Chapter III

Polyphenols from Juglans regia L. (Walnut) kernel modulate cigarette smoke extract induced acute inflammation, oxidative stress and lung injury in Wistar rats
**Introduction**

Many plants and plant products have been proved to have chemopreventive efficacies on different organs including lung. *Juglans regia* L. commonly known as walnut is grown in southeast Europe, southwest & central Asia and southwest China. Its dried fruits (kernels) are used as healthy food stuff. Walnuts are reported to have rich antioxidants content (Halvorsen et al., 2002). The major phenolics of *J. regia* are ellagic, caffeic, syringic acid and juglone (Colaric et al., 2005). Antioxidants are a group of compounds which are present in plant foods. They are able to counteract free radicals formed during the aging process and have a potential role in preventing the commencement of some chronic diseases such as cardiovascular disease, neurological disorders and inflammatory processes (Halliwell, 1997; Stanner et al., 2004).

Recent studies has determined the antioxidant capacity of different nuts, such as almonds, hazelnuts, Brazil nuts, macadamias, peanuts, pecans, pistachios, pine nuts and walnuts (Li et al., 2006; Pellegrini et al., 2006; Wu et al., 2004). These studies concluded that walnut (*Juglans regia* L.) exhibits greater antioxidant capacity than any other nuts (Pellegrini et al., 2006; Wu et al., 2004) and that this antioxidant is presumably a product of phenolic compounds (Fukuda et al., 2003). In *in-vivo* studies on rodents, walnut extract has shown antioxidant properties and caused a significant decrease in oxidative stress markers (Fukuda et al., 2004). It is also reported to have maintained the phenotype of endothelial cells (Cortés et al., 2006). On the basis of these reports it can be hypothesized that *J. regia* may have protective effects on lung against toxicities. Present study was designed to assess the protective effects of *Juglans regia* kernel extract against cigarette smoke extract (CSE) induced acute lung toxicity in female Wistar rats. The
doses of *J. regia* extract in the present study were taken as 50 and 100 mg/kg b.wt. and were chosen on the basis of preliminary studies conducted in our laboratory (data not shown).

**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., Mumbai, 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) Chennai, India.

**Results**

**J. regia antioxidant activity**

Total polyphenolic content of *J. regia* extract was found to be 96 ± 0.81 mg gallic acid equivalent (GAE)/g dry weight. In ferric reducing potential assay, the extract shows reducing potential with an increase in absorbance by 264 ± 1.46 % when compared with control. Figure 1 shows the free radical scavenging activity of the extract. It shows a gradual increase in free radical scavenging with the increase in concentration of the extract; 100 µg of the *J. regia* kernel extract scavenges 40.54% DPPH radicals.
**Inflammatory response and edema**

CSE administration caused significant increases (p<0.01) in total cell count of BALF of the rats when compared with control group (Group I). Dose 1 and 2 of *J. regia* (50 and 100 mg/kg b.wt.) significantly decreased (p<0.01) the levels of total cell count (Group III and IV) when compared with CSE group (Group II) animals (Fig. 1). Intratracheal instillation of CSE led to discernible edema after 24 h in terms of protein content of BALF (Fig. 2). CSE administration caused significant edema formation when compared with group I animals (p < 0.01). Group IV animals which were pre-treated with *J. regia* extract (100 mg/kg b.wt.) show reduced lung edema response in CSE exposed rats and the changes in response was found to be significant (p < 0.05) when compared with CSE-treated animals (Group II). Changes in Group III were not found to be significant.

**Lung cellular injury**

CSE administration in Group II animals showed significant enhancement in levels of cytotoxicity marker LDH (Fig. 3) in BALF when compared with Group I (control group). Group III and IV on *J. regia* extract pre-treatment showed a significant decrease in cytotoxicity indicating protective effect of *J. regia* against CSE lung toxicity.

**Lung antioxidants**
The intratracheal instillation of CSE (Group II) caused a significant reduction in the activities of glutathione metabolizing enzyme GR ($p < 0.01$), and catalase activity ($p < 0.01$) when compared with control (Group I) animals (Table 1). No significant change was observed in only *J. regia* extract treated animals (Group V) when compared with control group. Administration of both the doses of *J. regia* (50 and 100 mg/kg b.wt.) + CSE in Group III and IV animals showed a significant increase in the activities of GR ($p<0.01$) and catalase ($p<0.01$) as compared to CSE-treated animals (Group II). A significant ($p<0.01$) decrease in GSH was observed from the BALF in CSE-treated animals (Group II) when compared with Group I animals (Table 1). *J. regia* extract dose 1 (50 mg/kg b.wt.) + CSE (Group III) and dose 2 (100 mg/kg b.wt.) + CSE (Group IV) treatments showed a significant increase in GSH when compared with Group II animals. Only dose 2 (100 mg/kg b.wt.) (Group V) did not show any significant changes in GSH levels in BALF when compared with Group I.

*Xanthine oxidase (XO) activity*

CSE administration significantly increased ($p<0.01$) xanthine oxidase (XO) activities in lung tissue when compared with Group I (Fig. 4). Both of the doses of *J. regia* extract significantly reduced ($p<0.01$) the levels of XO in Group III and IV when compared with Group II (Fig. 4).

**Discussion**

Lung epithelial cells are the first line of defense against the inhalant toxicants. Injuries to lung epithelium, by cigarette smoke toxicants, initiate inflammatory cells infiltration and
edema formation. The most damaging effect of the cigarette smoke on the lung epithelium is cell death (Aoshiba et al., 2003). In-vitro studies show that aqueous cigarette smoke extract induces apoptosis at low concentration and necrosis at higher concentration in A549 cells. It is suggested that these cytotoxic effects are the result of free radicals and aldehydes present in volatile phase of cigarette smoke (Hoshino et al., 2001). In the present study results clearly demonstrate cytotoxic effects in lungs of CSE exposed Wistar rats in terms of increased levels of LDH in BALF. Further, results also suggest that higher dose of J. regia extract (100 mg/kg b.wt.) significantly reduces the cytotoxicity which defines protective role in lung epithelium.

Both of the doses of J. regia extract abrogates the inflammatory responses and edema shown by a reduction in total cell count and total protein in BALF, which were induced by CSE exposure. Reduction in the recruitment of inflammatory cells also reduces the chances of elastin degradation by neutrophil elastase, which is a hallmark of emphysema; a cigarette smoke associated disease (Aoshiba et al., 2003). So it can be hypothesized that J. regia kernel decreases the risk of emphysema by reducing the inflammatory cell recruitment. A significant reduction was also observed in XO activity in lung tissue by both of the doses of J. regia extract that suggests its role against free radical generating processes in lungs of the rats. Induction of catalase and GR activities explains its role against CSE induced oxidative damage to lung cells (Nakayama et al., 1985). Reduced glutathione (GSH) play a major role in lung cellular defenses (Kode et al., 2006) and our results show a marked depletion in BALF GSH levels in CSE exposed rat lungs and it is well correlated with the literature (Joshi et al., 1988). J. regia extract significantly restored the GSH levels in BALF. High content of polyphenols in J. regia kernel extract,
that are known to play a major role against oxidative damages, is suggested to play protective role (Rahman et al., 2006). The antioxidant potential of *J. regia* kernel extract is proved by its ferric reducing potential and DPPH free radical scavenging activity. Our results indicate that lung protective properties of *J. regia* extract against CSE induced lung injuries in rats is probably due to its polyphenolic antioxidants content.

**Conclusion:**

On the basis of results it is concluded that *J. regia* kernel extract is capable of minimizing the toxic manifestations of CSE on lung of the Wistar rats. It has protective effects on rat lungs against oxidative damage caused by cigarette smoke toxicants. The present investigation gives a path to study the effects of *J. regia* kernel polyphenols more deeply on the lung toxicities and provide the insight for chemopreventive tool.
Figure 1: Effect of *J. regia* and cigarette smoke extract (CSE) on Total cell count in BALF

Fig. 1. Effect of *J. regia* and CSE on total cell count in BALF of rats. Values are expressed as means±S.D. (*n* = 6) measured as m n 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 when compared with control.
Figure 2: Effect of *J. regia* and cigarette smoke extract (CSE) on Total protein in BALF

Fig. 2. Effect of *J. regia* and CSE on total protein in BALF of rats. Values are expressed as means±S.D. (*n* = 6) measured as mg protein/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 when compared with Group II; ns = not significant when compared with control.
Effect of *J. regia* and cigarette smoke extract (CSE) on LDH levels in BALF

Fig. 3. Effect of *J. regia* 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 when compared with control.
Figure 4: Effect of *J. regia* and cigarette smoke extract (CSE) on Xanthine oxidase activity in lung tissue

![Bar chart showing the effect of *J. regia* and CSE on xanthine oxidase activity in lung tissue. Values are expressed as means±S.D. (n=6) measured as mg uric acid formed/mg protein. 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 when compared with control.]
Table 1: Effect of *J. regia* kernel extract and cigarette smoke extract (CSE) on lung antioxidants

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glutathione reductase (GR) (nmol NADPH oxidized/min/mg protein)</th>
<th>Catalase (nmol H₂O₂ consumed/min/mg protein)</th>
<th>Reduced glutathione (GSH) (nmol GSH conjugates/ml BALF)</th>
</tr>
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<tbody>
<tr>
<td>I [Only vehicle]</td>
<td>26.83 ± 0.28</td>
<td>8.108 ± 0.35</td>
<td>89.56 ± 9.1</td>
</tr>
<tr>
<td>II [CSE]</td>
<td>15.22 ± 0.13**</td>
<td>6.786 ± 0.27**</td>
<td>46.15 ± 11.58**</td>
</tr>
<tr>
<td>III [D1 + CSE]]</td>
<td>26.23 ± 0.31##</td>
<td>8.849 ± 0.49##</td>
<td>113.96 ± 16.84##</td>
</tr>
<tr>
<td>IV [D2 + CSE]</td>
<td>29.53 ± 0.79##</td>
<td>8.88 ± 0.65##</td>
<td>121.31 ± 11.0##</td>
</tr>
<tr>
<td>V [D2]</td>
<td>27.68 ± 0.34 ns</td>
<td>7.91 ± 0.73 ns</td>
<td>91.9 ± 4.65 ns</td>
</tr>
</tbody>
</table>

Values are means ± S.D. (*n = 6*). 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). CSE = cigarette smoke extract; D1 = *J. regia* kernel extract 50 mg/kg b.wt.; D2 = *J. regia* kernel extract 100 mg/kg b.wt. ns = not significant when compared with control.