acid derivatives, prostaglandins and leukotrienes, complement, kinins etc also play an important role in acute inflammation.

**Inflammatory response and the role of macrophages**

One of the key players in inflammation are macrophages, cells of the reticuloendothelial system. Their development takes in the bone marrow and passes through the following steps: stem cell - committed stem cell - monoblast - promonocyte - monocyte (bone marrow) - monocyte (peripheral blood) - macrophage (tissues). Their development takes place in 1.5 to 3 days. Macrophages can be divided into normal and inflammatory macrophages. Normal macrophages include macrophages in connective tissue (histiocytes), liver (Kupffer's cells), lung (alveolar macrophages), lymph nodes (free and fixed macrophages), spleen (free and fixed macrophages), bone marrow (fixed macrophages), serous fluids (pleural and peritoneal macrophages), skin (histiocytes, Langerhans's cell) and in other tissues. Inflammatory macrophages are present in various exudates and are characterized by specific markers, eg. peroxidase activity. They are of vital importance in both innate and adaptive immunity and play a pivotal role in inflammation by producing various mediators. Besides their phagocytic role, macrophages produce a number of reactive oxygen and nitrogen intermediates to promote killing of pathogens (Chaplin, 2006). They also act as antigen presenting cells after incorporation of pathogen and secrete inflammatory cytokines, such as IL-1, IL-12, TNF-α etc. in response to microbes or their products such as lipopolysaccharide (LPS).
APC, an anti-inflammatory agent

Therefore, macrophage activation contributes to the pathology of many inflammatory diseases. In the hemophagocytic syndrome, activated macrophages cause excessive cytokine secretion i.e. the "cytokine storm" and devour erythrocytes (McDonald & Cassatella, 1997). In cancer, cachexia, macrophages secrete IL-6 and TNF-α while in inflammatory bowel disease, it is mainly TNF-α (Strassmann et al., 1992, Costelli et al., 1992, Andreakos et al., 2002). Periodontitis may be caused by macrophages activated by LPS (Deschner et al., 2003). Inhibition of inflammatory mediators produced by macrophages is thereby believed to be crucial for managing inflammatory diseases. Many investigators have therefore focused either on identifying anti-inflammatory agents from natural resources or on developing synthetic anti-inflammatory compounds (Lin et al., 2008, Chiou & Liu, 1999).

Role of nitric oxide in inflammation

One of the important pro-inflammatory mediators produced by activated macrophages is nitric oxide (NO). It is an important regulatory molecule with a wide range of physiological functions including neurotransmission and vasodilatation. It also plays an important part in host defense (Macmicking et al., 1997) and pathophysiological actions (Moncada et al., 1991, Nathan & Xie 1994). The diverse functions are mediated by its action on most cells of the body through interaction with different molecular targets, which can either be activated or inhibited (Xie K, 1998).

NO is a short lived molecule generated as a free radical during the conversion of L-arginine to citrulline, the reaction being catalyzed by nitric oxide synthase (NOS). Molecular cloning and sequencing analysis has revealed that NOS exists in three forms, namely endothelial NOS (eNOS) released from vascular endothelium, neuronal NOS (nNOS) released from central and peripheral neurons and inducible NOS (iNOS) (Andrew & Mayer, 1999) released from activated macrophages. The first two NOS isoforms are constitutive (cNOS) and are calcium/calmodulin dependent. When stimulated by agonists that increase intracellular calcium, these isoforms produce small amounts of NO only for short periods. On the other hand, LPS activated macrophages, hepatocytes and endothelial cells secrete the calcium independent high output or inducible NOS following its transcriptional induction and protein synthesis (Nathan & Xie, 1994). The iNOS catalyzes the formation and release of a large amount of NO which then plays a key role in disease pathophysiology (Liu & Hotchkiss, 1995, Tunctan et al., 1998). Low concentrations of NO produced by iNOS have beneficial roles such as the anti-microbial activity of macrophages against pathogens (Moncada et al., 1991,
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Lowenstein et al., 1993, Cook & Cattell, 1996, Hibbs et al., 1988). However, overproduction of NO and its derivatives such as peroxynitrite and nitrogen dioxide, mutagenic in vivo, provoke the pathogenesis of septic shock and cause diverse autoimmune disorders (Liu & Hotchkiss, 1995, Wink et al., 1991, Nguyen et al., 1992, Kilbourn et al., 1990, Miller et al., 1993). Increased expression of iNOS and concomitant NO levels have been reported in several inflammatory diseases such as Crohn's disease, asthma and rheumatoid arthritis (Boughton-Smith et al., 1993, Kharitinov & Barnes, 2004, Yasuda et al., 2004). Moreover, NO and its oxidized forms have also been shown to be carcinogenic (Halliwell, 1994, Ohshima & Bartsch, 1994).

Materials and Methods

Isolation and characterization of APC (kindly gifted by Dr. S. Chattopadhyay, Bhabha Atomic Research Centre, Mumbai, India):
The air-dried leaves of Piper betle (250 g) were chopped into fine pieces, soaked in 95% ethanol (1 L) for two days and the supernatant decanted. The entire process was repeated thrice and the chemical constituents of Piper betle were analyzed by HPTLC (Jasco model, PU-2080) plus chromatogram using Hypersil GOLD (250 x 4.6 mm, particle size 5μm, Thermo Electron Corporation) column as described in Materials and Methods and peaks were detected at 254 nm (Rathee et al., 2006).

Animals:
Male Sprague Dawley rats (100 - 120 g) were used; animals were housed under standard conditions, temperature being 25 ± 5°C, fed on a standard pellet diet and provided water ad libitum. All experimental protocols were performed with prior approval from the Institutional Animal Ethical Committee.

Cell culture:
The mouse monocyte-macrophage cell line RAW 264.7, was maintained in DMEM medium containing 10% FCS, penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were subcultured every 72 h, 1 x 10^6 cells being the initial inoculum and maintained at 37°C, 5% CO₂ atmosphere.

Determination of the acute inflammatory response:
Acute inflammatory response was measured using dextran (2% in 0.9% NaCl w/v) (Rowley & Benditt, 1956) wherein the experimental animals were divided into 3 groups (n = 6); APC (10 mg/kg b.w., p.o.) or Indomethacin (10 mg/kg b.w., p.o.) were
APC, an anti-inflammatory agent administered and oedema volume was measured plethysmographically consecutively for 3 h as described in Materials and Methods and percent inhibition was calculated.

**Cytotoxicity assay:**

RAW 264.7 cells (2x10⁵/200 μl/well) were treated with APC (0 - 25 μg/ml) for 48 h, assayed for viability using the MTS- PMS assay as described in Materials and Methods and specific absorbances measured spectrophotometrically at 490 nm (Sen et al., 2007).

**Determination of extracellular nitric oxide (NO):**

RAW 264.7 cells (1 x 10⁶/ml/well) were incubated with LPS (10 μg/ml) in the presence or absence of APC (0 - 5 μg/ml) for 48 h, assayed for extracellular NO using Griess reagent (mixture of 0.1% NED in water and 1% sulphanilamide in 5% phosphoric acid) as described in Materials and Methods and absorbances measured at 546 nm (Hibbs et al., 1988).

**Determination of intracellular nitric oxide (NO):**

RAW 264.7 cells (1 x 10⁶/ml/well) were incubated with LPS (10 μg/ml) in the presence or absence of APC (5 μg/ml) for 2 h, assayed for intracellular NO using diaminofluorescein diacetate (DAFDA) as described in Materials and Methods and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, Dikshit & Sharma, 2002). Forward scatter vs. side scatter was used to gate the macrophage population and a FL1 histogram to quantify fluorescence of viable macrophages; analyses were done using BD Cell Quest Pro software.

**Reverse Transcriptase polymerase chain reaction analysis of iNOS mRNA:**

RAW 264.7 cells were treated with LPS (10 μg/ml) in conjunction with APC (2.5 μg/ml, or 5 μg/ml) for 24 h. RNA was isolated and subjected to RT-PCR using the primers listed below as described in Materials and Methods. RT-PCR products of β-actin and iNOS mRNA was resolved on an agarose gel (1.5%) and visualized by ethidium bromide staining. The expression of IL-12 p40 was quantified densitometrically with Total lab software (Ganguly et al., 2007).
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<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Name</th>
<th>Primer</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>β-actin</td>
<td>Sense: 5'-TGGAATCCTGTGGCATCCATGAAAC-3' Anti-sense: 5'-TAAAACGCAGCTCAGTAACAGTCG-3'</td>
</tr>
<tr>
<td>2</td>
<td>iNOS</td>
<td>Sense: 5'-CATGGCTTGCCCCCTTGAAGTCTTTCTCCTCAAAG-3' Anti-sense: 5'-GCAGCATTCCCCCTGATGCTGCGCATCG-3'</td>
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Statistical Analysis:
Results were expressed as mean ± S.D./S.E.M. Statistical analysis was evaluated using Graph Pad Prism software (version 4) and student's t test, p<0.05 was considered as statistically significant.

Results

APC reduced dextran mediated acute inflammatory response:
Dextran, an established phlogistic agent induces an inflammatory response resulting in oedema; therefore, the reduction of oedema volume is a measure of the anti-inflammatory potential of a compound. APC (10 mg/kg b.w., p.o.) effectively reduced dextran induced oedema and caused a reduction of 74.19% which was comparable with Indomethacin, an established anti-inflammatory drug that caused 83.87 % reduction in oedema volume as compared to control animals (Table 1).

Table 1: Anti-inflammatory activity of APC in a dextran induced model of acute inflammation

<table>
<thead>
<tr>
<th>Group</th>
<th>*Oedema volume (ml)</th>
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<tr>
<td></td>
<td>(% inhibition)</td>
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<tr>
<td></td>
<td>1st hr</td>
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<tr>
<td>Control</td>
<td>0.78 ± 0.09</td>
</tr>
<tr>
<td>Indomethacin (10 mg/kg bw)</td>
<td>*0.24 ± 0.03</td>
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<td></td>
<td>(69.35%)</td>
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<tr>
<td>APC (10 mg/kg bw)</td>
<td>^0.33 ± 0.13</td>
</tr>
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<td></td>
<td>(58.06%)</td>
</tr>
</tbody>
</table>

*Acute inflammation was induced using dextran; oedema volume (ml) was measured plethysmometrically for three hours and % inhibition was calculated as described in Materials and Methods. Each value represents the mean ± S.D. of at least three experiments. *p<0.001 and ^p<0.005 as compared to controls.
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APC causes reduction of production of nitric oxide (NO):
LPS, a major component of the cell wall of gram-negative bacteria inducing a strong innate and adaptive immune response, was used to elicit increased synthesis of NO. The half-life of NO being very short, nitrite production served as an indicator of NO released; the nitrite produced reacted with Griess reagent in acidic medium to form an azo compound; therefore the reduction in the formation of the azo compound is a measure of anti-inflammatory potential. LPS caused a >4 fold increase in NO production as compared to normal macrophages (66.32 ± 0.73 vs. 16.17 ± 0.15, p<0.005, Figure 2A). The addition of APC resulted in a dose dependent decrease in LPS induced NO production as APC (2.5 and 5 μg/ml) caused a 69.31% (6 fold, 20.35 ± 0.11, p<0.01), and 82.92% (7 fold, 11.33 ± 0.33, p<0.001) decrease in NO production respectively as compared to LPS (Figure 2A). DMSO, the vehicle control, had no effect on NO production (61.54 ± 1.71, data not shown), which reconfirmed its immunological inertness.

The decrease in extracellular NO was corroborated by measurement of intracellular NO using DAFDA. DAFDA is a lipid soluble, membrane permeable compound that following esterase mediated cleavage is subsequently oxidized by intracellular NO and the resultant fluorescent compound serves as a measure of the quantum of NO generated. LPS caused a 76.50% increase in intracellular NO as compared to background fluorescence (155.46 ± 7.49 vs. 88.08 ± 2.97, p<0.05, Figure 2B); the addition of APC (5 μg/ml) effectively decreased LPS induced increase in fluorescence (80.08 ± 2.58, p<0.05), with levels of NO reaching control values by 2 h. A cell viability assay verified that the APC induced decrease in NO at the above mentioned concentration was not due to general cellular toxicity (Figure 2C).
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Figure 2: Effect of APC on NO production

A: RAW 264.7 cells (1 x 10⁶/ml/well, 1) were incubated with LPS (10 μg/ml, 2) in the presence of APC [2.5 μg/ml, (3) or 5 μg/ml, (4)] and assayed for extracellular NO as described in Materials and Methods. Each point represents the mean ± S.D. of NO₂⁻ (μM) of at least three experiments, *p<0.005 and **p<0.001 as compared to LPS.

B: RAW 264.7 cells (1 x 10⁶/ml/well) were incubated with LPS (10 μg/ml, 3) in the presence of APC (5 μg/ml, —) and assayed for levels of intracellular NO as described in Materials and Methods. The data is a representative profile of 3 experiments. Inset: RAW 264.7 cells were incubated with LPS, in the absence (1) or presence of APC (5 μg/ml, 2). The results are mean ± SEM of the Geometric mean fluorescence channel (GMFC) of at least three experiments in duplicate, p<0.05 as compared to LPS.

C: RAW 264.7 cells were incubated in the presence of APC (0 – 25 μg/ml) and assayed for viability as described in Materials and Methods. Each point represents % viable cells of at least 3 experiments in duplicate.
APC causes inhibition of iNOS:

To elucidate the mechanisms involved in the inhibition of NO generation by APC in macrophages, the effect of APC on LPS-induced iNOS gene expression was analyzed. RAW 264.7 cells in normal conditions express practically non detectable levels of iNOS mRNA; after 24 h of LPS stimulation, the iNOS expression was markedly increased (Figure 3); with the addition of APC (0 – 5 µg/ml), a dose dependent inhibition of iNOS expression was demonstrated indicating that APC modulated expression of iNOS at a transcriptional level which accounted for its inhibition of NO production.

**Figure 3: Effect of APC on iNOS**

RAW 264.7 cells (1) were treated with LPS (10 µg/ml, 2) along with APC [2.5 µg/ml, (3) or 5 µg/ml, (4)] for 24 h. RNA was isolated and subjected to RT-PCR as described in Materials and Methods. RT-PCR products of β-actin and iNOS were resolved on an agarose gel (1.5%) and visualized by ethidium bromide staining. The expression of iNOS (■) was quantified densitometrically with Total lab software. The data is a representative profile.

**Discussion**

For centuries, dietary and medicinal phytochemicals have been used as anti-inflammatory remedies. Phytochemicals derived from plants or microbes serve as a valuable source for isolating and characterising lead molecules with specific functions. In recent times, considerable attention has been focused towards identifying the active component(s) of these traditional preparations. This approach will help in identifying compounds that show specific bioactivity. The leaves of *Piper betle* have been reported to have a wide range of pharmacological activities ranging from anti-inflammatory, anti-fungal and anti-microbial amongst others but the pharmacological activities of the key
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Phytoconstituents have not been pinpointed. The cytoprotective and anti-oxidant activity of APC was demonstrated to cause attenuation of indomethacin induced gastric ulceration in rats (Bhattacharya et al., 2007) prompting further pharmacological elaboration of the underlying pathways involved.

Acute inflammation can be induced by the injection of carrageenin, dextran, kaolin, histamine, serotonin etc (Winter et al., 1962, Nakamura et al., 1988, Rovati et al., 1979). Dextran is a complex polysaccharide obtained from Leuconostoc mesenteroides consisting of a (1→6)-linked glucan with side chains attached to the 3-positions of the backbone glucose units. It is freely soluble in water, dimethyl sulphoxide, formamide, ethylene glycol, glycerol, 4-methylmorpholine-4-oxide, and hexamethylphosphoramide (a carcinogen). Dextran induced paw oedema is known to be mediated by histamine and serotonin, known mediators of inflammation (Lo et al., 1982) but does not involve leucocytic migration (Nakamura et al., 1988). Dextran induces fluid accumulation, which contains little protein and few neutrophils (Kumar & Robin, 1995). The effectiveness of APC (10 mg/kg bw) in reducing dextran induced acute inflammation (Table 1) prompted us to delineate the underlying molecular mechanisms contributing to the observed anti-inflammatory action of APC.

NO plays a pivotal role in many body functions and is essential for the elimination of invasive antigens but chronic production damages normal cells and tissues leading to cytotoxicity, carcinogenicity and a variety of other inflammatory disorders including rheumatoid arthritis and many other autoimmune diseases (Liu & Hotchkiss, 1995, Nguyen et al., 1992, Weinberg, 1998). NO is often overproduced by activated macrophages. The activation of macrophages leads to increased expression of iNOS and the production of NO through an intracellular reaction with L-arginine. Multiple studies implicated that elevated NO production and oxidative stress in activated macrophages is closely related with inflammation, the development of atherosclerosis and cancer. (Berliner et al., 1995, Gobert et al., 2002, Lee et al., 2003). Therefore, inhibitors of NO are required for the prevention of inflammatory diseases. APC caused a dose dependent decrease in extracellular and intracellular production of NO (Figures 2A and B), which was attributed to APC down regulating the mRNA expression of iNOS (Figure 3).