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To cite this article: Varsha Hinge, Jaykiran Tidke, Biswadeep Das, Shrikant Bhute, Pradeep Parab & Kishori Apte (2016): Air pollution exacerbates effect of allergenic pollen proteins of *Cassia siamea*: a preliminary report, Grana, DOI: [10.1080/00173134.2016.1193221](https://doi.org/10.1080/00173134.2016.1193221)

To link to this article: <http://dx.doi.org/10.1080/00173134.2016.1193221>



Published online: 25 Jul 2016.



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Air pollution exacerbates effect of allergenic pollen proteins of *Cassia siamea*: a preliminary report

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Abstract

Pollinosis caused by several allergenic proteins in pollen grains has been a major problem of healthcare in most parts of the world. Environmental factors such as air pollution are known to alter the release of allergenic pollen proteins from a variety of the plant species. *Cassia siamea* is commonly planted along roadsides and in industrialised areas in many parts of India. The present study reports the findings of animal experiments demonstrating the effect of air pollution on the allergenicity of *Cassia siamea* pollen proteins. Total white blood cell (WBC) count and lymphocyte count were significantly higher in animals that received protein extract of pollen collected from a polluted site compared to those that received protein extract of pollen collected from a non-polluted site. This was concomitant with increased production of IgE antibodies; followed by marked degranulation of mast cell leading to heightened type I hypersensitivity in these animals. These results are important for the development of a consensus linking ever-increasing pollution due to industrialisation and an increase in associated pollinosis.

Keywords: air pollution, *Cassia siamea*, pollen proteins, allergenicity

The pollen of many trees, weeds and grasses are major causes of allergies in several parts of the world (D'Amato et al. 2013) including India (Singh & Shahi 2008). Although pollen themselves are not allergenic, they carry several proteins, glycoproteins and non-protein substances such as pollen associated lipid mediators (PALMs) within them during their flight that make them allergenic (Gilles et al. 2009; Songnuan 2013). Several proteins essential to make pollen a male gametophyte are strategically located and released from various sites of pollen like exine, intine or cytoplasm (Behrendt & Becker 2001). Some of these proteins belonging to protein families such as profilin (Tehrani et al. 2011); polcalcin and expansin (Radauer & Breiteneder 2006) act as potent aeroallergens. Similarly, by using one-dimensional (1D) and two-dimensional (2D) electrophoresis and Western blotting, allergenic pollen glycoprotein have been characterised (Chow et al. 2005; Manduzio et al. 2012). In addition, it has

been demonstrated that pollen release excessive amounts of lipids called PALMs upon hydration, which independent of proteins present in pollen shows immuno-stimulatory and/or immune-modulatory properties (Behrendt et al. 2001; Gilles et al. 2009). Pollen particles thus act as a carrier of complex allergenic substances, which may lead to allergic rhinitis (Taketomi et al. 2006).

The effect of air pollution on human health has a long history; there has been a rapid increase in air pollution during the last century with increased global population, excessive energy consumption in the form of petroleum and its derivatives in motor vehicles and industrial activities (Bartra et al. 2007). The prevalence of atopy and other allergenic disorders has been found rising parallel with increased air pollution, especially in industrialised countries (D'Amato et al. 2014). Pollen grains of gymnosperms and angiosperms are major contributors of aeroallergens (Sénéchal et al. 2015) and some studies have indicated the

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(Received 4 December 2015; accepted 30 April 2016)

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possible role of air pollution in increased allergenicity of pollen under polluted conditions over non-polluted conditions (Armentia et al. 2002; Cortegano et al. 2004). This rise in plant derived respiratory allergies could be partly explained by the fact that different air pollutants interact with pollen and alter their antigenic potency (Arbabian et al. 2011; Rezanejad et al. 2003). Air pollutants such as carbon dioxide (CO₂) present in the air is known to affect fertilisation and pollen production as well as allergen contents of pollen grains (Reid & Gamble 2009). Diesel exhaust and nitrogen oxide have been observed to act as adjuvant to pollen allergen both in animals (Zijverden et al. 2000) as well as humans (Diaz-Sanchez et al. 2000). Traffic-related pollutants such as nitrogen dioxide (NO₂) and ozone (O₃) are linked with the release of pollen cytoplasmic granules (PCGs) which are likely to contain allergens (Motta et al. 2006). Thus, there is a direct link between air pollution and altered and/or enhanced pollen allergies of pollen of many plants.

Cassia siamea L. (Fabaceae) has attracted ethnopharmacology for various medicinally important components purified from different parts of the plant (Kumar et al. 2010; Nsonde et al. 2010; Deguchi et al. 2012). Medium size trees of *C. siamea* are often planted along roadsides and in industrialised areas as afforestation programmes to control pollution. These plants densely flower during winter, thus increased pollen loads are observed near plantations during the flowering period (Singh & Shahi 2008). Using skin prick tests, Parui et al. (2002) have shown the presence of three potent allergenic proteins in the pollen of *C. siamea* corresponding to the molecular weight of 45, 53 and 97 kDa. Hussain et al. (2013) extended the work of identifying pollen allergens from three different *Cassia* species, namely *C. fistula* L., *C. occidentalis* L. and *C. tora* L., and showed that the pollen of these plants are important sources of aero-allergens near to plantations. However, to date, specific reports on the effects of air pollution on the allergenicity of *C. siamea* pollen are not available. The present study was therefore carried out to evaluate the effect of air pollution on the allergenicity of *C. siamea* pollen proteins.

Material and methods

Collection of *Cassia siamea* pollen

Two sites, one polluted and one non-polluted, were selected according to the data on ambient air quality, monitored by the Pune City Maharashtra Pollution Control Board. Several hundred anthers of *Cassia siamea* were collected from nearby plants for both sites at the time of anthesis. Anthers were immediately transported to the laboratory, crushed to obtain

pollen and sieved through different grade of meshes (100, 200 and 300 µm). Purity of pollen was checked by microscope observation (shape and size; Parui et al. 2002) and stored at -80 °C until the protein extraction.

Pollen protein extraction and quantification

Pollen proteins were extracted as described following the procedures of Noir et al. (2005). Briefly, 100 mg of pollen from polluted and non-polluted sites were carefully ground in liquid nitrogen and mixed with Tris-HCl buffer containing 50 mM Tris-HCl (pH 8.8), EDTA 5 mM, 20 mM DTT, 100 mM KCl and 2mM PMSF. The mixture was further ground for 20 min and centrifuged at 12 000 RPM for 30 min at 4 °C. Total proteins in the supernatant were precipitated with five volumes of acetone and incubated at -20 °C for 2 h. After the incubation a protein pellet was obtained by centrifugation at 12 000 RPM for 30 min at 4 °C. The resulting protein concentration was quantified using the Bradford method (Bradford 1976).

One-dimensional (1D)-gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) and Parui et al. (2002). Briefly, the concentration of protein extracts from polluted and non-polluted sites was adjusted to 50 µg in 10 µl mixed with sample buffer [0.06 M Tris-HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol, 0.01% bromophenol blue] and heated at 100 °C for 3 min. This mixture was loaded onto a 12% SDS-PAGE gel along with the PageRuler Plus Pre-stained Protein Ladder (Thermo Scientific, Waltham, MA, USA) and electrophoresed in buffer containing 0.05 M Tris-HCl, 0.192 M glycine and 0.1% SDS (pH 8.4) at 70 V for 150 min. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R250 and destained with methanol/acetic acid/water (4:1:5).

Animals used

All animal experiments were performed at the National Toxicology Centre, Pune, India. The experimental protocols (No. 201 on 19 January 2013) were approved by the Institutional Animal Ethical Committee (IAEC) following the guideline of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest and Climate Change, Government of India, New Dehli, India. Total of 24 Swiss albino mice (18–20 g) and six Wistar rats (150–170 g) were obtained from the in-house animal facility of the

National Toxicology Centre, Pune. Throughout the experiment, animals were fed with standard pellet food and water *ad libitum* and maintained at an optimum temperature and relative humidity with 12 h light/dark cycle. The mice were randomly divided into four experimental groups having six animals in each group as follows:

- Group 1. – Normal control (NC), received 100 µl/ml saline.
- Group 2. – Positive control (PC), received triple antigen.
- Group 3. – Polluted (P), received 100 µg/ml protein extract from the polluted site.
- Group 4. – Non-polluted (NP), received 100 µg/ml protein extract from the non-polluted site.

Haematological assays

During the 14-day study, blood samples were withdrawn at an interval of 0, 7 and 14 days from the retro orbital plexus of every animal in each group. Haematological assays were performed to record the differences in percentage changes of each cell type in response to allergenic challenge with polluted and non-polluted pollen extracts. All haematological assay were performed on Mindray BC-2800 Hematological Analyser.

In vitro mast cell degranulation (MCD)

On day 14, sera from active anaphylaxis induced animals from all groups was obtained except for the positive control group. Animals in the positive control group were injected intraperitoneally with a compound 48/80 at the concentration of 8 mg per kg of body weight. Mast cells were immediately obtained from these animals and used as positive control. In addition, mast cells were obtained from a donor Wistar rat by intraperitoneally injecting 10 ml normal saline and used for mast cell degranulation (MCD) assays as described by Das and Chauhan (2013). Fifty microlitres peritoneal mast cell suspension was mixed with 100 µl blood sera of each animal from all four groups and 10 µl antigen from the polluted and the non-polluted sites, respectively. This mixture was incubated for 3 min at 37 °C. After incubation, freshly prepared 2% glutaraldehyde solution in 0.2 M sodium phosphate buffer was added to the mixture and the cell pellet was obtained by centrifugation at 300g for 15 min. The remaining pellet was re-suspended in a minimum amount of supernatant and smear was made with 50 µl of the suspension. The smear was air-dried and stained with 0.1% toluidine blue and mast cells were counted. Final results were noted as a proportion

of morphologically altered cells to that of the total number of cells counted using the following formula

$$\%MCD = \frac{\text{Number of degranulated mast cells}}{\text{Total number of mast cells}} \times 100$$

Quantification of pollen allergen-specific antibodies

Three Wistar rats in each group (polluted and non-polluted) were used for antibody production as per the following dose: On the first day, a subcutaneous injection of 10 µl of pollen protein extract containing 10 µg of total proteins + 490 µl saline + 500 µl FCA (Freund's Complete Adjuvant) was given to each animal in the group. On the seventh day, a booster dose was given containing 5 µl of pollen protein extract containing 10 µg of total proteins + 495 µl saline + 500 µl FCA. After the similar booster dose on the tenth day, immune sera were collected and stored at –20 °C and were used for confirming antibody production using ELISA (enzyme-linked immunosorbent assay). Levels of polyclonal antibody production were measured by comparing antibody levels on the tenth day of the immune sera to the pre-immune blood sera from the first day as described in Sridhara et al. (2002). Thus, 10 µg/ml pollen protein extract from polluted and non-polluted samples were applied to a microtiter plate by overnight incubation at 4 °C and blocked with 100 µl 1% BSA (bovine serum albumin) at room temperature for 1 h. The wells were washed in 0.05% Tween in PBS (phosphate-buffered saline) three times and 1:100 dilution of primary antibodies (anti-sera obtained from animals) was added to respective wells and incubated for 3 h at 37 °C for antibodies to bind to the antigen. After incubation, the plate was washed with 0.05% Tween in PBS three times to remove any unbound antibodies and 1:1000 dilution of anti-rat immunoglobulin G (IgG) antibodies as secondary antibodies labelled with horseradish peroxidase was added to each well. The plates were incubated for 3 h at 37 °C for secondary antibodies to bind to primary antibodies and washed with 0.05% Tween in PBS three times to remove any unbound antibodies. A substrate 2 mg/ml ABTS was added and incubated for 10 min to form a colour complex and optic density (OD) was taken at 410 nm using a spectrophotometer. Fold increase in antibody productions was calculated using the following formula:

$$\text{fold increase} = \frac{\text{OD of polluted sample}}{\text{OD of non-polluted sample}} \times 100$$

Statistical analysis

Statistical analysis was performed in GraphPad Prism (version 5). Analysis of variance (one-way ANOVA)

test was used to compare both the blood cell types and MCD among the four study groups.

Results

SDS-PAGE

An SDS-PAGE profile revealed a pattern of around 10 bands in both pollen extracts ranging in molecular weight 25–120 kDa. We observed the qualitative difference in band intensities of polluted and non-polluted pollen extracts, polluted pollen extracts showed intense bands as compared to non-polluted pollen extracts (Figure 1). Notably, a band at around 50 kDa was found to be highly intense in pollen extract from the polluted site.

Polluted pollen extract has profound effect on haematological counts

We applied one-way ANOVA test followed by Tukey's HSD (honest significant difference) *post hoc* test to check whether pollen extracts from the polluted site and the non-polluted site have any effect on haematological cell counts (Figure 2, Table I). We observed no effect on monocyte and granulocyte counts throughout the study in any of the animal groups. However, on day 14, the total white blood cell (WBC) count (p -value = 0.00074) and lymphocyte count (p -value = 0.00021) were found significantly elevated in mice that received pollen protein extract from the polluted site as compared to the mice that received pollen protein extract from the non-polluted site as well as with positive and negative control.

Polluted pollen extract enhanced mast cell degranulation (MCD)

Mast cells are among the first cells to come in contact with inhaled particulate antigens and play an important role in immediate hypersensitivity. Therefore, we hypothesised that pollen extract collected from the polluted site to have profound effects on activation of mast cells. We observed that in normal control group the percentage MCD was $17.1 \pm 3.2\%$, whereas in the positive control group, it was $83.0 \pm 2.7\%$. Whereas, percentage MCD observed with non-polluted and polluted pollen extracts were $69.5 \pm 2.9\%$ and $79.0 \pm 2.5\%$, respectively. A t -test revealed that the percentage degranulation of mast cell was significantly higher in the pollen extract from polluted site than from non-polluted (p value < 0.01; Figure 3, Table II). These results suggest that pollution is responsible for the release of more allergenic components from pollen

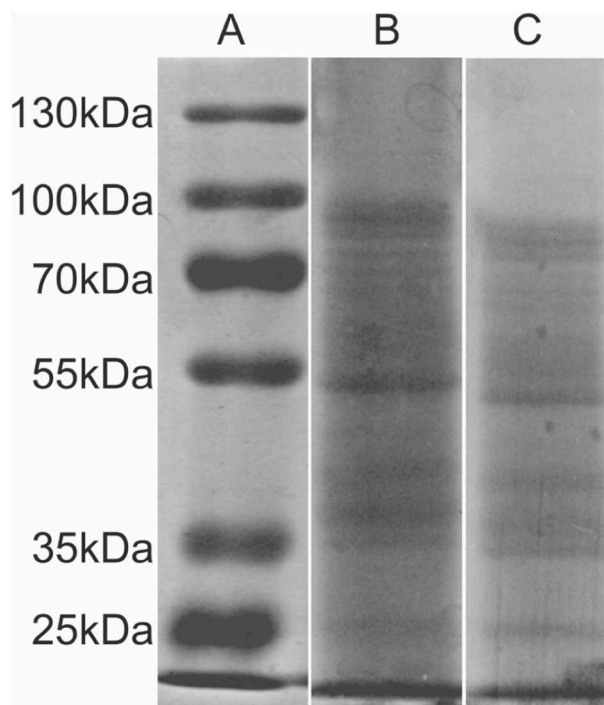


Figure 1. SDS-PAGE protein profile of *Cassia siamea* – **A**. Molecular weight marker. **B**. Pollen extract collected from polluted site. **C**. Pollen extract collected from non-polluted site.

and subsequent rise in mast cell activation/degranulation.

Antibodies against pollen allergen increased in polluted samples

We next compared the fold increase in antibody production of the polluted site and the non-polluted site. We observed 2.98 and 2.25 fold increase in antibody production for the protein samples collected from the polluted site and the non-polluted site, respectively. Thus, our results confirm that the protein extract collected from the polluted site led to a substantial increase in humoral immune response compared to protein extract collected from the non-polluted site.

Discussion

Our results demonstrate that pollen extract of *Cassia siamea* collected from both the polluted site and the non-polluted site elicits type I hypersensitivity. But the extent of hypersensitivity observed in animals receiving pollen extract from the polluted site was higher. We base our conclusion on two facts: first, pollen extracts from the polluted site evoked production of IgE antibodies, and second, the MCD was higher upon stimulation with pollen extract from this

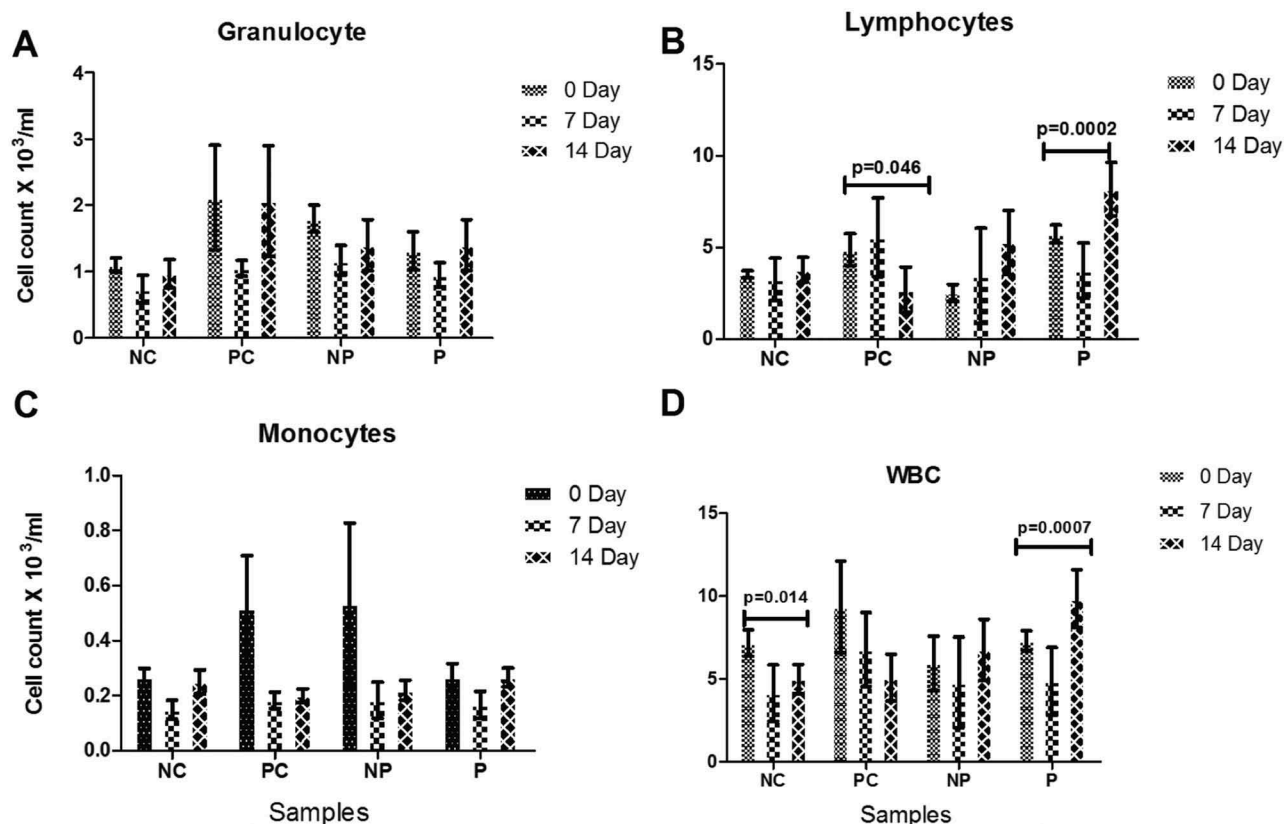


Figure 2. Variation in haematological parameters. **A.** Granulocyte. **B.** Lymphocytes. **C.** Monocytes. **D.** Total white blood cell (WBC) count in different groups of animals. Abbreviations: NC, negative control; PC, positive control; NP, non-polluted pollen extract; P, polluted pollen extract; at 0, 7 and 14 days.

site. Both features are the hallmark of type I hypersensitivity reactions marked by two phases: the sensitisation phase and the effector phase. The sensitisation phase is marked by the production of allergen-specific IgE antibodies that eventually bind to Fc receptors on mast cells. The effector phase is the pathological phase, which is marked by the degranulation of mast cells if a Fab region of antibodies cross-linked with the specific allergen during the subsequent exposure. Our results are in accordance with previous studies reporting similar findings with the pollen extract of other plant species (Klein et al. 2000; Katayama et al. 2005). We further speculate that this exacerbation of the effect of air pollutant on allergenicity of pollen proteins could be due to altered morphological features of pollen (such as breaks in exine wall) leading to the excessive release of pollen proteins as evident from our SDS-PAGE analysis.

The severity and prevalence of pollen associated respiratory allergic diseases is more common in industrialised versus non-industrialised and urban versus rural areas (Amato 2000). Several studies conducted on pollen allergies from industrialised

and non-industrialised areas have highlighted the possible role of air pollution in heightened pollinosis for several plant species (Arbabian et al. 2011; Armentia et al. 2002; Ghiani et al. 2012). The pollen proteins from *Cassia siamea*, a plant which is abundantly found in most parts of India, have been shown to possess allergenic properties (Parui et al. 2002). Out of the 11 bands observed on the 1D-gel, Parui et al. (2002) found a total of nine protein bands to be allergenic in susceptible individuals, of which three bands were major and the rest were minor allergenic proteins. In contrast to the 11 bands reported in an earlier study, we were able to observe ten distinct protein bands on the 1D-gel. This difference could be attributed to the minor differences during the protein extraction steps or due the differences in sensitivity of staining dye used. Other members of genus *Cassia* (*C. tora*, *C. occidentalis* and *C. fistula*) were found to have 10–11 protein bands on the 1D-gels, some of which found to be allergenic during the skin prick test in susceptible individuals (Hussain et al. 2013). In addition, a study based on light and scanning electron microscopy has revealed morphological changes in pollen of two *Cassia* species

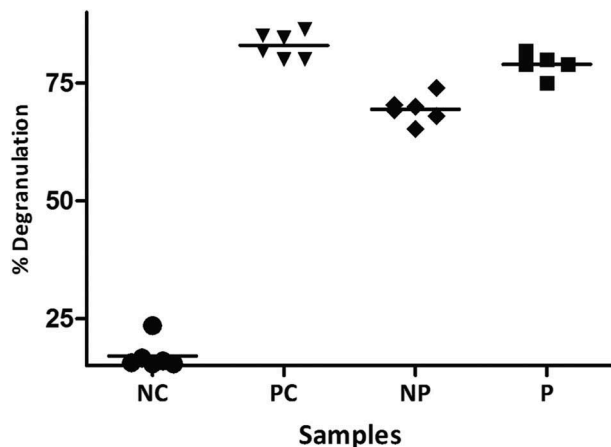


Figure 3. Percentage mast cell degranulation (MCD) observed per group. Abbreviations: NC, negative control; PC, positive control; NP, non-polluted pollen extract; P, polluted pollen extract.

(*C. siamea*, *C. fistula*; Kalkar & Jaiswal 2014). The study reports that the pollen from the polluted site was shrunken with broken exine and deposition of particulate matters on their surface as compared to pollen from the non-polluted site. Hence, the result presented here, are in accordance with these previous studies and suggests the allergenic nature of *C. siamea* pollen, which further exacerbates due to pollution.

Strictly controlled laboratory experiments are generally conducted to assess the effect of air pollutants on allergenicity of pollen (Motta et al. 2006). However, extrapolation of the results of such experiments to atmospheric *in situ* conditions is often limited due

to the use of a single pollutant at a time and use of matured dehiscent pollen. In the present study, we have adopted a more realistic field approach to reveal the effect of air pollution on allergenicity of *Cassia siamea* pollen protein extracts. Such an approach is now becoming more popular in the relevant studies (Armentia et al. 2002; Ghiani et al. 2012).

While the use of crude pollen protein extracts can be viewed as a limitation of our study, the broader aim of our work was to check the effect of air pollution on the allergenicity of pollen as a whole and not of specific pollen protein derived from *Cassia siamea*. Furthermore, many components in crude protein extracts such as carbohydrates, lipids and leptins, etc. may act as allergens themselves or as an adjuvant to the allergenic action of the protein fraction (Bashir et al. 2013). Thus, the heightened allergenic response that we observed could be a cumulative one largely due to the allergenic pollen proteins and partly due to the other components of pollen such as pollen associated lipid mediators (Gilles et al. 2009).

Conclusion

Our results suggest an association between traffic related or industry associated air pollution and the allergenicity of pollen proteins. Considering the ever increasing pollution and the extent of *Cassia siamea* plantation in India, our result may facilitate policy-makers to adopt new strategies to control air pollution and associated allergies.

Table I. Summary of a one-way ANOVA of different white blood cell (WBC) types with Tukey's HSD *post hoc* test.

Cells and group	SS	MS	F	p-Value
Monocyte				
NC	0.047778	0.023889	2.905405	0.085806*
PC	0.423333	0.211667	2.744957	0.096413*
NP	0.435039	0.21752	1.099509	0.360143
P	0.04	0.02	1.666667	0.222011
Lymphocyte				
NC	0.347778	0.173889	0.289333	0.752849
PC	27.00444	13.50222	3.806183	0.046035**
NP	21.04651	10.52325	2.815067	0.09385*
P	59.61	29.805	15.60199	0.000217**
Granulocyte				
NC	0.413333	0.206667	1.660714	0.223095
PC	4.853333	2.426667	1.599883	0.234526
NP	1.231111	0.615556	2.613208	0.106241
P	0.687778	0.343889	1.238991	0.317687
Total WBC				
NC	29.03444	14.51722	5.799414	0.013621**
PC	55.76333	27.88167	1.575255	0.239342
NP	10.93525	5.467627	0.579745	0.572934
P	75.00444	37.50222	12.08753	0.000747**

*p-Value below 0.1. **p-Value below 0.05. Values in bold indicate p-values below 0.05.

Table II. Summary of *t*-test between different groups for mast cell degranulation (MCD)

Pair	Paired differences			<i>t</i>	df	<i>p</i> -Value
	Mean	Standard deviation	Standard error of the mean			
NC	17.09	3.18	1.31	−38.4347	10	3.39E-12
PC	82.96	2.7	1.11			
NC	17.09	3.18	1.30	−35.2094	9	5.94E-11
P	78.94	2.49	1.11			
NC	17.09	3.18	1.31	−29.9093	10	4.08E-11
NP	69.45	2.68	1.17			
NP	69.45	3.18	1.31	−5.78288	9	0.000265
P	78.94	2.49	1.11			
PC	82.96	2.7	1.11	8.355218	10	8.03E-06
NP	69.45	2.68	1.17			
PC	82.96	2.7	1.11	2.526686	9	0.032411
P	78.94	2.49	1.11			

Acknowledgements

The authors are thankful to Tania Paul (APT Research Foundation), and the APT Research Foundation, Pune, India, for helping in the animal experiments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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