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PROTEIN ESTIMATION AND PALYNLOGICAL STUDIES OF *CANNABIS SATIVA* L. POLLEN IN RELATION TO RESPIRATORY ALLERGIES

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Abstract

Airborne pollen allergies and asthma are on a rise in the metropolitan city of Islamabad. Knowledge of allergenic pollen is limited in the area. *Cannabis sativa* L. or commonly known as Hemp is widely spread weed in the city. Morphological studies performed via light microscopy and SEM have shown that the pollen of *Cannabis sativa* are 21µm long having triporate aperture, spheroidal in shape and scaberate exine. Quantitative and qualitative analysis of pollen proteins has also be done in to recognize allergenic protein bands. Bradford's analysis for proteins quantification has shown that the hemp pollen has 30.69 mg/g protein in fresh weight of pollen. While SDS-PAGE analysis showed 11 bands of various protein size ranging from 17kDa to 150kDa. The research findings indicate that *Cannabis sativa*, could be a potent allergenic pollen-producing weed that might cause serious health problems in the population of Islamabad.

Key words: *Cannabis*, Pollen, Allergy, Protein, Pollen morphology

Introduction

Biological invasions especially plant invasive species gained much importance during the last decades. The study of invasive plant species is getting indispensable owing to the emergence of new health problems. Especially, a widespread increase in prevalence and severity of respiratory allergic diseases like asthma (Hussain *et al.,* 2013) is generally being correlated with the advancement of civilization (Puc, 2003). It is said that allergic rhinitis and asthma showing the greatest rising trend, and anaphylaxis being the most severe type (Armentia *et al.,* 2011). Prospective studies suggested that allergic diseases such as allergic rhinitis, bronchial asthma, and atopic dermatitis have increased progressively throughout the world as well as in developing countries like Pakistan. However, for the last few years, this problem of allergic diseases in our country especially in twin cities of Rawalpindi and Islamabad has escalated. It is known that various factors are considered to be responsible to cause allergy, foremost contributing agents toward allergy are pollen grains, house dust mites, spores, insect debris, animal epithelia, etc (Singh & Kumar, 2003; Platts-Mills *et al*., 2011).

Formerly, not much research work was carried out regarding harmful effects of invasive species in Islamabad in relation to pollen allergy. Fourteen invasive plant species were identified as the "Species of Concern". Those included *Broussonetia papyrifera, Lantana camara, Prosopis juliflora, Xanthium strumarium, Cannabis sativa* and *Parthenium hysterophorus* (Fatimah & Ahmad, 2012). These plant species are responsible in aggravating pollen allergy. Pollen from monocotyledonic (grasses) and dicotyledonic (trees, weeds) considered to be one of the most important and frequent potent inhalant allergen sources (Perveen *et al.,* 2012; Hayek *et al*., 2014). Likewise, weed pollens are common sources of allergens worldwide so as in Islamabad *Cannabis sativa* is present in almost all the sectors of Islamabad, and its pollens are considered to be the main cause of pollen allergy in residents of Islamabad during fall season.

Pollen allergens represent low molecular weight proteins or glycoproteins (5-70 kDa) that surprisingly are located mainly intracellularly in the pollen grains (Hayek *et al*., 2014). Most of the allergens purified since 1960 were mostly proteins or glyco-proteins (Platts-Mills *et al*., 2011). Allergenic pollens or proteins usually have molecular weights ranging from 10–70 kDa and are water soluble or glyco-proteins. These proteins are highly stable and can withstand high temperature (even up to 100°C), pH changes (Puc, 2003).

Invasiveness of *Cannabis sativa* in Pakistan: In Pakistan *Cannabis sativa* is distributed in northern Punjab and Khyber Pakhtunkhwa. Although, it is not much aggressive and has a moderate degree of invasiveness but still is responsible for second most pollens counts in Islamabad after Paper mulberry. In northern Punjab and KPK, it is found along the roadside and invades waste areas, fence rows around farm building usually on bottomland soil. It is very adaptable herb because it is present from plains to 1000 ft. The history of *Cannabis sativa* invasion in Pakistan is not very clear but it appears to be an ancient introduction. It is assumed that it was cultivated initially for fiber use and for medicinal importance, which later escaped from cultivation and became wild (Marwat *et al*., 2010).

Hypersensitivity reactions to *Cannabis sativa*: Allergy to *Cannabis sativa* (hemp) pollen was recognized in 1980. Although, a lot of work has been done on various aspects of *C. sativa* as biologically active plant but its potential for allergenicity has scarcely been studied till date. The studies indicating clinical significance of its pollen as an aeroallergen and its management have been sparse. It was being documented first time over 40 years ago that *Cannabis sativa* cause allergy, despite of the fact, to date, little is known about the causal allergens associated with *C. sativa* (Nayak *et al.*, 2013).

Materials and Methods

Pollen collection: Pure pollen grains were obtained from buds of *Cannabis sativa*. The buds were collected in bulk amount and were allowed to air dry. After 24 hours, air dried buds were crushed gently so that pollen were collected with the help of brushes and physically purified by sieving of mesh size 100 µm so that debris was removed and only pollens were left. Collected pollens were then stored at -80^{o}C in air tight bags to avoid any contamination.

Palynological studies: *Cannabis sativa* pollen morphological studies were done by means of two techniques viz. light microscopy (LM) and Scanning electron microscopy (SEM). The slides of pollen grains for light (LM) and scanning electron microscopy (SEM) were prepared by the method as described by Erdtman, (1952). For Scanning electron microscopy (SEM) purified pollen grains were directly transferred to a metallic stub having double sided cello tape. Pollen grains were then coated with gold in a sputtering chamber (Ion-sputter JFC-1100). Coating was restricted to 150 A. The S.E.M examination was carried out on a Jeol microscope JSM-2.

Protein extraction and quantitative estimation by bradford assay

Extraction of pollen proteins: Pollen grains were defatted acetone in order to remove lipids or fats. 2 g of pollen powder was weighed and put into 10 ml acetone and allowed to shake and dry for 3 to 4 hours in fume hood. Again fresh acetone was added and the process was repeated two to three times so that clear acetone was observed. The pollen were finally air dried.

After defating pollen grains, proteins were extracted by in two extracting buffers *i.e.* Bicarbonate-buffered saline (Coca's extract) and Phosphate buffered Saline solution (pH 7.4). Extraction of proteins was carried out for 24 hours at low temperature (4^{o}C) with continuous mixing. The extracts were stored at 4^{o}C.

Lyophilization / Freeze drying: The extracted proteins were first frozen at -80^{o}C and in order to lyophilize the sample protein extract was shifted to vacuum cup. The protein samples were lyophilized in order to get solid, dried components of the protein after subliming water from it (Nireesha *et al.,* 2013).

Protein estimation by bradford assay: Protein quantification was performed by standard method described by Bradford, (1976). It is a colorimetric assay which involves the binding of a dye (Coomassie Brilliant Blue G-250) to protein which is monitored at 595 nm. Under acidic conditions the dye converts its color from red into its bluer form which is an indication of dye that as it has bound to the protein in the solution.

Characterization of pollen proteins using SDS-PAGE: 15% resolving gel and 4% stacking gel was used for isolation of protein bands of *Cannabis* pollen according to method described by Laemmli, (1970) with some modifications in it. About 20 µl of sample was loaded into sample wells with micropipette. A voltage of 80 Volts was set to run the gel. After the separation of bands through electropherosis, staining solution was used to stain the gel in order to make bands visible (100 g of Ammonium sulphate in 200 mililitre of distilled water stir with 100 ml phosphoric acid. 1.2 g Coomassie Brilliant Blue G-250 into 200 ml methanol. Before use both stocks were mixed and final volume was made up to 1000 ml with distilled water). Afterwards the gel was placed in to a destaining solution for 24 hours to remove on shaker (50 ml of Methanol with 70ml of Acetic acid in 1000 ml of water). The destaining solution was changed frequently until clear gel was obtained.

Results

This study was carried out to conduct survey in order to identify pollen morphological of *Cannabis saliva's* using light and scanning electron microscopy and protein allergens present in pollen of *Cannabis sativa* was identified and characterized by using the SDS-PAGE.

Pollen morphology: P/E ratio: 100; Shape: Spheriodal; Apertures: Triporate, annulate; Pore diameter: 7.89 µm; Polar Length: 21.04µm (18.41µm -23.67µm); Equatorial diameter 21.04µm (18.41µm -23.67µm); Exine: Scaberate (Fig. 1).

Concentration of Protein in pollen extracts: For measuring the quantity of protein present in extracts, Bradford's assay was used. A standard curve was plotted for standard concentration of bovine serum albumin (BSA). Likewise, the optical density (O.D) of sample was taken at 595 nm and the value was compared with standard curve. The quantity of protein in Coca's solution was found to be 7.69µg/ml and pollen protein extracted in PBS (pH 7.4) buffer showed concentration of 1.54 µg/ml and 30.69 mg/g fresh weight of pollen.

Protein Extraction and Quantitative Estimation by Bradford Assay

SDS-PAGE characterization of pollen proteins of *Cannabis sativa*: SDS-PAGE was done by the method of Laemmli, (1970) with slight modifications. 15% polyacrylamide resolving gel and 20 ml of sample was loaded. The findings of current study revealed a range of protein bands from 17 kDa to 150 kDa. A total of 11 protein bands were observed of molecular weights viz. 150 kDa, 105 kDa, 85 kDa, 75kDa, 63 kDa, 55 kDa,, 50 kDa, 46 kDa, 32 kDa, 20 kDa, 17 kDa. It was noteworthy that 4 protein bands were less than 50 KDa which can be characterized as allergenic protein bands (Fig. 2).

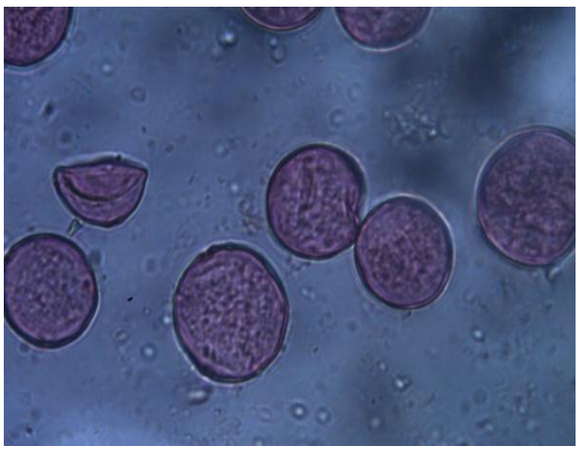

Fig. 1a

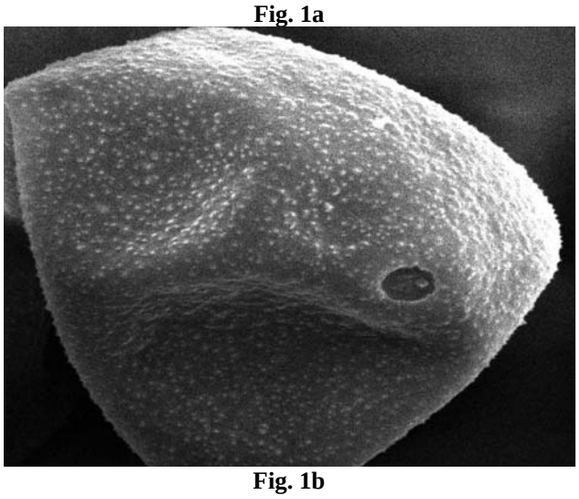

Fig. 1b

Fig. 1. Pollen grain of *Cannabis sativa*; Light microscopic photo-micrographs (1a), Scanning electron photo-micrograph (1b)

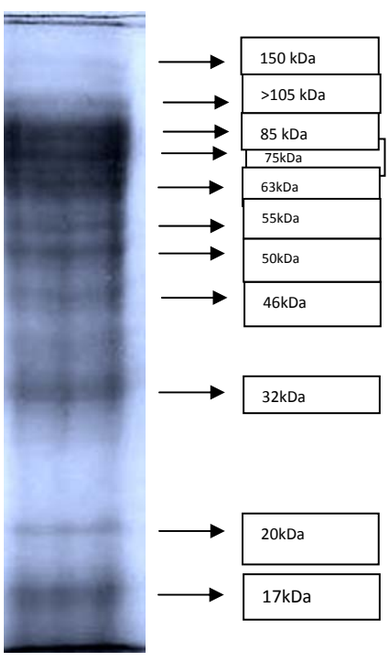

Fig. 2. SDS-PAGE analysis of *Cannabis sativa* pollen proteins.

Discussion

In past few years, worldwide rapid increase in respiratory allergic diseases was due to environmental factors including pollution, climate change and modernization. It is known that pollen allergy is caused by pollens that are wind pollinated i.e. from trees, grasses and weeds (Hussain *et al.*, 2013). According to a survey published by Pakistan Medical and Research Council in 1995, 45% of the residents of Rawalpindi and Islamabad were found to be allergic to pollen of *Cannabis* and paper mulberry. The dilemma of pollen allergy has not been studied extensively in Pakistan but few studies are there in which allergy to pollen was found to be a crucially important risk factor.

Therefore, the aim of present study was to identify pollen allergy patients on the basis of prevalence of various clinical symptoms. As the second aim of this study was to explain various morphological characters of *Cannabis sativa* pollens so palynological features of pollens were studied using light and scanning electron microscopic techniques. At last, but not the least allergenic proteins present in the pollen of *Cannabis sativa* was characterized using SDS-PAGE.

Palynological studies of allergenic pollen is indispensable as these studies are important in order to understand the underlying reasons of pollen being allergic and influence of environmental factors on allergenicity of pollens. Thus, during present study salient morphological features of *Cannabis* pollens were studied. In this context, several authors such as Walker (1955), Godwin, (1967), French & Moore, (1986), Whittington & Gordon, (1987), and Whittington & Edwards, (1989) described and sketched pollen grains of Cannabaceae. Studies by Zavada, (1986), Pehlivan, (1987), Devarkar, (2011) revealed that *Cannabis* pollens are triporate. Likewise, in present study the photo micrographs of *cannabis* pollens confirmed that pollens are triporate

The characterization of allergenic protein in pollens is significantly important in order to analyze the allergenic profile of sensitized individuals. Although, a lot of work has been done on various aspects of *C. sativa* as biologically active plant but its allergenicity has scarcely been studied. Various studies regarding characterization of allergens in *C. sativa* have been done but most of studies were subjected to allergens in other parts of plant other than pollen. Studies by Tanaka *et al.* (1998) and Mayoral *et al.* (2008) are specifically target towards the characterization of *C. sativa* pollen allergens. Hence, it is very first time in Pakistan that characterization of allergens in pollen of *Cannabis sativa* has been done. The results of present study are in accordance from Tanaka *et al.* (1998), and Mayoral *et al.* (2008). Tanaka *et al.* (1998) revealed the presence of six allergic bands i.e.10, 14, 20, 45, 60, and 68 kDa and Mayoral *et al.* (2008) study reported two allergens of molecular weights 37 and 70-80 kDa. According to present study findings shows 11 protein bands which have been reported for the first time. Stokes *et al.* (2000) hypothesized that *Cannabis* might be clinically important allergen especially in patients complaining of asthma and allergic rhinitis symptoms (Kumar & Gupta, 2013).

Conclusion

Prevalence of asthma and allergic rhinitis is on rise and shows an alarming situation in the studied area. Moreover, lower molecular weight protein bands have been detected in the pollen grains of *Cannabis sativa*. This property of studied pollen type might be responsible for eliciting bronchial allergies problems in the population of Islamabad city. This study shows that among other allergenic plants of Islamabad, *Cannabis sativa* is also a potent allergy causing weed that could not be overlooked.

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