Antioxidant and ACE enhancing potential of Pankajakasthuri in fluoride toxicity: An in vitro study on mammalian lungs

Rupal A Vasant, Mona C Khajuria and AVRL Narasimhacharya

Toxicol Ind Health 2011 27: 793 originally published online 30 March 2011
DOI: 10.1177/0748233711399308

The online version of this article can be found at:
http://tih.sagepub.com/content/27/9/793
Antioxidant and ACE enhancing potential of Pankajakasthuri in fluoride toxicity: An in vitro study on mammalian lungs

Rupal A Vasant, Mona C Khajuria and AVRL Narasimhacharya

Abstract
Fluoride toxicity occurs due to high concentrations of fluoride in water sources or anthropogenic causes. The aim of the present study was to investigate the effects of an Ayurvedic drug—Pankajakasthuri (PK)—in relation to fluoride-induced toxicity in mammalian lungs. The results indicated that sodium fluoride increased lipid peroxidation and decreased enzymatic and non-enzymatic antioxidants in a concentration-dependent manner in lungs. The antioxidant potential of the lungs was suppressed maximally at 10 ppm fluoride concentration and PK at all three dose levels (i.e., 100, 200 and 300 μl) decreased fluoride induced lipid peroxidation (p < 0.05) and increased the levels of total ascorbic acid, superoxide dismutase, catalase, reduced glutathione, glutathione peroxidise and FRAP values significantly (p < 0.05) in a dose-dependent manner. When PK was examined for its effects on angiotensin-converting enzyme (ACE) activity, in fluoride-induced toxicity, the ACE activity was found to increase (p < 0.0001) in lung homogenates with all three doses. This study indicates that PK, an Ayurvedic drug, improves mammalian lung function by increasing antioxidant potential and ACE activity under the conditions of fluoride toxicity.

Keywords
Fluoride toxicity, oxidative stress, ACE, Pankajakasthuri, antioxidants

Introduction
Fluorosis is caused by a long-term intake of high levels of fluoride and is characterized by clinical manifestations in bones and teeth (Bhussry et al., 1970). Intake of high levels of fluoride is known to cause structural changes (Shivarajashankara et al., 2002; Zhavoronkov, 1977), altered activities of enzymes (Vani and Reddy, 2000) and metabolic lesions (Shashi 1992; Shashi et al., 1994). It has been reported that generation of free radicals, altered antioxidant defence system and increased lipid peroxidation play an important role in the toxic effects of fluoride (Rzeuski et al., 1998; Zhi-Zhong et al., 1989). Further, long-term exposure to high fluoride content in early developmental stages was shown to enhance oxidative stress in blood and decreases the antioxidant defense systems in liver with declined activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPX), glutathione (GSH), glutathione transferase (GST) and total ascorbic acid (TAA; Shanthakumari et al., 2004; Shivarajashankara et al., 2003). A chronic exposure to higher doses of fluoride is reported to bring about cloudy swellings, tubular epithelial degeneration, tissue necrosis, tubular vacuolization, glomerular hypertrophy and interstitial edema in kidneys result in nephritis (Shashi et al., 2002). Long-term or acute intake of fluoride is known to cause alveolar haemorrhage, congestion, edema, necrosis of alveolar epithelium, distortion of alveolar architecture and desquamation of respiratory tract with damage to...
tracheal cartilage (Oncu et al., 2004, 2006; Purohit et al., 1999). Fluoride administration to several generations of laboratory animals indicated that lung tissue is a target organ where several changes were observed such as emphysema and inflammation of lung parenchyma associated with loss of alveolar architecture, bronchiolitis, followed by progressive exudation of oedema fluid, congestion and hyperplasia of alveolar cell nuclei (Aydin et al., 2003; Shashi et al., 1988). Sodium fluoride-induced toxicity is reported to induce apoptosis in lung epithelial cells and alveolar macrophages (Hirano and Ando, 1996; Refsnes et al., 2002, 2003). Further, fluoride toxicity also suppresses the antioxidant levels of the lungs while increasing the lipid peroxidation (Aydin et al., 2003; Oncu et al., 2006). However, no reports are available referring to the toxic effects of fluoride on the angiotensin-converting enzyme (ACE). ACE is a zinc-metallo-peptidase and plays an important role in the regulation of blood pressure at least in part by cleaving the inactive angiotensin I to the vasoconstrictor angiotensin II (Skeggs et al., 1955) and inactivating the vasodilator bradykinin through cleavage (Dorer et al., 1974). Originally, ACE has been discovered from horse plasma by Skeggs et al. (1955). ACE activity is also found in tissues, highest activity being in lungs and testis, moderate activities in gastrointestinal tissues and in kidneys and low activities in brain and liver (Roth et al., 1969; Yang et al., 2003).

Plant-based dietary therapies are recognized as having potential for therapeutic applications as they either have minimal or no side effects (Singh et al., 2003). In the recent years, there has been a growing interest in natural antioxidants of the plants and their use is gaining importance as nutraceuticals and phytocuticals as they have significant impact on the status of human health and disease prevention (Noguchi and Nikki, 2000). An increased production of superoxide anions and metabolites and/or a reduced bioavailability of antioxidants cause oxidative damage to cells and tissues (Dhalla et al., 2000). The polyphenols and flavonoids have beneficial effects in preventing atherosclerosis, stimulate catalase and SOD gene transcription and decrease MDA concentration (Fitzpatrick et al., 1998; Ranaivo et al., 2004; Toyokuni et al., 2003).

Pankajakasthuri (PK; Pankajakasthuri Herbals India Pvt. Ltd., Poovachal, Trivendrum, Kerala, India), a herbal preparation containing 14 medicinal plants (Table 1), is recommended for bronchitis, eosinophilia, cough and for building resistance to diseases. This Ayurvedic drug was chosen for the present work to investigate its antioxidant properties and its effect on ACE activity in mammalian lung tissue exposed to sodium fluoride in *in vitro* system.

**Materials and methods**

**PK and its extraction**

PK was purchased from a local Ayurvedic medicine shop, finely powdered and stored in an air-tight container till use. The extract was prepared by homogenizing 5 gm of powder in 50 ml of 5 mM borate buffer (pH 8.3). The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was collected and dialyzed for 12 h against 20 volumes of the same buffer at 4°C. This extract was further used for profiling the antioxidant potential and ACE activity in lung homogenates.

**Phytochemical analyses**

A quantitative analysis of PK was carried out for proteins, fibers, saponins, total phytosterols, polyphenols, flavonoids, total ascorbic acid and total antioxidant potential (FRAP). Protein content was determined by Lowry et al. (1951) using Folin-Ciocalteu reagent. PK was extracted in petroleum ether to remove fat and subjected to acid and alkaline treatment; the fibre content was estimated by gravimetric analysis (Thimmaiah, 1999). Saponin and total phytosterol contents of the powder were estimated using Vanillin-sulfuric acid and ferric chloride-sulfuric acid methods, respectively (Ebrahimzadeh and Niknam, 1998; Goad and Akihisha, 1997). The polyphenol and flavonoid contents of PK were analyzed using Folin-Ciocalteu and Vanillin sulfuric acid reagents, respectively (Thimmaiah, 1999). The total ascorbic acid content was estimated using 2, 4-dinitrophenyl hydrazine reagent (Schaffert and Kingsley, 1955). Total antioxidant power in terms of FRAP value was determined using TPTZ (2, 4, 6- Tri (2’-pyridyl) 1,3,5- triazine) reagent (Benzie and Strain, 1996).

**Collection of lung tissue**

Lung tissue was collected from a freshly killed male goat at a local slaughter house and brought immediately to the laboratory in frozen condition and stored at lower temperature until used.
Effects of sodium fluoride on lung homogenates

Different concentrations of sodium fluoride (2.5, 5.0, 7.5 and 10.0 ppm) were tested on goat lung homogenate to profile the thiobarbituric acid reactive substances (TBARS), total ascorbic acid (TAA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GPx), FRAP and ACE activity. Suitable concentration of NaF was determined as 10 ppm with maximum effect on these parameters. Therefore, 10.0 ppm NaF was selected for further experimentation with three different doses of PK (100, 200 and 300 ?l).

Biochemical analyses

The lung lipid peroxidation (malondialdehyde concentration) was determined by the thiobarbituric acid reactive substances (TBA) assay (Niehauß and Samuelsson, 1968). Total ascorbic acid was estimated using 2, 4-dinitrophenyl hydrazine reagent (Schaffert and Kingsley, 1955). Superoxide dismutase (SOD; EC 1.15.1.1) was measured using the nitroblue tetrazolium reduction method (Kakkar et al., 1984). Catalase (EC 1.11.1.6) was assayed spectrophotometrically as decomposition of H₂O₂ at 240 nm according to the method described by Aebi (1974). Assay of glutathione peroxidase (GPx; EC 1.11.1.9) was based on GSH consumption as described by Flohe and Gunzler (1984). Reduced glutathione (GSH) was measured by reduction of DTNB as prescribed by Jollow et al. (1974). Total antioxidant power in terms of FRAP value was determined using TPTZ (2, 4, 6- Tris (2- pyridyl) 1, 3, 5- triazine) reagent (Benzie and Strain, 1996). ACE activity in the lung homogenates was assayed according to the method of Cushman and Cheung (1971). All the chemicals used were of analytical grade (SISCO Research Laboratories, Mumbai, India).

Statistical analysis

Results are expressed as means ± SEM. The statistical significance of differences between the treatment groups was calculated by ANOVA using SPSS software. p < 0.05, 0.0001 were considered significant.
**Results**

**Phytochemical analyses of PK**

The phytochemical analyses revealed that PK contains 241.11 mg% protein and 0.036 gm% crude fiber in addition to polyphenols, flavonoids, phytosterols, saponins and ascorbic acid. The total antioxidant potential of PK (FRAP) is found to be 20.15 mmole/gm (Table 2).

**Fluoride-exposure affects the lipid peroxidation and antioxidant profile in lung tissue**

When the lung homogenates were exposed to various concentrations of NaF (2.5, 5.0, 7.5 and 10.0 ppm) a concentration-dependent increase in lipid peroxidation was observed. The levels of enzymatic antioxidants—SOD, CAT and GPX decreased significantly with increasing concentrations of NaF. Similarly, when the lung homogenates were exposed to different concentrations of NaF, the content of non-enzymatic antioxidants—TAA and GSH—also decreased significantly. The total antioxidant capacity of the lung tissue decreased with increasing exposure to fluoride concentrations (Figures 1 and 2).

**PK extract decreases lipid peroxidation and improves the antioxidant profile in lung tissue**

A significant reduction in LPO levels were observed in lung homogenates exposed to NaF (2.5, 5.0, 7.5 and 10.0 ppm) when treated with PK extracts (100, 200 and 300 µl). Besides, the PK extracts were also found to decrease the LPO levels in lung extracts even when they were not exposed to NaF. On the other hand, significant increases in the levels of enzymatic and non-enzymatic antioxidants occurred in a dose-dependent manner in presence as well as in absence of NaF (Figures 1 and 2).

**Discussion**

In recent decades, extensive information has been accumulated on the role of fluoride in cellular respiratory processes and associated free radical reactions (Rzeuski et al., 1998). It has been shown that chronic intake of fluoride enhances lipid peroxidation and decreases antioxidant levels in soft tissues of mice (Chinoy and Patel, 1998; Rzeuski et al., 1998; Shashi, 1992; Shivarajashankara et al., 2001).

In the present study, when lung homogenates were treated with 2.5 to 10.0 ppm of sodium fluoride, lipid peroxidation increased up to 64% at 10.0 ppm. PK at all three doses was found to counteract fluoride-induced lipid peroxidation, and the highest dose (300 µl) reduced the LPO levels by 30%. Even when untreated (NaF) lung homogenates were incubated with PK extracts, a dose-dependent reduction in lipid peroxidation (by 18%) was observed. These observations indicate that (i) PK can reduce the oxidative stress caused by the cellular metabolism and (ii) PK can reduce the oxidative stress significantly in presence of NaF. This property of PK could be attributed to its high antioxidant potential. Besides, the lung extracts also showed higher FRAP values when incubated with PK in presence / absence of NaF (Figures 1 and 2).

Vitamin C (ascorbic acid) is a powerful inhibitor of lipid peroxidation and regenerates vitamin E in lipoproteins and membranes. Being an anti-stress factor, Vitamin C acts as an antioxidant; scavenges free radicals and reduces the fluoride levels in the body (Cesari et al., 2004). Ascorbic acid was found to have a dual effect in brain—at low concentration

### Table 2. Phytochemical analyses of Pankajakasthuri

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values (triplicate mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (mg %)</td>
<td>241.11 ± 0.69</td>
</tr>
<tr>
<td>Crude fiber (gm %)</td>
<td>0.036 ± 0.02</td>
</tr>
<tr>
<td>Polyphenols (mg %)</td>
<td>28.42 ± 0.92</td>
</tr>
<tr>
<td>Total phytosterols (mg %)</td>
<td>9.21 ± 0.58</td>
</tr>
<tr>
<td>Flavonoids (mg %)</td>
<td>24.29 ± 0.72</td>
</tr>
<tr>
<td>Saponins (gm %)</td>
<td>19.36 ± 0.42</td>
</tr>
<tr>
<td>Total ascorbic acid (mg/gm)</td>
<td>144.24 ± 3.70</td>
</tr>
<tr>
<td>FRAP µmole/ mg</td>
<td>20.15 ± 0.08</td>
</tr>
</tbody>
</table>

**Fluoride-exposure affects ACE activity in lung tissue**

When the lung homogenates were subjected to different concentrations of NaF (2.5, 5.0, 7.5 and 10.0 ppm) a concentration-dependent reduction was found in ACE activity (Table 3).

**PK extract restores the ACE activity in lung tissue**

In the presence of both dialyzed and undialyzed extracts of PK, a significant elevation in ACE activity occurred in lung homogenates exposed to 10 ppm NaF. Undialyzed extract was however found to be more potent in restoring the ACE activity in a dose-dependent manner as compared to the dialyzed extract (Table 3).
Figure 1. Effects of NaF and Pankajakasturi in lung homogenates on lipid peroxidation (TBARS), total ascorbic acid (TAA), superoxide dismutase (SOD) and catalase (CAT).

Figure 2. Effects of NaF and Pankajakasturi in lung homogenates on glutathione (GSH), glutathione peroxidase (GPX) and total antioxidant capacity (FRAP).
promoting lipid peroxidation and at high concentration acting as an antioxidant (Halliwell and Gutteridge, 1985). In the present context, a significant reduction in ascorbic acid content was found in lung homogenates treated with fluoride and when lung extracts were incubated with PK, the ascorbic acid content increased significantly with all doses.

The antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) also play important roles in reducing the cellular stress. SOD scavenges the superoxide radical by converting it to H2O2 and molecular oxygen (Robinson, 1998) while catalase brings about the reduction of hydrogen peroxides and protects tissues from the highly reactive hydroxyl radicals (Brioukhanov and Netrusov, 2004). Presently, fluoride at all concentrations significantly decreased the levels of both SOD and CAT activities. At 10.0 ppm, NaF exerted maximum inhibition on SOD and CAT. On the other hand, PK at all doses increased the activities of both SOD and CAT; especially the highest dose of PK brought about increased activation of the enzymes. When the lung homogenates were incubated with PK extracts alone, the activities of both SOD and CAT increased significantly indicating the antioxidant capacity of PK and its ability to reduce the NaF toxicity on both these enzymes (Figure 1).

Glutathione (GSH), a tripeptide normally present at high concentrations intracellularly, constitutes the major reducing capacity of the cytoplasm (Lu, 1999) and protects the cellular system against toxic effects of lipid peroxidation (Nicotera and Orrenius, 1986). Glutathione peroxidase (GPX) is selenium-containing enzyme and acts on reduced glutathione and H2O2 to produce oxidized glutathione and H2O. It provides second line of defense against hydro-peroxides before they can damage membranes and other cell components. A reduced GPX activity with a concurrent reduction in GSH indicates that GPX activity is dependent on GSH content (Illing et al., 1951). In the present context, all the NaF concentrations used (2.5 to 10.0 ppm) reduced the levels of both GSH and GPX significantly and PK improved these levels significantly in a dose-dependent manner. The nascent lung extracts too exhibited an increase in GSH and GPX levels when incubated with PK extracts. These observations reiterate the antioxidant potential of PK that could be related to the phytoconstituents namely fibers, sterols, saponins, phenols, flavonoids, and ascorbic acid (Table 2) as all these have been shown to be antioxidants (Bhattacharya et al., 1997; Kang et al., 1998a, 1998b, 1999, 2000; Lemcke-Norojarvi et al., 2001; Lindequist et al., 2005; Nozaki et al., 2006; Vinson et al., 1998).

ACE is a metallopeptide that plays a major role in blood pressure regulation and electrolyte homeostasis by acting on two major vasoactive peptides (Skeggs et al., 1955; Dorer et al., 1974). In the present study, NaF at all concentrations (2.5 to 10.0 ppm) suppressed ACE activity significantly and the lung homogenates registered increasing ACE activity in presence of both undialyzed and dialyzed PK extracts indicating the counteraction of PK in reducing the inhibition of ACE brought about by NaF. Interestingly, all three doses of the undialyzed PK extracts were found to be more potent than the dialyzed extracts in improving the ACE activity (Table 3).

In conclusion, the results of the present study revealed that sodium fluoride depresses both antioxidant profiles and ACE activity in lung homogenates. PK at different doses increases the enzymatic and non-enzymatic antioxidants in a dose-dependent manner with a reduction in the levels of lipid peroxidation and significantly increases ACE activity in presence

### Table 3. ACE activity in lung homogenates incubated with fluoride ions and PK extracts

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>ACE units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Control (lung homogenate)</td>
<td>59.71 ± 0.19</td>
</tr>
<tr>
<td>2 FC- 2.5 ppm</td>
<td>14.77 ± 0.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3 FC- 5.0 ppm</td>
<td>14.52 ± 1.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4 FC- 7.5 ppm</td>
<td>13.79 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 FC- 10.0 ppm</td>
<td>13.00 ± 0.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaF ppm PK extract (µl) UDPKE DPKE</td>
<td></td>
</tr>
<tr>
<td>6 10.0 100</td>
<td>20.22 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 10.0 200</td>
<td>40.25 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6 10.0 300</td>
<td>49.66 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

DPKE: dialyzed extract of Pankajakasthuri, ns: non significant, UDPKE: undialyzed extract of Pankajakasthuri.

<sup>a</sup> FC-2.5, 5, 7.5 and 10 ppm: Lung homogenates (fluoride controls) treated with 2.5, 5, 7.5 and 10 ppm of sodium fluoride.

<sup>b</sup> p < 0.0001.
and absence of sodium fluoride. It can be concluded that the aforementioned effects of PK is owing to the phytoconstituents present in both dialyzed and undialyzed extracts.

Acknowledgement
We are thankful to Head, Department of Biosciences, for providing the facilities required for the present work.

Funding
Financial assistance in the form of a Research Fellowship to RAV from UGC, New Delhi, India is gratefully acknowledged.

References


