Chapter IV

Intratracheally instilled cigarette smoke extract induces acute inflammation and cytotoxicity in lungs of Wistar rats: Reversal by Cinnamomum zeylanicum Lin. bark extract
Introduction

*Cinnamomum zeylanicum* is a tropical tree (family *Lauraceae*) grown in India and Sri Lanka. Its bark commonly known as 'cinnamon' is widely used as spice worldwide. For centuries in traditional system of medicine it is used as a remedy against various inflammatory diseases. The major constituent of the cinnamon bark is cinnamaldehyde. other constituents are phenols and terpenes, including eugenol, trans-cinnamic acid, o-methoxycinnamaldehyde, hydroxycinnamaldehyde, alpha-terpineol, tannins, oligomeric procyanidins, and trace amounts of coumarin (Bisset, 1994; Trease and Evans, 1989). Cinnamon constituents possess antioxidant properties and may prove beneficial against free radical damage to cell membranes (Dragland et al., 2003; Jayaprakasha et al., 2003). *C. zeylanicum* bark is reported to have medicinal properties (Kamath et al., 2003). Cinnamaldehyde is the main bioactive compound of the cinnamon (Al-Bayati and Mohammed, 2009) and reported to possess anti-inflammatory properties (Cao et al., 2008). It is also reported to show inhibitory effects against cigarette smoke carcinogen induced lung tumors in mice model (Imai et al., 2002). Lung being the primary target for injuries by inhalant toxicants is prone to various diseases. Most of the lethal lung diseases are chronic inflammatory in nature that may arise as a result of repeated acute episodes of inflammation. *C. zeylanicum* that is reported to have anti-inflammatory and antioxidant properties may be proved as a preventive agent against various lung insults and consequent debilities. On the basis of all these facts the present study was designed to assess the protective role of *C. zeylanicum* bark extract against intratrachealy administered cigarette smoke extract induced acute lung injury in Wistar rats. Low doses of *C. zeylanicum* extract (20 and 50 mg/kg b.w.t.) were selected on the basis of previous reports (Qin et al., 2000; Qin et al., 2003; Kanniappan et al., 2008) and to avoid probable toxic effects higher doses.
**Animals**

Female rats of Wistar strain were used in this study. Animals were obtained from Central Animal House Facility of Hamdard University, New Delhi, India. The rats were approximately 10 weeks old at starting of study (weights in the range of 150-200 grams). They were housed in polypropylene cages in groups of six rats per cage and were kept in a room maintained at 25±2°C with a 12 hour light/dark cycle, and were allowed to acclimatize for one week before the experiments. They were given free access to standard laboratory animal feed (Hindustan Lever Ltd., Bombay, India) and water *ad libitum*.

All procedures using animals were reviewed and approved by the Institutional Animal Ethical Committee that is fully accredited by the Committee for Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

**Treatment regimen**

To study the effect of pretreatment of animals with *C. zeylanicum* bark methanolic extract on CSE induced acute lung toxicity, 30 female Wistar rats were randomly allocated to five groups of six rats in each. The animals of group I & II served as control and toxicant (CSE) respectively, received normal saline orally (0.15 M NaCl, once daily, for 7 days). Group III received pretreatment with *C. zeylanicum* extract by gavages once daily for 7 days at a dose of 20 mg/kg b.wt. (D1). Group IV and V received pretreatment with *C. zeylanicum* extract once daily for seven consecutive days at a dose level of 50 mg/kg b.wt. (D2). On day 7, one hour after the last treatment with *C. zeylanicum* extract or normal saline the animals of group II, III and IV were administered with a single i.t. dose of CSE (1.3 ml/kg b.wt.), group I (control group) and group V (only D2 group) was administered with DDW i.t. (1.3 mL/kg b.wt.). All the animals were sacrificed 24 h after last treatment.
Results

Antioxidant potential of *C. zeylanicum* extract

*C. zeylanicum* extract shows anti-radical activity in DPPH assay. It shows a gradual increase in the activity with the increase in concentration of *C. zeylanicum* extract; at 100 µg it scavenges 87.38 % DPPH radicals. Total polyphenolic content of *C. zeylanicum* extract was found to be 56.52 ± 0.069 mg gallic acid equivalent (GAE)/g dry weight.

Cytotoxicity assay

Figure 1 shows that CSE administration increases the lung cytotoxicity marker, LDH level in BALF. It significantly increases (p<0.01) lactate dehydrogenase (LDH) (Group II) when compared with control group (Group I). Administration of *C. zeylanicum* extract (50 mg/kg b.wt.) significantly decreased (p<0.01) the levels of LDH (Group IV), effects of lower dose of *C. zeylanicum* extract (20 mg/kg b.wt.) was not found to be significant (Group III) when compared with Group II. Only *C. zeylanicum* extract (50 mg/kg b.wt.) (Group V) does not show any significant alterations when compared with control group.

Acute inflammation and edema

Figure 2 shows the effects of *C. zeylanicum* extract and CSE on the acute inflammation in terms of total cell count in BALF of different treatment groups. *C. zeylanicum* extract (50 mg/kg b.wt.) (Group IV) significantly reduces (p<0.01) inflammation when compared with CSE group (Group II). In Group III results of inflammation was insignificant. Intratracheal instillation of CSE led to discernible edema after 24 h in terms of protein content of BALF (Fig. 3). CSE administration caused significant edema formation when compared with group I animals (p < 0.01). Group IV animals which were pre-treated with *C. zeylanicum* extract (50
mg/kg b.wt.) show reduced lung edema response in CSE exposed rats and the changes in response was significant ($p < 0.05$) when compared with CSE-treated animals (Group II). Effects of lower dose of *C. zeylanicum* extract (20 mg/kg b.wt.) were not found to be significant (Group III) when compared with Group II. Group V does not show any significant alterations when compared with control group.

**Membrane damage (LPO)**

Figure 4 shows the effects of *C. zeylanicum* extract and CSE on the membrane lipid peroxidation in terms of MDA formation in different treatment groups. *C. zeylanicum* extract (50 mg/kg b.wt.) (Group IV) significantly reduced ($p<0.01$) LPO when compared with CSE group (Group II). *C. zeylanicum* extract (20 mg/kg b.wt.) (Group III) did not reduce LPO significantly.

**Antioxidant enzyme activity**

Table 1 shows that intratracheal administration of CSE (Group II) significantly decreased the lung GR ($p<0.01$), GPx ($p<0.05$) and catalase ($p<0.01$) when compared with Group I. *C. zeylanicum* extract (50 mg/kg b.wt.) (Group IV) significantly restored ($p<0.01$) the activity of GR, GPx and catalase (Table 1). Changes in Group III (*C. zeylanicum* extract, 20 mg/kg b.wt.) were not found to be significant in GR and GPx but in catalase changes were significant ($p<0.05$) when compared with Group II. *C. zeylanicum* extract (50 mg/kg b.wt.) when administered alone show a slight increase in antioxidant enzymes level that was not found to be significant and do not show any toxic effect on the lung.
Discussion

Recently research has been focused on the effects of plant-based medicines on human health. It is well known that polyphenols and other phytochemicals in fruits and vegetables possess antioxidant properties and protect biological system from oxidative damages (Rahman et al., 2006). They are reported to ameliorate the development of many disorders caused by environmental toxicants (Eastwood, 1999). In the present study methanolic extract of *C. zeylanicum* bark was found to contain a fare amount of polyphenols. Moreover the extract, in DPPH assay, showed high free radical scavenging potential even at low doses.

Cigarette smoke exposure is known to impose debilitating effects on the lung and other body organs lading to development of various disorders. Presence of a large number of free radicals in cigarette smoke initially inflicts an undue oxidative burden on lungs. Overwhelming effects of these oxidants causes oxidative injury. In the present investigation intratracheal administration of CSE caused cytotoxicity, inflammation and edema in lungs of the rats. Further our results show that CSE imposes oxidative stress on the lung as a whole. These alterations are in accordance to the literature (Aoshiba et al., 2003). Oral administration of *C. zeylanicum* extract (50 mg/kg b.wt.) exhibited significant amelioration of lung injury in terms of reduced cytotoxicity, early inflammation and edema. Reduced levels of LPO in lung tissue of *C. zeylanicum* treated animals also show its cytoprotective role.

Inflammation plays an important role in the development of various lung disorders and amelioration of inflammation in early stages may be a better strategy to check the development of inflammatory lung diseases. During early inflammation various types of inflammatory cells are recruited, neutrophils being major one. These cells generate reactive oxygen species and protease enzymes that cause cytotoxic damages to lung tissue (Taraseviciene-Stewart and Voelkel, 2008). *C. zeylanicum* bark extract significantly reduced early inflammation and edema in terms of reduced total cell count and total protein in BALF.
*Cinnamomum* polyphenols are reported to have anti-inflammatory activities during early immune responses (Cao et al., 2008). These activities may be attributed to cinnamaldehyde, main active principle of *C. zeylanicum* bark, which is recently reported to inhibit pro-inflammatory cytokines release from macrophages (Cao et al., 2008). This may be the probable mechanism in the protection of lung injuries caused by cigarette smoke toxicants. Cinnamaldehyde is also reported to have chemopreventive properties against lung cancer development by cigarette smoke carcinogen (Imai et al., 2002). At lower dose *C. zeylanicum* bark extract was unable to show any significant protective role against cigarette smoke toxicant induced lung injury. This shows a dose response relationship in this investigation.

Cigarette smoke contains billions of free radicals (Church et al., 1985) and causes formation of ROS *in-vivo*. This increased burden of endogenous and exogenous free radicals causes exhaustion of antioxidant enzymes and cell damage (Aoshiba et al., 2003). In the present investigation it was found that CSE significantly reduced antioxidant enzymes activities in rat lungs. It indicates that the lung damage in CSE exposed Wistar rats might be due to oxidative burden, and prophylactic treatment of *C. zeylanicum* bark extract significantly restored the levels of antioxidant enzymes GR, GPx and catalase. These properties are probably due to eugenol that is reported to be the major antioxidant compound in *C. zeylanicum* bark (Chericoni et al., 2005). It can be suggested that *C. zeylanicum* extract reversed the damage most probably by scavenging the exogenous free radicals and minimizing the formation of endogenous one. Reversal of lung injury might be due to antioxidant and anti-inflammatory properties of compounds in *C. zeylanicum* bark extract.

**Conclusion**

On the basis of present findings it can be concluded that *C. zeylanicum* bark has antioxidant and anti-inflammatory effects on rat lungs and shows protective effects against oxidative
stress, inflammation and consequent cytotoxicity caused by cigarette smoke toxicants. So the present investigation gives a path to further studies on components of *C. zeylanicum* bark against cigarette smoke induced lung ailments including cancer.
Figure 1: Effect of cigarette smoke extract (CSE) and *Cinnamomum zeylanicum* bark extract on LDH levels in bronchoalveolar lavage fluid (BALF).

Fig. 1. Effect of *C. zeylanicum* and CSE on cytotoxicity marker LDH in BALF of rats. Values are expressed as means ± S.D. (n = 6) measured as μmol NADH oxidized/ml BALF. Significant differences are indicated by **p < 0.01** when compared with control animals (Group I) and ***p < 0.01*** when compared with CSE-treated animals (Group II), NS = not significant.
Figure 2: Effect of cigarette smoke extract (CSE) and *Cinnamomum zeylanicum* bark extract on Total cell count in bronchoalveolar lavage fluid (BALF)

![Bar Chart]

**Treatment groups**

Fig. 2. Effect of *C. zeylanicum* and CSE on total cell count in BALF of rats. Values are expressed as means ± S.D. (n = 6) measured as mn cells/ml BALF. Significant differences are indicated by **p < 0.01** when compared with control animals (Group I) and **##p < 0.01** when compared with CSE-treated animals (Group II), NS = not significant.
Figure 3: Effect of cigarette smoke extract (CSE) and Cinnamomum zeylanicum bark extract on Total protein in bronchoalveolar lavage fluid (BALF)

![Graph showing the effect of CSE and Cinnamomum zeylanicum bark extract on total protein content in BALF of rats. Values are expressed as means ± S.D. (n = 6) measured as mg/dl BALF. Significant differences are indicated by **p < 0.01 when compared with control animals (Group I) and #p < 0.05 when compared with CSE-treated animals (Group II), NS = not significant.]

Fig. 3. Effect of C. zeylanicum and CSE on total total protein content in BALF of rats. Values are expressed as means ± S.D. (n = 6) measured as mg/dl BALF. Significant differences are indicated by **p < 0.01 when compared with control animals (Group I) and #p < 0.05 when compared with CSE-treated animals (Group II), NS = not significant.
Figure 4: Effect of cigarette smoke extract (CSE) and Cinnamomum zeylanicum bark extract on MDA levels in bronchoalveolar lavage fluid (BALF)

Fig. 4. Effect of C.zeylanicum and CSE on lipid peroxidation in lung tissue of rats. Values are expressed as means ± S.D. (n = 6) measured as μmol MDA/g tissue. Significant differences are indicated by **p < 0.01 when compared with control animals (Group I) and #p < 0.05 when compared with CSE-treated animals (Group II), NS = not significant.
Table 1: Effect of cigarette smoke extract (CSE) and Cinnamomum zeylanicum bark extract on lung antioxidants

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glutathione reductase (nmol NADPH oxidized/min/mg protein)</th>
<th>Glutathione peroxidase (nmol NADPH oxidized /min / mg protein)</th>
<th>Catalase (nmol H₂O₂ consumed/min/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>I [Only vehicle]</td>
<td>26.835 ± 0.77</td>
<td>189.26 ± 3.85</td>
<td>8.12 ± 0.33</td>
</tr>
<tr>
<td>II [CSE]</td>
<td>15.25 ± 0.36 **</td>
<td>176.24 ± 1.8 *</td>
<td>6.89 ± 0.37 **</td>
</tr>
<tr>
<td>III [D1 + CSE]</td>
<td>14.42 ± 1.76 NS</td>
<td>177.33 ± 5.13 NS</td>
<td>8.92 ± 0.31 ***</td>
</tr>
<tr>
<td>IV [D2 + CSE]</td>
<td>31.27 ± 2.55 **</td>
<td>193.78 ± 8.59 **</td>
<td>9.00 ± 0.44 **</td>
</tr>
<tr>
<td>V [D2]</td>
<td>27.027 ± 1.5</td>
<td>188.19 ± 3.46</td>
<td>8.76 ± 0.72</td>
</tr>
</tbody>
</table>

Values are means ± S.D. (n = 6). Significant differences are indicated by *p < 0.05 and **p < 0.01 when compared with control animals (Group I), and ###p < 0.01 when compared with CSE-treated animals (Group II), NS is not significant. CSE = cigarette smoke extract; D1 = C. zeylanicum 20 mg/kg b.wt.; D2 = C. zeylanicum extract 50 mg/kg b.wt.