CHARACTERIZATION OF THE ALLERGENIC COMPONENTS OF PAPER MULBERRY'S (*BROUSSONETIA PAPYRIFERA L.*) POLLENS

Islamabad

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In The Name Of Allah, The Most Gracious, The Most Compassionate

CERTIFICATE

This thesis submitted by Ms. Sumia Khan is accepted in its present form by the Department of Biotechnology, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, as satisfying the thesis requirements for the degree of Master of Philosophy in Biotechnology.

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Declaration of Originality

I hereby declare that the work accomplished in this thesis is the result research carried out in the Molecular Systematics and Applied Ethnobotany Laboratory, Department of Biotechnology, Quaid-i-Azam University, Islamabad. This thesis has not been published previously nor does it contain any materials from the published resources that can be considered as the violation of international copyright law. Furthermore, I also declare that I am aware of the terms 'copy right' and 'plagiarism'. If, any copyright violation is found in this research work I will be responsible for the consequences of any such violation.

Signature: 

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Date: 16 July, 2014

DEDICATED TO MY BELOVED PARENTS

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Sumia Khan

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List of Abbreviations

%	Percentage
µl	Microliter
°C	Degree centigrade
APS	Ammonium per-sulfate
BSA	Bovine serum albumin
cm	Centimeter
DTT	Dithiothreitol
g/L	Gram per liter
HCl	Hydrogen chloride
IAS	Invasive alien species
IgE	An allergen-specific immunoglobulin E
kDa	Kilodalton
LM	Light microscopy
m^2	square metre
m^3	Cubic metre
mg	Miligram
ml	Mililiter
mM	Milli molar
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogen carbonate
nm	Nanometer
O.D	Optical Density
PBS	Phosphate buffer saline
P/E	Ratio of the length of the polar axis to the equatorial diameter
rpm	Revolution per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
TEMED	N,N,N',N'-Tetramethylethylenediamine
v/v	Volume/Volume
w/v	Weight/Volume

ABSTRACT

Broussonetia papyrifera is considered as the world's worst alien invasive species. It is listed as one of the six worst plant invaders in Pakistan by The International Union for the Conservation of Nature. During spring season, mainly from February until April proximity, paper mulberry peak pollen count value goes above 55000 per m^3 which is considered as a the main contributor towards allergy problem of Islamabad. With the changing climatic conditions adverse effects of *Broussonetia papyrifera* are also increasing. As a consequence symptoms related to pollen hypersensitivity reaction are also changing. Allergy patients are directly showing aggravated symptoms of persistent cough and difficulty in breathing at their early stage of disease. In order to explore the aspects related to the allergenicity of *Broussonetia papyrifera*, three different methodologies were applied. Firstly a survey based study was conducted to identify the patterns of allergy symptoms in allergy patients. Survey based study showed that allergy patients were having seasonal rhinitis and symptoms of asthma. Additionally a high percentage of patients up to 71.4% were having aggravated symptoms in the month of March. Which is mostly reported for the peak pollen values of *Broussonetia papyrifera*. Secondly pollen palynological studies were conducted to identify different features of *Broussonetia papyrifera* pollen. To study morphology of pollen light and scanning electron microscopy was used. Study has shown that pollen size was very small and pore size was large as compare to previous reports. Finally protein characterization study for *Brossonetia papyrifera* pollen was conducted using sodium dodecyl sulfate polyacrylamide gel electrophoresis. Pollen protein extraction was carried out using PBS buffer and Coca's extract. Protein concentration was very low as maximum value obtain with PBS buffer was 2.4 µg per 50 µl. Polyacrylamide gel electrophoresis shown a notable band of 75 kDa. Presences of such higher molecular weight protein band indicates the high proteolytic stability of pollen protein. It is evident from the results that there is strong connection between the allergy symptoms in the pollination month of *Broussonetia papyrifera*. Variations in the pollen morphology and presence of highly stable protein in the pollen of *Broussonetia papyrifera* can contribute to allergenicity. However, extensive research is required to fully characterize all the pollen proteins of *Broussonetia papyrifera*, which can lead to the development of rational strategies to control allergy issue in Islamabad.

INTRODUCTION

Plants are the basis and backbone of all types of life on earth and are essential for people's livelihoods. They offer natural conservation, ecological balance and benefits. They also contribute towards the aesthetic values of the nature. Plants were first to contribute towards basic conditions for present day life that is why people have very close relationship with plants and live in harmony with nature (Rap, 2008). There are many wetland plants that are known as invasive plants. These are the plant species that quickly expands and enhance their spatial distribution into native plant communities (Richardson *et al.*, 2000).

Globally, invasive alien species are considered the greatest threat for biodiversity (Reddy, 2008), as they can invade nearly every sort of native ecosystem. Therefore, may results in the extinction of many wild type plants (Khan *et al.*, 2011). Similarly, in Pakistan due to lack of cultivation practices of medicinal plants, native flora is moving towards extinction. Therefore, to fill up the deforested areas, we have introduced new plant species. Hence, most of these plant species do not belong to our regional ecosystem. Ultimately, these alien plants are invading and disturbing natural habitats.

Crucially these species have serious human health implications, as pollen from these species are one of the most significant elicitors of allergy. During spring season, grass, weed and tree species release large quantities of pollen. These pollens are the main factor for causing seasonal rhino-conjunctivitis and bronchial asthma in hypersensitive individuals. As an example, various invasive species in Pakistan are causing unrest in the society i.e. Paper mulberry (*Broussonetia papyrifera*), Parthenium (*Parthenium hysterophorus*), Marijuana (*Cannabis sativa*) and *Conocarpus erectus* (Fatimah and Ahmad, 2012). It has been reported that the pollens from these invasive species are the major contributors to various allergy problems and found to be associated with increased cases of asthma and seasonal allergies in different cities of Pakistan, especially in Islamabad. Pollen and mold allergies are growing into a serious concern and found to be highly problematic in capital city, Islamabad (Abbas *et al.*, 2012).

Pollen grains are considered to be one of the most important allergen bodies found in air. Pollen of various invasive plant species are reported to cause respiratory allergies and bronchial asthma (Parveen *et al.*, 2012). *Broussonetia papyrifera* is mostly reported as a vital source of inhalant allergy in Islamabad (Malik and Hussain, 2007). Pollen of this major invader is known to be allergenic. Additionally, these allergenic pollens release in huge amounts and boost the allergen content in atmosphere (Chen *et al.*, 2013).

1.1. Background and context of the problem

Broussonetia papyrifera commonly named as Paper mulberry, is mainly native to East Asia. In Pakistan, it was introduced in nineteen century (1960). Seeds of paper mulberry were dispersed through helicopters. Basic purpose of planting paper mulberry was to beautify the capital, Islamabad. Consequently, paper mulberry with a percentage of 60-70 % is prevailing all the other trees in Islamabad. Even, it has been proclaimed as highly invasive in less than 30 years. *Broussonetia papyrifera* grows aggressively by replacing the native flora and above all induces severe allergenic pollen reactions in the local communities. This tree is major contributor to pollen allergy in Islamabad, Pakistan.

During spring season, mainly from February until April proximity, paper mulberry peak pollen count value goes above 55000 per m^3 which according to PMD, (2012) is the main contributor towards allergy problem of Islamabad. With the changing climatic conditions adverse effects of *Broussonetia papyrifera* are also increasing. As a consequence symptoms related to pollen hypersensitivity reaction are also changing. Allergy patients are directly showing aggravated symptoms of persistent coughing and difficulty in breathing at their early stage of disease (Rashid *et al.*, 2014).

Despite of the fact that allergenicity of paper mulberry is increasing day by day, there are very few studies related to patterns of allergy symptoms as well as to pollen protein allergens. Therefore, extensive research is instantly required to develop rational strategies to overcome this critical spring issue of Islamabad.

1.2. Review of Literature

1.2.1 Invasive species

Non-native species of a particular area that arrived in a new area outside their natural range are basically known as Alien exotic plant species (Thompson *et al.*, 1995). These plant species arrived outside their natural distribution ranges by help of intentional or unintentional human related activities. These species establish themselves in new natural or semi natural ecosystem as wild. Therefore, possess great threats against native biological diversity (Arroyo *et al.*, 2000; IUCN, 2004). According to GIPS these are also referred to as exotic organisms that has the potential to cause harm to the natural environment, social economies and human health (Qureshi *et al.*, 2014).

1.2.2 History of invasive species

Historically speaking exploration and colonization played an important role in the spread of IAS (Invasive Alien Species) (Qureshi *et al.*, 2014). All newly introduced plants are not invasive and it is very difficult for scientists to estimate whether a new arrival would become invasive or not. Alien species generally need long periods, may take decades and even centuries in acclimatization before becoming invasive. For an example *Passiflora ligularis* invasion in the forest gaps of Indonesia started in 1984-85. However, it existed there until 19th Century (Sherley, 2000).

1.2.3 Factors associated with successful invasive plants

There are many factors which play vital role in successful hasty spread of invasive plants in a non-native environment. These factors include their evergreen nature and immense capability of adaptation such as production of high quantities of new plants each season, tolerating different soil type and weather conditions. Invasive species spread easily and efficiently when they are free of the natural checks and balances like absence of natural enemies found in their native range. There frequent growth allows them to compete and displace slower growing plants. According to Hashim and Marwat, (2002) *Prosopis juliflora* is considered an invasive species due its prolific reproduction, wide adoptability and large capability to escape from common control measures.

Invasive species are not restricted to specific areas. These can be found throughout the world in all kind of ecosystems including all categories of living organisms. In terrestrial environment plant, mammals and insects are the most common types of invasives (Hoenicka and Fladung, 2006). Among all invasives, plants due to their huge biomass are considered one of the worst invaders in the world (Holm *et al.*, 1991).

1.2.4. Impact of invasive alien species (IAS)

The impact of the invasive alien species on local environment is very damaging. These not only cause disturbance of habitat but also play a key role in the biodiversity loss, economic loss and ecological imbalance. These destructive impacts are even greater due to frequency and magnitude of their introduction along with their tremendous ability to grow and proliferate. Biological invasion is more alarming than the chemical one (Khan *et al.*, 2010). Therefore, globally biological invasion is regarded as the second greatest risk for the biodiversity of an ecosystem (Reddy, 2008). This fact is supported by Gause (1934) competitive exclusion principle. According to Gause's principal when two species inhabit the same niche in the ecosystem, they cannot survive simultaneously for ever alongside. The more aggressive one flourishes and the poor competitor vanishes. For co-existence of two species they must have separate niche.

However, these Invasive species are not only a prominent threat to the local biodiversity but also responsible to affect environment, social economy and human health throughout the world (Adkins and Navie, 2006; Schmidt and Drake, 2011). According to one of the review, just 79 invasive species in USA caused $97 billion loss from 1906-1991. Moreover, 15 more species with invasive potential were estimated to cause about $134 billion loss (Randal and Marnelli, 1996).

1.2.5. Invasive plant problem in Pakistan

Pakistan is remarkably rich in biodiversity. It possesses a range of landscapes, ecological regions and climatic conditions. When we look geographically it possess varying landscapes from plains to deserts, forests, hills, plateaus and from sea level to the second highest point (K-2 at 8611 m) of the world. With these varying landscapes, climatic conditions also vary which collectively create new ecological regions.

These new ecological regions constitute 18 distinct habitats. Thus, this rich variety of habitats support various species of plants, mammals, reptiles, amphibians, fishes and invertebrates which overall contribute towards the biodiversity of this region (Khalid, 2000). Moreover, it contains 6,000 species of flowering plants. These flowering plant species include both native and introduced species, representing 22 families and about 150 genera. High rate of gene diversity is indicated in one of the preliminary analysis of the flora of Pakistan. Where it has been found that in Pakistan species number per genus is very low as compare to the global average (Shinwari and Shinwari, 2010). There are four monotypic genera of flowering plants and about 400 species are endemic to Pakistan. New endemics are still not known and needs to be discovered (GOP, WWF-P and IUCN-P, 2000).

Despite the fact that Pakistan is very rich in biodiversity, there exist very severe threats like over harvesting, over grazing, water logging, deforestation, salinization, land conversion, desertification, soil erosion, and introduction of high yield varieties, alien invasive species and chemical effluence (Baig and Al-Subaiee, 2009).

Pakistan possesses an extended history of introduction of alien plant and animal species. According to Marwat *et al.*, (2010), there are 16 invasive weeds present in the northwest province of Pakistan. In Pakistan the magnitude of IAS (Invasive Alien species) is less as compared to other countries but unfortunately the effects of these alien exotic species have not been well documented on the native biodiversity (GOP, WWF-P and IUCN-P, 2000; Shinwari and Shinwari, 2010). There is no appropriate cataloging of invasive species in Pakistan. Dataset is not available that could give comprehensive information about the effect of alien invasive species on native species composition and diversity. Inadequate and insufficient research efforts resulted in data deficit in the invasion biology literature. So far list of 700 alien species of vascular plants has been obtained by merging insufficient studies related to invasive species (Khatoon and Ali, 1999).

In Pakistan most of recent alien invasives were introduced with main objective behind to fill the gap between demand and supply of timber, fuel wood and fodder (Hussain and Zarif, 2003). However, many trees like *Robinia pseudo-acacia, Broussonetia papyrifera and Ailanthus altissima* were intentionally introduced which

later on became invasive. While all others were introduced unintentionally which spread further from invaded areas to uninvaded areas (Marwat *et al.*, 2010). Out of these, *Broussonetia papyrifera, Prosopis juliflora, Parthenium hysterophorus* and *Lantana camara* can be regarded as high impact invasives (Hussain *et al.*, 2003).

1.3. *Broussonetia papyrifera* (L.) Vent.

Broussonetia papyrifera (L.) Vent. (Syn. *Morus papyrifera* L.) is commonly known as Jangli toot, paper mulberry and tapa cloth tree. It belongs to family Moraceae. Native range of *Broussonetia papyrifera* is Southeast Asia (Qureshi *et al.*, 2014).

Broussonetia papyrifera is a dioecious, deciduous tree with milky secretions. Maximum growth height of *Broussonetia papyrifera* is about 45 ft. (15 m). It has hairy reddish brown twigs. Bark of Paper mulberry is tan which is smooth to equitably furrow. The soft and brittle wood of the tree has conical buds. The leaves are alternate, opposite or whorled along the stem and often lobed or mitten-shaped. The leaf base is heart-shaped with pointed tip and margin of leaf is sharply toothed. The upper leaf surface is rough feeling.

In the spring season separate female and male flowers appears. Male flower are pendulous, elongate each about 2 ½ to 3 in. (6-8 cm) long and present in the form of cluster which is composed of many separate flowers. However, female flowers of paper mulberry are globular in shape and nearly 1 in. (2cm) in diameter. The fruits appear in summer and are reddish purple to orange, which is ¾-1 in. (1.5-2.0 cm) in diameter. It may be confuse with young Flame tree (*Brachychiton acerifolius*), edible Fig fruit tree (*Ficus carisa*) and White mulberry (*Morus alba*) (Swearingen, 2005).

1.3.1 Success of *Broussonetia papyrifera* as an invasive plant species

Broussonetia papyrifera is native to Eastern Asia but it has become an invasive plant species in several continents including dozens of countries. It is a significant invasive weed particularly in Pakistan, Argentina, Ghana and Uganda. *Broussonetia papyrifera* is listed among the six worst plant invaders in Pakistan (Khatoon and Ali, 1999). Recent studies have shown that it is also one of the top alien invasive in Pampa plains of Argentina (Ghersa *et al.*, 2002).

Broussonetia papyrifera success in non-native environment is due to its rapid growth rate, vegetative regeneration strategy and effective dispersal by birds (Malik and Hussain, 2007). *Broussonetia papyrifera* also spreads by means of its fruits which are transported by local wildlife to significant distances. This also helps it to arrive and occupy gaps deep within an undisturbed areas. It also produces large number of pollens and at specific temperature and humidity these emitted pollens can form a fine visible white mist due to which it is also known as "smoke tree". According to Chen *et al.*, (2013), an individual male inflorescence can produce about 6×10^8 pollen grains which are wind pollinated and play significant role in wide distribution of these plants. Its shallow root system and high seed production helps it to grow aggressively in an area (Anon, 2004). *Broussonetia papyrifera* grows along streams and drains where its vigorous growth is ensured by high moisture content (Khatoon and Ali, 1999). However, it has ability to grow in dry land conditions.

Along with other characteristics, the ability to respout from root plays an important role in promoting the invasiveness of *Broussonetia papyrifera*. Tree can shoot up randomly from a dense mat root system at short interval of time when growing conditions are right. It not only has the highest growth rate but also prevents the regeneration of most native species competitively (Bosu and Apetorgbor, 2007). Once *Broussonetia papyrifera* gets established in an area, it can easily spreads from its dense thick root system, which is often thirty feet across (Eric and Overholt, 2004).

Additionally, when on one hand scientists are concerned and working to eradicate *Broussonetia papyrifea*, on the other hand a group of scientists is working to make it more salt and drought resistance. Na^{+}/H^{+} antiporter (NHX) gene is well documented for its role in the enhancement of plant salt tolerance (Apse *et al.*, 1999; Quintero *et al.*, 2000; Blumwald, 2000; Zhang and Blumwald, 2001; Zhang *et al.*, 2001).

In one of the studies by Li *et al.*, (2011), paper mulberry plant was made high drought tolerant by overexpressing Na^{+}/H^{+} antiporter (AtNHX5) gene. It was also proved that under high salt stress *Broussonetia papyrifera* remained alive while all wild type plants died. Under drought stress survival rate of the resistant *B. papyrifera* was greater than 66%. In AtNHX5 overexpressing leaves ion level of Na^{+} and K^{+} was higher as compared to wild type leaves under saline conditions (Li *et al.*, 2011).

Plants with AtNHX5 gene exhibited higher chlorophyll and water leaf contents. Under high salt stress and water deficiency, these plants were capable of accumulating more soluble sugars. Moreover, membranes in AtNHX5 plants were less damaged than the wild type plants. Thus the study clearly indicated that *Broussonetia papyrifera* with AtNHX5 gene could tolerate multiple environmental stresses and can counter the osmotic stress by accumulating effective osmolytes (Li *et al.*, 2011).

If such a resistant plant gets an access to non-native environment where no natural pests and killers are present, it can easily overcome the natural flora. Where such deliberate efforts can add to invasive success of *Broussonetia papyrifera* at the same time these can cause irreversible changes in the natural biodiversity of a region.

1.3.2 Introduction of *Broussonetia papyrifera* in Pakistan

Broussonetia papyrifera was introduced in subcontinent about more than 100 years ago (Marwat *et al.*, 2010). First it was planted in 1889 at Saharanpur but until 1924, it increased its growth and spread till Lahore. In Lahore, *Broussonetia papyrifera* can be found in Shahdara plantation and along the irrigation channels (Parker, 1956). *Broussonetia papyrifera* was deliberately introduced in Islamabad to make capital green in a short period of time. In 1960 *Broussonetia papyrifera* seeds were sprayed over Islamabad with the help of helicopters (Malik and Hussain, 2007).

1.3.3. Current distribution in Pakistan

Broussonetia papyrifera is present in metropolitan and northern areas of Pakistan as a most problematic invasive plant species (Marwat *et al.*, 2010; Ashraf *et al.*, 2012). In the Himalayan foothills of Pakistan and subtropical forests, *Broussonetia papyrifera* is the major type of woody species invasion ever known (Malik and Hussain, 2007).

Although it is native of South East Asia but in Pakistan it is dispersed from Lahore to Peshawar. It is also distributed in the salt ranges of Pakistan. Capital area of Islamabad is one of the worst affected area by this notorious invasive weed. According to Abbas *et al.*, (2012) *Broussonetia papyrifera* is existing in almost every sector of Islamabad. It is also distributed among the different areas of Rawalpindi and Peshawar (Marwat *et al.*, 2010).

1.3.4. Major impacts of *Broussonetia papyrifera*

Broussonetia papyrifera is a threat to biodiversity as well as to human health. Among 700 alien invasive species, 5 or 6 species can be distinguished as high impact invasives. These high impact invasive species comprise of Paper mulberry (*Broussonetia papyrifera*), Water hyacinth (*Eichhornia crassipes*), Congress grass (*Parthenium hysterophorus*), and Mesquite (*Prosopis juliflora*). Out of these species, *Broussonetia papyrifera* is not only known for causing financial loss to agriculture communities but also plays a significant role in reducing the land value. In Peshawar and Islamabad *Broussonetia papyrifera* is known to be a major source of allergy and associated health problems (Hussain *et al.*, 2000).

1.3.5. Threat to natural biodiversity

Broussonetia papyrifera is considered as the world's worst alien invasive species (Ali and Malik, 2010) and listed as one of the six worst plant invaders in Pakistan by The International Union for the Conservation of Nature. *Broussonetia papyrifera* has immense ability to thrive in various climatic conditions throughout the world. It also covers a tremendous range by quickly colonizing an area (Eric and Overholt, 2004). However, growth of *Broussonetia papyrifera* is mostly associated with the areas of greater moisture content but it is also capable of invading drier sites (Malik and Husain, 2006). This species was introduced some years back in Islamabad and eventually it flourished quickly in the whole area putting drastic effect on local vegetation. Invaded areas of paper mulberry are known to be associated with significantly lower richness and diversity of herbaceous and woody species (Malik and Husain, 2007).

According to Ali and Malik, (2010), *Broussonetia papyrifera* has played a key role in deteriorating natural landscapes of Islamabad. It is present abundantly and taking hold over the indigenous species of *Dalbergia sisso*. In and around Islamabad, paper mulberry has changed the xerophytic flora to mesophytic flora (Malik and Husain, 2007). This invasive species is not only a growing threat for the natural vegetation of National park and the Eastern valleys of Islamabad but also to the natural vegetation of Azad Jammu and Kashmir (Marwat *et al.*, 2010).

1.3.6. Human health hazards related to *Broussonetia papyrifera*

Broussonetia papyrifera belongs to the family Moraceae and has catkins, which contain about 200 flowers/catkin. Moreover, each catkin may contain as many as about 3 to 6 million pollens. Moraceae pollen is almost 14 to 15 mm in size and is an important cause of asthma and allergic rhinitis in susceptible persons (Abbas *et al.*, 2012). According Royal Botanic Gardens database, Kew, (2011) pollen of *Broussonetia papyrifera* is allergenic. As it produces large quantities of pollen during the pollination season which becomes a vital source of allergens (Chen *et al.*, 2013). It is reported as a main culprit of inhalant allergy in Islamabad, where this species is a very common urban weed (Malik and Husain, 2007). According to khan *et al.*, (2011), Invasive *Broussonetia pepyrifera* of Islamabad and Peshawar University campus is very allergenic.

According to one of the reports by Pakistan Medical Research Council (1995), about 45.5% of allergy patients are found to be sensitive to *Broussonetia papyrifera* pollen in Islamabad and Rawalpindi (Marwat *et al.*, 2010). *Broussonetia papyrifera* contributes to severe pollen allergy from February to April. (Birsel, 2007). During this period, pollen count of *Broussonetia papyrifera* reaches approx. 40000 per m^3 which becomes a major cause of severe asthma related problems in the native population of Islamabad (Ali and Malik, 2010).

1.4. Allergy

Allergic diseases are one of the most common and chronic disorders worldwide (Prabhakar *et al.*, 2013). Over 20% of the total world population is suffering from immunoglobulin E-mediated allergic diseases such as eczema, allergic rhinitis, asthma and anaphylaxis (Johansson and Haahtela, 2004). Moreover, more than 25% of the population in industrialized countries is suffering from chronic allergy disease (Valenta, 2002). According to the Asthma and Allergic Foundation of America, (2002) allergies are the sixth important cause of chronic disorders in USA. The annual cost of dealing with chronic allergies in USA is estimated up to $18 Billion. Basically allergy can be recognized as a specific counter response of body's immune system towards a typically harmless substance.

1.4.1. What causes Allergy?

The allergenic content of the atmosphere varies according to climate, geography and vegetation (D'Amato *et al.*, 2007). Additionally, individuals with allergies are usually hypersensitive to more than one substance. Therefore, apart from plant pollens other airborne allergens can cause allergic reactions. These allergens include house dust, pet dander, cockroaches, allergens of some food products and insect venom, food additives, metals, latex and aldehydes (NIAID, 2012).

However, of all the related things that can cause allergy, pollens are one of the major cause found to be associated with allergic reactions (NIAID, 2012). Trees and grass pollens are recognized as the most effective inhalants for causing hay fever, rhinitis and asthma (Liu *et al.*, 2010).

1.4.2. Pollen Allergy

Today allergy to airborne pollen is a very common disease. 2% to 10% of the USA population is affected by pollinosis. Currently 20% of Europe population is suffering from pollinosis while the prevalence was 1% at the beginning of the 20th century (Dai and Lu, 2000) In China, approximately 0.5%–5% of the populations have particular serum antibodies to pollen (Ye *et al.*,1998).

Mostly people recognize pollen allergy as hay fever, but health specialists typically name it as "seasonal allergic rhinitis". Primarily this means a hypersensitivity reaction against certain pollen. During a specific season pollen allergy induce mucous membrane excretions. Pollen allergy involves a particular immune response of an organism's body towards harmless pollens (NIAID, 2012). Symptoms of pollen allergy consist of runny nose, itchy eyes, sneezing, congestion of the nose, red and watery eyes (NIAID, 2012).

Immunoglobulin (Ig) E mediated Allergic disorders are frequently increasing around the globe. Study of these allergic reactions shows a solid connection between disease symptoms and airborne pollens (Aberg *et al.*, 1995; Beasly *et al.*, 1998). Pollens in relation to allergic reactions also vary with the variations in the local flora and fauna of a region (Moverare *et al.*, 2002).

Moreover, as a result of climatic changes, plants are becoming abundant in new geographical areas where they were not existing previously. In Pakistan, *Broussonetia papyrifera* pollen is a main reason of respiratory allergy. Where it is considered as a great impact invader (Pallewatta *et al.*, 2003). Previously, *Broussonetia papyrifera* tree pollen was considered to elicit respirational allergies in the US (Targow, 1971). It has been reported that pollen counts of *Broussonetia papyrifera* pollen have been increasing over the last few decades (Sneller *et al.*, 1993). Studies have shown that in India pollens from *Broussonetia papyrifera* tree are a major aeroallergen towards the north regions (Singh and kumar, 2003; Singh and Shahi, 2008). While in Madrid, Spain pollens of *Broussonetia papyrifera* are known as predominant aeroallergens from January to April (Subiza *et al.*, 1995).

Broussonetia papyrifera pollen is the most abundant pollen type present in the air of Islamabad. Highest pollen values of mulberry start from mid-March and finally decreases in mid-April. This time period is very critical for allergy patients of Islamabad (Haroon and Rasul, 2008). Due to *Broussonetia papyrifera* pollens there are severe allergic diseases in spring season. These include allergic diseases like allergic rhinitis, asthma and urticarial. During a single day of spring when the pollen count of *Broussonetia papyrifera* reaches above tan 30,000/ m^3 there are deaths due to asthma. For which pollen of *Broussonetia papyrifera* is regarded as the main culprit (Abbas *et al*, 2012).

Not only *Broussonetia papyrifera* but exposure to pollens from same family plant like *Morus alba*, is also reported to elicit asthma, allergic rhinitis, allergic conjunctivitis and urticaria in susceptible individuals (Munoz *et al.*, 1995; Navarro *et al.*, 1997). A case has been reported from Italy where a Spanish patients with artichoke allergy and pollinosis also showed sensitization to *Broussonetia papyrifera* (Romano *et al.*, 2000; Zanforlin and Incorvaia, 2004). Despite the frequently increasing allergy disease due to invasive *Broussonetia papyrifera* pollen, the causative pollen allergens are yet to be categorized.

1.4.3. What is a pollen?

A pollen is a fine dusty substance which is usually yellow in color. Pollens are microscopic grains released from the male cone or male part of the flower. Each pollen grain has a male gamete, which has all the necessary information to develop into a complete plant (Johnstone and Adam, 2001). Pollen can fertilize a female ovule to form an embryo. It can travel long distances by means of air, insects, wind, bird or other animals.

1.4.4. Types of pollen

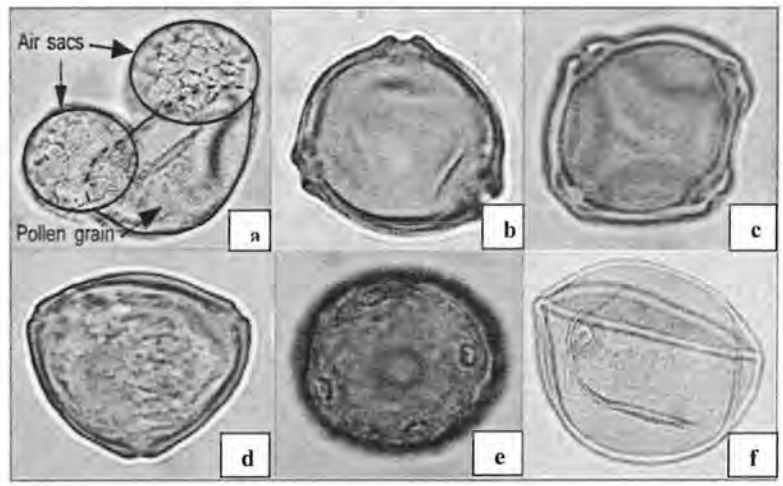

Fig 1.1: Different pollen types: a, Pine pollen; b, Birch pollen; c, Alder pollen; d, Hazel pollen; e, Ribwort plantain pollen; 5, Cereal pollen

1.4.4.1. Pine pollen

Pine pollen contains two air sacs which are attached to the pollen grain. Pollen grains look like Mickey Mouse due to the presences of air sacs. These air sacs help pollen to travel great distances in the presences of wind.

1.4.4.2. Birch pollen

It contains three pores. These pores stick out from the apparent surface of the pollen grain. In birch pollen number and shape of the pores plays a significant role in its identification.

1.4.4.3. Alder pollen

It contains three to five pores. The existence of arc-shaped shadows between the pores and the shape of the pores are among the key characteristics of this grain.

1.4.4.4. Hazel pollen

It contains three pores. Hazel pollen pores are much smaller than those of Alder and Birch. Pores number and shape are important to detect these grains.

1.4.4.5. Ribwort plantain pollen

Ribwwort plantain pollen contains eight to fourteen pores, but no more than this. All the pores cannot be seen at the same time as some pores remains hidden behind the grain. The pores are randomly distributed over its surface. Center of the pores sticks out slightly due the presence of a "plug" of pollen material. The plug presence feature and the number of pores are used to identify this grain.

1.4.4.6. Cereal pollen

It appears to be very similar to grass pollen. It contains one pore. It does not have furrows. It is a very delicate grain which gets folded easily. Its size is three times the size of grass pollen and its pore contains a thickened edge.

1.4.5. Basic structure of pollen

Except in the case of some submerged aquatic plants, the developed pollen grain has a double wall. The inner delicate thin wall of intact cellulose is known as intine or endospore. Endospore surrounds the vegetative and reproductive cells in the pollen grain. The outer resistant and cuticularized wall of pollen is known as exine or exospore. Exospore is largely composed of sporopollenin which is a resistance biopolymer (Simpson, 2011). The exine contains warts or spines. Exine also possess various sculpture which can be used to identify genus, specie, individual or even a cultivar. If the spines are less than a micron in length than these are known as spinulose (scabrate) while spines longer than a micron are referred as echinate. Sculpturing is called reticulate if it gives a net like appearance (Singh, 2004).

The outer pollen wall is composed of two layers. These two layers are tectum and the foot layer. Foot layer is present just above the intine. Columella is the region which separates tectum and foot layer and it is composed of strengthening rods (Simpson, 2011).

Various pollen wall modifications are known as apertures. Aperture may contain thinnings, ridges and pores. Aperture allows the pollen tube to pass through the wall of pollen. When there is change in the moisture content aperture allows the shrinking and swelling of the pollen grain. The elongated apertures are known as colpi while apertures with furrows are referred to sulci. In order to classify pollen grains as colpate or sulcate the orientation of furrows in relation to the original tetrad of microspores is very important (Sporne, 1972). Pollen with three colpi are referred to tricolpate (Judd and Olmstead, 2004) and pollen that contains a single sulcus is described as monosulcate (Singh, 2004; Simpson, 2011). In the absence of aperture pollens are known as inaperturate while in the presence of aperture they are termed as aperturate (Furness and Rudall, 2003). If aperture contains a lid it is described as operculate. These different aperture structures along with pores can be regarded as a major criterion for the identification of pollen classes.

1.4.6. Pollen number related to allergy

There are different types of pollen but the pollen most commonly associated with allergy are produced by trees, grasses and weeds. These plants do not contain very showy flowers. These plants produce large number of very small pollens which can cover long distances by wind. As ragweed pollen grains has been collected two miles high in air and 400 miles out at sea (NIAID, 2012).

Allergenic pollen plants are also indicated as anemophilous plants. These are wind pollinated plants. Pollen wall of these anemophilous plants contains specific proteins which induce hypersensitivity reaction in susceptible individuals (Last and Guidotti, 1990; Durham, 1998; Ye *et al.*, 1998). Pollinosis patients show the clinical symptoms in their breathing system. Along the annual pollen exposure, symptoms in patients could become more severe (Ye *et al.*, 1998). Usually most allergenic pollens come from flowering plants that release these in enormous amounts. For an example, a single ragweed plant can produce a million of pollen grains a day (NIAID, 2012).

It is reported that large amount of airborne allergenic pollen can cause pollinosis, hence these are threatening for human health (Ye *et al.*, 1998). Pollen has a very important role to trigger allergic respiratory diseases. Human health is directly affected due to the presence of their high concentration in the atmosphere (Haroon and Rasul, 2008). Pollen amount in the atmosphere has great impact on the health issues like pollinosis (Mandal *et al.*, 2008). It is revealed that percentage of woody taxa in the atmosphere results in pollen allergy related diseases (Parveen *et al.*, 2012; Ozturk *et al.*, 2013). Pollen from grasses and trees are regarded as the most effective inhalants for causing asthma, pollinosis and hay fever (Liu *et al.*, 2010).

From February until April, *Broussonetia papyrifera* tree is known to induce severe pollen allergy in the residents of Islamabad (Birsel, 2007). Pollen count of *Broussonetia papyrifera* recorded in this period reaches approx. 40000 per m^3. Due to the production of such a large quantities of airborne pollen *B*roussonetia *papyrifera* is the major contributor to the sever allergy problems of the local population (Ali and Malik, 2010).

1.4.7. Pollen counts of *Broussonetia papyrifera* in relation to allergy patients

Dispersal of pollen grains completely depends on water availability, mechanism to avoid desiccation, types of carbohydrate and the type of pollen (Pacini and Hesse, 2004). Production and release of pollen is mostly affected by meteorological factors. Among these atmospheric factors, rainfall, temperature, wind speed and direction are the major moderators (Lattore and Caccvari, 2009). Temperature effect has been widely studied on the pollen count of woody species and it revealed to have marked influence on the reproductive phenology of early spring flowering trees (Menzel *et al.*, 2006).

Pollen number in relation to pollinosis has great significance (Mandal *et al.*, 2008). Pollinosis is one of the most emerging health problems in the city of Islamabad. Various plant species are responsible for eliciting allergic reactions by producing large quantities of pollens. These species includes *Broussonetia papyrifera, Cannabis sativa, Alternanthera pungens, Eucalyptus globules,* Grasses and *Pinus sp.* (Ghufran *et al.*, 2013).

In one of the studies by Abbas *et al.*, (2012), pollen count of main flowering trees of Islamabad was recorded. The trees included were *Broussonetia papyrifera, Eucalyptus species, Pinus species, Acacia species, Morus alba,* and *Schinus molle.* These trees are mostly related to producing allergenic pollens in Islamabad. During the consecutive three years of study (2005-2007), pollen of these trees was monitored (Fig 1.2). In 2005 the total number of pollen recorded was 81.98%, among these *Broussonetia papyrifera's* pollens trapped were 71.3%. During 2006 pollens trapped were 82.64%. Again, among these *Broussonetia papyrifera* pollen was present with the highest pollen count of 75.06%. In 2007 the same previous two years trend was observed. In the last year of study total tree pollens collected were 80.85% and *Broussonetia papyrifera* pollen percentage was 74.4% (Abbas *et al.*, 2012).

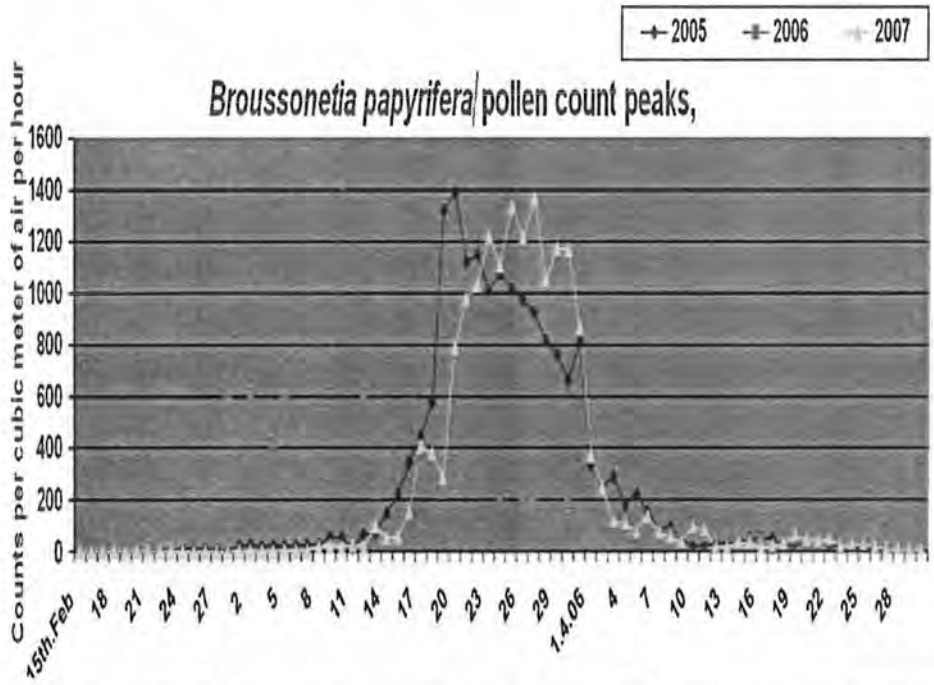

Figure 1.2: Daily graph of *Broussonetia papyrifera* pollen count (15th February-30th April) for 2005, 2006, and 2007.

During 2009-2010, the annual trend of *Broussonetia papyrifera* pollen production in relation to temperature fluctuations was studied which indicated that climatological factors mainly optimum average temperature has directly affected the release of pollens in the air (Fig 1.3) (Ghufran *et al.*, 2013).

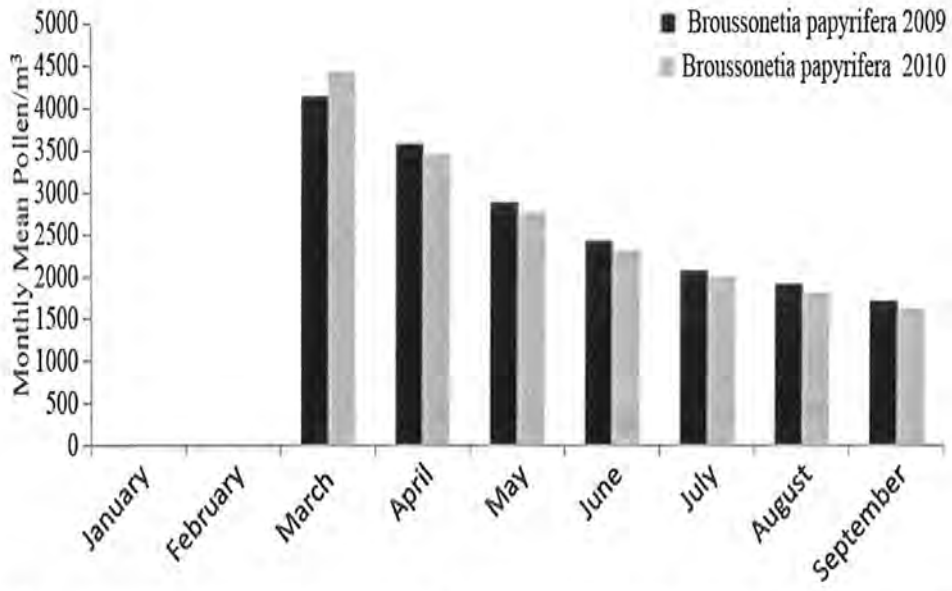

Fig 1.3: *Broussonetia papyrifera* pollen trend for Islamabad city during study years 2009-2010.

Normally, high temperature conditions enhance the pollen production (Davies and Smith, 1973). Therefore, in the initial time period of 2009, it was observed that there was increase in pollen quantities with an increase in temperature. During March, 2009 when optimum average temperature was 17.5 °C, peak pollen count observed was up to 4143.0 pollens/ m^2. Afterwards there was a decline in pollen quantities. However, average temperature increased gradually. Comparatively, in colder months of the year, lowest pollen count up to 1718.07 pollens/ m^2 was observed in September (Fig 1.4).

Parallel pattern was detected in the year 2010, till the mid of February pollen quantities were low with a value of 4431.6 pollens/ m^2. After that pollen counts gradually increased and in the middle of March at an optimal average temperature of 20.5 °C reached to its peak value of 4, 09,162/ m^3. Lowest Pollen count values were seen in September, a comparatively colder month of the year (Fig 1.4). It was noticed that the highest pollen quantities in the air of capital city was strongly supported by the optimum temperature (Ghufran *et al.*, 2013).

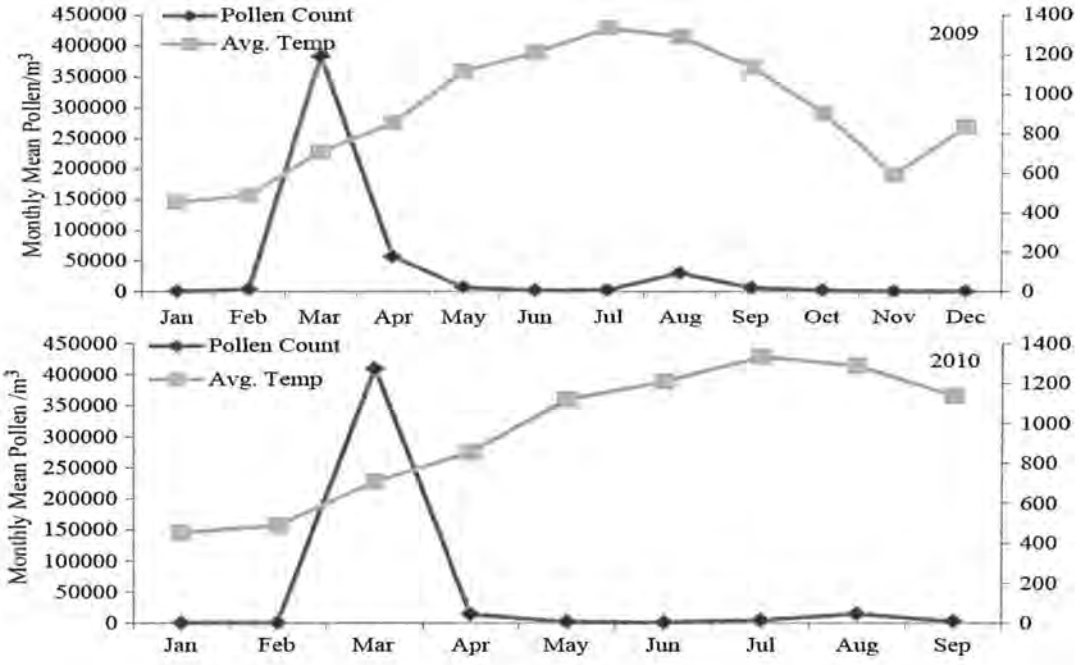

Fig 1.4: Relationship of monthly pollen count and temperature from 2009-2010.

Comparison of allergic patients with monthly pollen count from 2009-2010 showed that in the month of March highest number of patients were recorded. There were 1624 number of patients and pollen count values at that time were 4143.21 pollen/ m^2 (Fig 1.5). In March 2010, patient numbers were increased from 1624 to 1724 with the increase in pollen count of *Broussonetia payrifera* from 4143.21 pollen/ m^2 to 4431.5 pollen/ m^2. After March there was significant decrease in the number of allergy patients and 492 patients were recorded in June (Fig 1.5). The general pattern showed that during pollen season large number of patients registered in both years. Particularly, variations in pollen allergen production in relation to climatic changes has increased the occurrence of asthma and allergy related diseases (Shea *et al.*, 2008).

It is recommended that forest department should pay attention towards the conscious selection and plantation of exotic plants. Awareness campaigns regarding preventive strategies should be conducted through electronic media. Masses should be given knowledge about the serious health implication of invasive species in their areas (Ghufran *et al.*, 2013).

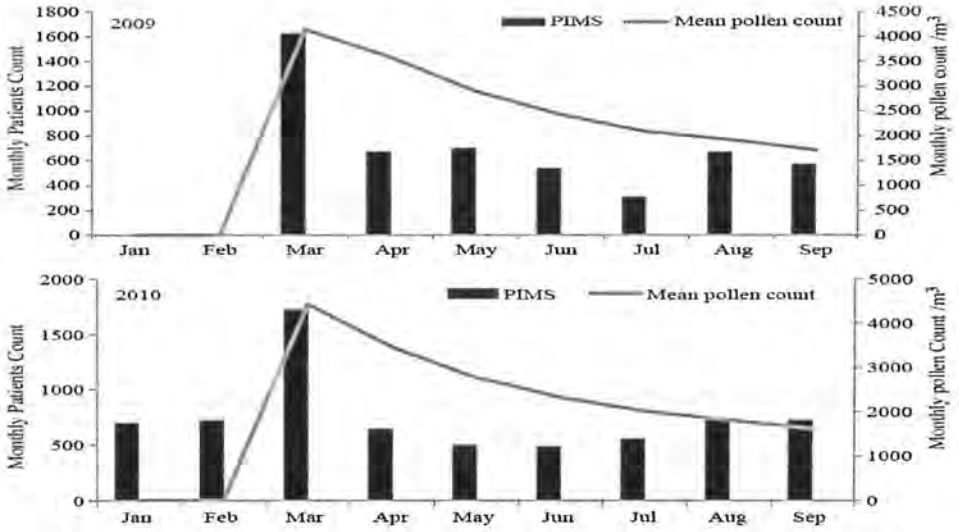

Fig 1.5: Monthly recorded allergic patients and mean pollen count for the study years 2009-2010.

1.4.8. Pollen allergens and immunology

Among the environmental allergens, mostly common are airborne allergens existing in pollen of different plants. The allergens of natural environment are usually proteins. These proteins generally have molecular weight higher than 10 kDa. Mostly pollen allergens are regarded as water soluble proteins or glycoproteins with a molecular weight ranging from 10–70 kDa (Belin, 1972; Esch and Klapper, 1989; Knox and Suphioglu, 1996; D'Amato, 2000). Pollen allergens are highly stable in the changing environment of pH and temperature, even these can tolerate high temperature up to 100 °C (Obtuluwicz *et al.*, 1996).

Other than humoral and cellular immunities, the humans have specialized system of local immunity. Local immunity occurs in the organs which are direct exposed in outer environment like immune system of mucous membranes of the respiratory system, gastrointestinal tract and epidermis. All these immune systems are associated with local immunity. Individuals with good health have a biological balance between the extent and applied conditions of the cell mediated immune system. Whereas, persons with allergic reaction lack this balanced state. According to systematic organization method of Gell and Coombs there are four types of allergic reactions (Gell and Coombs, 1968). Besides, pollinosis is more related to Type 1 hypersensitivity reaction. Hypersensitivity Type 1 reaction starts when an allergen induces the formulation of immunoglobulin E (IgE) antibodies in an organism (Knox

and Suphioglu, 1996; Sato *et al.*, 1997). In the initial stage of an allergic reaction, specific IgE antibodies induce mastocyes and basophiles to excrete inflammatory mediators. However, in late stages of Type 1 hypersensitivity reaction, long exposure to allergens can lead to chronic inflammation. In that case, release of inflammatory mediators is not only induced by specific antibodies but also by unspecified factors. Clinical symptoms in Type 1 hypersensitivity reaction includes, sneezing, burning sensation, runny nose, production of mucous or watery excretions, conjunctivitis and perhaps change condition of other organs (Buczylko, 1996). Above all, Type 1 reaction can cause anaphylactic shock, IgE- reliant swelling, urticaria and bronchial asthma. Therefore, extensive studies are required to investigate different allergens.

1.4.9. Allergens of family Moraceae

There are a number of studies on the allergenicity of the Moraceae family. A single protein of 35-50 kDa was detected in mulberry silk extract. This protein was shown to bind immunoglobulin (Ig) E from the sera of 41% mulberry silk allergy patients (Zaoming *et al.*, 1996). Jackfruit from the breadfruit tree (*Artocarpus integrifolia*), which belongs to the subfamily Artocapeae, has been stated to induce oral allergy disease. Allergens involved in IgE reactivity to jackfruit did not shown cross-reactivity with the Bet v 1 and Bet v 2 of birch pollen allergens (Wuthrich *et al.*, 1997). Fruits of the Moraceae family cause allergic reactions which are also associated with the latex-fruit syndrome. IgE cross-reactivity was observed between *Ficus carica* fruits and *Ficus benjamina* latex. Moreover, patients with Fig allergies were found to be sensitive for *Ficus* fruits and latex (Focke *et al.*, 2003). In *Ficus benjamina* latex IgE-binding proteins of 22 kDa and 28-34 kDa were identified. Cross reactivity in Moraceae family members has been demonstrated by a study in which three Italian Fig allergy patients were found to be associated with *Morus nigra* and *Morus alba* fruits (Caiaffa *et al.*, 2003).

Moreover, International Union of Immunological Societies has included a nonspecific lipid transfer protein (Mor n 3) of *Morus nigra* fruits into its allergen database. Despite of the significance of aeroallergens, only a limited number of studies are available on allergenicity of *Broussonetia papyrifera* pollen. In this field extensive research is required to identify pollen allergens (Micheal *et al.*, 2013).

1.5. Future perspective of the proposed research

As a matter of fact, it is known that Pakistan is a developing country and facing many challenges in every aspect of life. Especially, we are lagging far behind in the field of pollen allergy. Pollen and mold allergies are extremely challenging in Islamabad. Pollen grains are considered to be one of the most important allergen bodies found in air. In Pakistan, Pollen of various invasive plant species have been reported to cause respiratory allergies and bronchial asthma. Among all invasive species, *Broussonetia paprifera* is the most frequently reported invasive species responsible for severe pollen allergies in Islamabad. Despite of the fact that *Broussonetia papyrifera* pollens are highly allergenic and responsible of many deaths in every spring season in Islamabad, there are no comprehensive studies available on its pollen. Especially, related to proteins existing in pollen grains of *Broussonetia papyrifera.*

Currently natural or recombinant protein allergens are introduced into diagnostic tests and more likely can be used for allergen-specific immunotherapy. A systematic biochemical characterization of pollen protein where can facilitate the full grasp on the issue at the same time can offer the detection of cross reactivity. Thus, in a changing environment an extensive characterization of all pollen protein is required to develop a deeper understanding of pollen allergenicity. Furthermore, studies related to pollen, and pollen protein characterization will facilitate the development of rational strategies for prevention and management of pollen allergy. The aim of this study is, therefore, an identification and biochemical characterization of pollen allergens of invasive *Broussonetia papyrifera.*

1.6. Aims and Objectives

- Palynological studies of Invasive paper mulberry pollens.
- Extraction of pollen grains by Coca's extract and PBS buffer.
- Biochemical characterization of pollen proteins through SDS-PAGE.
- Determine the prevalence of pollen allergy and factors associated with it using survey methodology.

MATERIALS AND METHODS

A number of experiments were performed in Quaid-i-Azam University, Islamabad with collaboration of Centre for Plant Conservation, University of Karachi and School of Biological Sciences, University of the Punjab. Aim of this experimental work was to characterize the potential allergens present in pollens of *Broussonetia paprifera* as well as to perform palynological study of these pollens. Survey was also conducted under the supervision of Dr. Osman Yusuf (Chief consultant at The Institute of Asthma and Allergy, Pakistan) in order to evaluate and understand the symptoms closely related to pollen allergy disease. Overall experimental work was divided into three steps namely survey of allergy patients, palynological studies based on different microscopic techniques and protein characterization using SDS-PAGE. The details of methodology used for these studies are being discussed in this section.

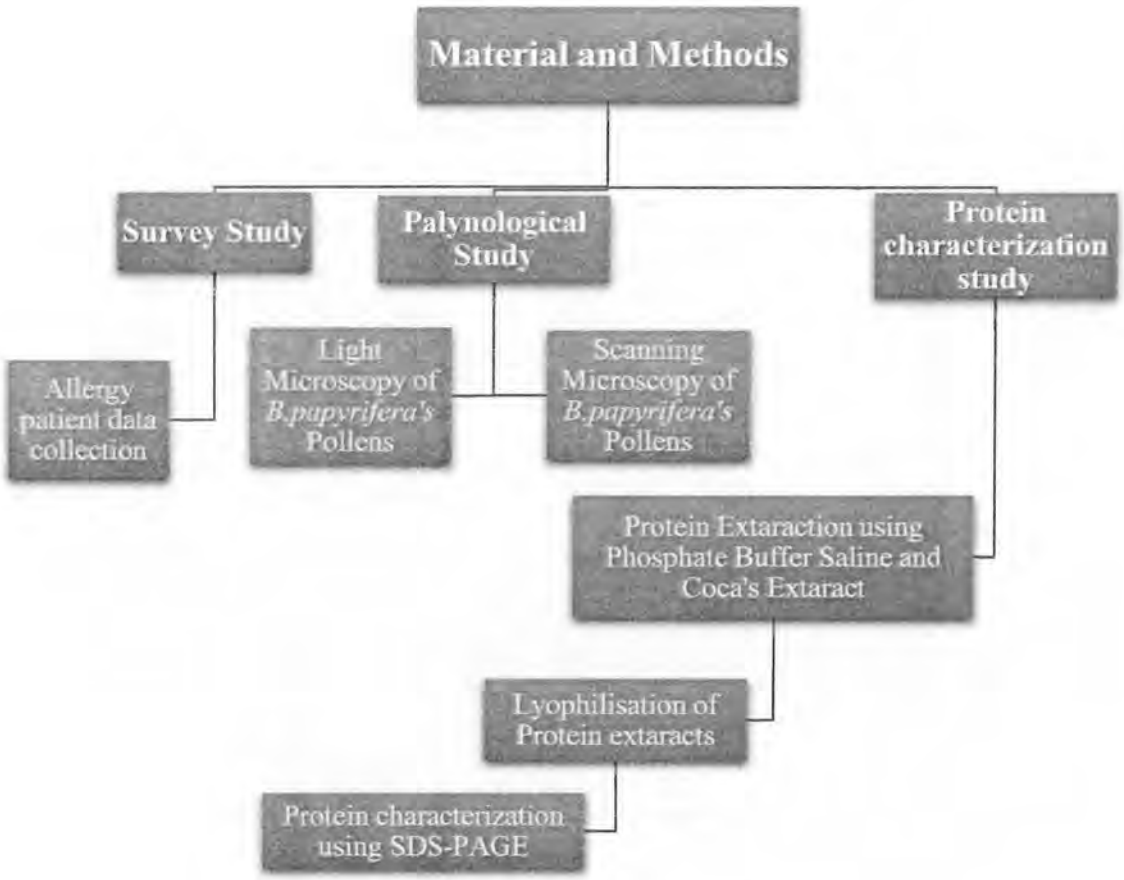

Fig 2.1. Detailed methodology used in study

Part-A: Survey of Allergy patients

2.1. Allergy camp

A survey was conducted at allergy camp arranged in Aabpara Community Centre, Islamabad in the month of March. Detailed questionnaire was used to gather both qualitative and qualitative information to determine the factors associated with pollen allergy. Total of 32 patients were interviewed in which 25 were female and 7 were male patients. Questionnaire was divided into four parts:

2.1.1. Consent of patient

2.1.2. Clinical features

2.1.3. Rhinitis

2.1.4. Asthma or Chronic cough

2.1.1. Consent of patient

Patients were informed in details about the purpose of questionnaire and with their permission and signature approval procedure was proceeded.

2.1.2. Clinical features

This section contain questions regarding symptoms related to allergy disease, history of allergy and asthma in patient's family and environment in which they spend most of their time.

Patients whose response were positive for symptoms related to rhinitis were further proceeded to the 3rd rhinitis section to investigate about their disease in details. In the same way patients whose response was positive for asthma or chronic cough were advanced towards 4th asthma section.

2.1.3. Rhinitis

This questionnaire section contained a separate set of specific questions for those who claimed to have suffered from allergy symptoms. To verify the type of allergies, whether pollen or other, the respondents were asked about detailed symptoms of allergy, duration of illness, seasonal as well as time variations in relation to

aggravation of symptoms, aggravation by other factors (house dust, smoke, cold etc.) treatment and effect with the change in environment.

2.1.4. Asthma or chronic cough

In last section respondents were asked about symptoms related to asthma and cough, family history, form of cough and other cough associated factors. Data was compiled and analysed using Microsoft word and excel.

Part-B: Palynological studies of *Broussonetia papyrifera's* pollen

2.2. Pollen collection and storage

Pollen were collected in March 2013. With the help of scissors catkins of male *Broussonetia papyrifera* (See fig 2.3) plant was removed. Then these catkins were placed in surgical trays covered with filtered paper for 2-3 days. After 2-3 days catkin shifted to new trays and shed pollens were collected and sieved 2-3 times. Collected pollens then shifted to air tight zipper bags and labelled (Fig 2.2) and stored at -80 °C.

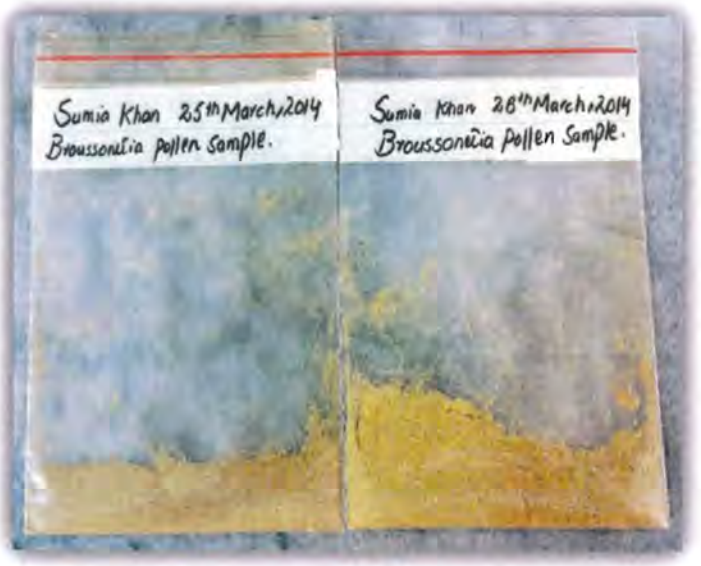

Fig 2.2: Pollen sample of *Broussonetia papyrifera.*

Fig 2.3: Catkin of *B. papyrifera*

2.2.1. Microscopy

Microscopy is the science of investigating microscopic objects using a microscope. Microscopic means invisible to the eye unless aided by a microscope. Different microscopic techniques are used to study the structure of *Broussonetia papyrifera's* pollens. Microscopic techniques are utilized for the identification and detail structural studies of pollens. For light microscopy (LM), pollen grains preparation was done by following the method of Erdtman, (1966). For scanning electron microscopy (SEM) standard methods designated by Erdtman, (1952) was followed.

Mainly two type of microscopy was used:

2.2.2. Light microscopy (LM)

2.2.3. Scanning Electron microscopy (SEM)

2.2.2. Light microscopy

2.2.2.1. Slide preparation

In order to perform light microscopy, first of all anthers were separated from the catkin by using dissecting needle and placed on a glass slide along with few drops of acetic acid. After that, anthers were crushed on glass slide in order to obtain pollen grains. Extra anther material was discarded and only pollens were allowed to remain on the slide. Excess of water was removed with the help of filter paper. Pollen grains were stained with glycerin-jelly mixed with 1% safranine. Bubbles were carefully removed by slightly heating the slide. After removal of bubbles cover slip was carefully placed on the glass slide. When cooled the cover slip edges were fixed with transparent nail varnish. After sealing glass slides were labelled.

2.2.2.2. Slide observation

The prepared *Broussoneia papyrifera* pollen slides were observed under the light microscope. Different pollen characters were observed which include such as type of pollen, shape of pollen in polar and equatorial view, polar and equatorial diameter, P/E ratio and length were measured. Photographs of pollens were taken at 40x with the help of Nickon FX-35 Camera equipped with photomicrograph system (Japan).

2.2.3. Scanning electron microscopy

2.2.3.1. Slide preparation

Firstly paper mulberry pollen were suspended in a small drop of water in order to perform scanning electron microscopy (SEM). Then suspended pollens were directly transferred with the help of a fine pipette to a metallic stub by using double sided cello tape. Gold coating of pollens were executed in a sputtering chamber (Ion-sputter JFC-1100). Besides, gold coating of pollen was restricted to 150 A.

2.2.3.2. Slide observation

The scanning electron microscopic study was carried out by using a Jeol microscope JSM-2. After 15-20 readings, final measurements for pollen were taken. Pollen characteristics under observation were pollen diameter, polar axis (P) /equatorial diameter (E) ratio, pollen length, pollen shape, no. of pores in pollen and their diameter.

Part-C: Protein characterization using SDS-PAGE

2.3. Protein extraction

Broussonetia papyrifera purified pollens were removed from the freezer and allowed to reach at room temperature without opening the air tight zipper bag. The purity of the pollen was verified using scanning electron microscopy. Proteins were extracted using 2 extraction buffers to compare the total protein pattern. Mainly two type of extraction buffer were used namely Phosphate buffer saline (PBS) and Bicarbonate buffer saline (Coca's extract 10%w/v). For composition of these buffers consult Table 2.1.

Table 2.1: Composition of extraction buffers.

Sr.	Name of Buffer	Composition
1	Phosphate buffer saline (PBS)	150 mM NaCl, 20 mM phosphate, pH 7.1
2	Bicarbonate buffer saline (Coca's extract 10% w/v).	$NaHCO_3$ 2 g/L, Glucose 50 g/L, Phenol 5 g/L

2.3.1. Protein extraction using phosphate buffer saline (PBS)

2.3.1.1. Defatting of pollens

B.papyrifera's pollen up to 2 gm was suspended in 10 ml ice cold acetone. Suspended pollens were stirred for 1 hr and centrifugation was done at 2200 g up to 15 min at 4 °C. Supernatant layer was discarded and remaining pellet was re-suspended in 5 ml mixture of ethanol/acetone in (1:3 v/v). Re-suspended pellet was centrifuged again at 2200 g for 15 min at 4 °C and followed by air drying in dust free environment.

2.3.1.2. Extraction of pollens

After removing all the moisture content pellet was suspended in 10 ml of PBS buffer (See composition in Table 2.1). Suspended pellet was stirred for 3 hr centrifuged and filtered using 2.5 µm pore size filtered paper. Pollen extract was stored at -20 °C.

2.3.2. Protein extraction using Bicarbonate buffer saline (Coca's extract)

2.3.2.1. Defatting of pollens

Pollen quantity of 2 gm was added to 10-20 ml of ice cold acetone and allowed to shake for 3-4 hr. After shaking supernatant acetone was discarded and same process was repeated until the supernatant acetone started to remain clear and no debris or fat droplets were visible. Then pollen grains were air dried at 29 °C in dust free environment until all the traces of the smell of acetone were removed.

2.3.2.2. Extraction of pollens

After defatting pollen pellet was suspended in 10 ml bicarbonate buffer saline (Coca's extract 10% w/v) as given in Table 2.1. Extraction was carried out for 24 hrs at ambient temperature (18-22 °C) with continuous mixing in shaker. The extract obtained were filtered through Whatman no.3 filter paper until clear. The same filter paper was repeatedly used for individual extract to avoid protein loss in the filter paper. Extract was stored at -20 °C.

2.4. Lyophilization

Lyophilization also known as freeze drying or cryo-desiccation. It is a dehydration process which involves the principle of sublimation. In this process, first the material is freeze and then by reducing the surrounding pressure the frozen water in the material is allow to pass directly from the solid phase to the gas phase.

2.4.1. Lyophilisation of pollen extracts

Pollen extracts were lyophilized to concentrate the protein content. Labconco 7670530 free zone bench top freeze dry system with a capacity of 2.5litters (See fig 2.4) was used to lyophilize the pollen extracts. Extracts were shifted to lyophilisation cups which were properly sealed using parafilm. Lyophilisation was carried out at very low temperature (-63 °C) and under high vacuum pressure (9 mbar).

Fig 2.4: Labconco freeze dry system

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a most commonly used technique in biochemistry in which the protein molecules are separated on the basis of their electrophoretic mobility by using polyacrylamide gels.

In SDS-PAGE, samples are treated with SDS combined with a reducing agent like dithiothreitol (DTT) and heat. SDS is a strong anionic detergent, basic function of which is to linearize the protein polypeptide chain by disrupting all the secondary, tertiary and quaternary conformations. SDS uniformly provides a negative charge to the protein molecules, irrespective of the protein's native charge. Protein molecules now migrate on the basis of their size. When electric field applied the negatively charged protein molecules in accordance to their size moves from cathode (-) to anode (+) under the influence of electric current. In SDS-PAGE two kinds of gel are used.

2.5.1. Stacking gel

2.5.2. Resolving gel

2.5.1. Stacking gel

Stacking gel is prepared and used up to 5%. It is casted on the top of resolving gel. The main function of stacking gel is to stack all the protein samples on the top of the face of resolving gel. Pore size of stacking gel are mostly large due to which it allows free movement of proteins and helps them to concentrate under the effect of electric field.

2.5.2. Resolving gel

It has variable percentage from 6-15%. The percentage depend upon the size of protein sample loaded in gel. It has higher pH which ionize the glycine as a result SDS-polypeptide complexes move freely through the resolving gel in uniform voltage and separate on the basis of size by sieving. The main function of resolving gel is to give resolution to the protein sample loaded according to its mass to charge ratio.

For protein study of *Broussonetia papyrifera's* pollens modified SDS-PAGE method was used as outlined by Laemmli, (1970).

2.5.3. Preparation of SDS-PAGE solutions

2.5.3.1. 30% Acrylamide mixture

Acrylamide, 29 gm and 1 gm of bisacrylamide were dissolved in little volume of deionized water. After that final volume of the solution was made up to 100 ml and stored in the dark bottle at 4 °C.

2.5.3.2. 1.5 M Tris resolving buffer (pH 8.8)

Into 150 ml of deionized water, 36.3 gm of Tris base was dissolved and pH 8.8 of solution was adjusted using 32% HCl. After adjusting pH final volume of buffer was made up to 200 ml by adding deionized water. Solution was stored at 4 °C.

2.5.3.3. 1 M Tris stacking buffer (pH 6.8)

For stacking buffer 12.1 gm of Tris base was mixed into 70 ml of deionized water. Solution pH 6.8 was adjusted by using 32% HCl. Adding deionized water final volume was maintained to 100 ml and finally stored at 4 °C.

2.5.3.4. 10% SDS solution (10 ml)

SDS up to 1 gm was dissolved in 10 ml of deionized water.

2.5.3.5. 10% APS solution (1 ml)

Ammonium per-sulfate 0.1 gm was dissolved in 1 ml of deionized water. Every time freshly prepared APS solution was used.

2.5.3.6. 1X Bromophenol blue dye (10 ml)

Tris-Cl 50 mM (0.06 gm), DTT 100 mM (0.15 gm), SDS 2% (0.2 gm), Bromophenol blue 0.1% (0.01 gm) and glycerol 10% (1 ml) was dissolved in 8.5 ml of deionized water. Finally dye was stored at -20 °C.

2.5.3.7. 5X Tris-glycine tank buffer (1000 ml)

Glycine 94 gm, 15.1 gm of Tris base and 50 ml of 10% SDS were mixed and final volume up to 1000 ml was made with distilled water.

2.5.3.8. 1X Tris-glycine tank buffer (1000 ml)

For glycine tank buffer 3.02 gm of Tris base, 18.8 gm of glycine and 10 ml of 10% SDS were mixed and final volume up to 1000 ml was made with distilled water.

2.5.3.9. Fixative solution (100 ml)

For fixative solution 10 ml of 10% acetic acid and 30 ml of 30% ethanol were mixed and final volume was made up to 100 ml by adding deionized water.

2.5.3.10. Staining solution (1000 ml)

For staining solution 100 gm of ammonium sulfate was mixed in 200 ml of distilled water and 100 ml of phosphoric acid was added. Stock solution was preserved.

Coomassie Brilliant Blue G-250, 1.2 gm was added into 200 ml of methanol. Solution was stirred and stock was preserved.

For making final solution, both stocks were mixed and volume of the final solution was made up to 1000 ml by adding distilled water.

2.5.3.11. Destain solution (1000 ml)

For destain solution 70 ml of Acetic acid was mixed with 50 ml of methanol and by adding distilled water final volume of destain solution was made up to 1000 ml.

2.5.3.12. Preservative solution (200 ml)

For preservative solution 50 ml of glycerol was mixed with 150 ml of deionized water and final volume was adjusted to 200 ml.

2.5.4. Preparation and pouring of polyacrylamide gel

Gel caster plates were washed with distilled water and air dried. Spacers were placed on the sides of the gel caster plates. After placing spacers gel caster plates were put into gel caster without notches which were then placed in gel caster with notches and closed. Leakage was checked by pouring distilled water. After checking leakage water was drain off and plates were dried by filter paper.

Resolving gel 12% (See composition in Table 2.2) was prepared in a falcon tube. First position for stacking gel was marked by inserting the comb between the spaces of two plates. After adding all the chemical ingredients TMED was added into the gel solution in the end. Gel was poured with the help of micropipette into the gap between the glass plates and overlay with water. 1 cm space was left below the mark position for stacking gel. Waited for 15-20 minutes until gel completely polymerized. After polymerization overlay water was drained off and remaining water was carefully removed with the help of filter paper. Stacking gel of 5% was prepared by adding all the chemical ingredients as given in Table 2.2 into a falcon tube. To avoid frequent polymerization TEMED was added in the end. After complete polymerization of resolving gel, stacking gel was poured on its surface and Teflon comb was immediately inserted into the stacking gel. Gel assembly was left until completely polymerized.

Table 2.2: Composition of 12% resolving and 5% stacking gel.

Chemical ingredients	12% Resolving gel (10 ml)	5% Stacking gel (5 ml)
Distilled water	3.3 ml = 3300 µl	3.4 ml = 3400 µl
30% Acrylamide Mix	4 ml = 4000 µl	0.83 ml = 830 µl
1.5 M Tris (pH8.8)	2.5 ml = 2500 µl	_
1M Tris (pH6.8)	_	0.63 ml = 630 µl
10% SDS	0.1 m = 100 µl	0.05 ml = 50 µl
10% APS	0.1 ml = 100 µl	0.05 ml = 50 µl
TMED	0.004 ml = 4 µl	0.005 ml = 5 µl
Bromophenol Blue Dye	_	0.04 ml = 40 µl

2.5.5. Pollen sample Preparation

Lyophilized pollen extract powder weight 50 mg was added into 50 µl of water, PBS and Coca's extract in three separate Eppendorf.

2.5.6. Protein estimation of pollen samples

For protein estimation Bradford assay was performed and optical density (OD) of each sample was recorded at 595 nm by using spectrophotometer. Protein concentration of each sample was estimated by comparing its OD with the BSA standard curve (As shown in Fig 2.5).

2.5.6.1. Plotting standard curve of Bovine serum Album (BSA)

Bovine serum Album (BSA) Stock solution was prepared by taking 1ml distilled water in falcon tube and mixing 1 mg of BSA into it. Using BSA stock solution dilutions were prepared from 1 µg to 10 µg each of 1 ml final volume as shown in Table 2.3. Absorption of each BSA dilution was recorded at 595 nm (See Table 2.4). Based on OD of each dilution standard curve of Bovine serum Album (BSA) was plotted (See fig 2.5) using Microsoft excel.

Table 2.3: Preparation of the dilution of the given protein sample (BSA).

BSA Dilutions (µg)	Vol. of BSA solution Stock (µl)	Vol. of Distilled water (µl)	Vol. of Assay Reagent (µl)	Final Vol. (µl)
1 µg	1 µl	199 µl	800 µl	1 ml
2 µg	2 µl	198 µl	800 µl	1 ml
3 µg	3 µl	197 µl	800 µl	1 ml
4 µg	4 µl	196 µl	800 µl	1 ml
5 µg	5 µl	195 µl	800 µl	1 ml
6 µg	6 µl	194 µl	800 µl	1 ml
7 µg	7 µl	193 µl	800 µl	1 ml
8 µg	8 µl	192 µl	800 µl	1 ml
9 µg	9 µl	191 µl	800 µl	1 ml
10 µg	10 µl	190 µl	800 µl	1 ml

Table 2.4: Absorbance values of BSA dilutions.

Sr.	Conc. Of BSA dilutions (µg)	Absorbance at 595 nm (OD)
1	1 µg	0.036
2	2 µg	0.06
3	3 µg	0.095
4	4 µg	0.131
5	5 µg	0.162
6	6 µg	0.192
7	7 µg	0.223
8	8 µg	0.258
9	9 µg	0.301
10	10 µg	0.337

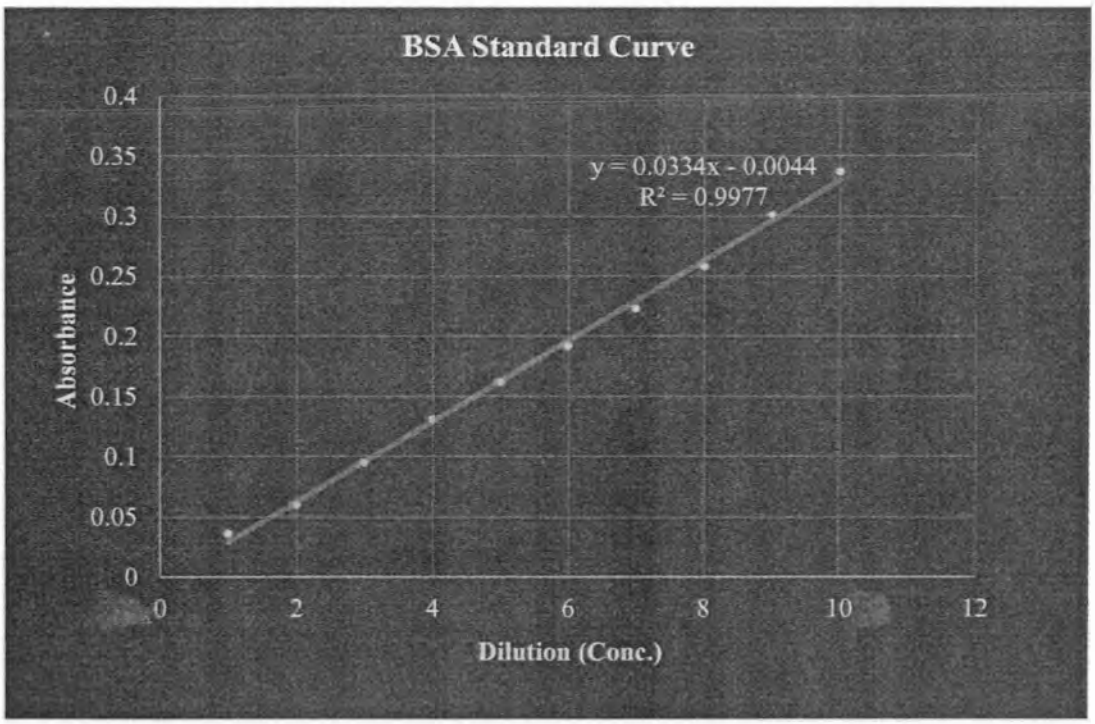

Fig 2.5: Standard curve of BSA

2.5.7. Loading of Samples

Before loading samples were heated at 95 °C for 15 min in water bath. 20 µl of each sample was loaded in polyacrylamide gel along with PageRuler Unstained Protein Ladder (Thermo Scientific).

2.5.8. Running SDS-PAGE

Gel caster containing completely polymerized gel was placed and tightened in PAGE apparatus. 1X Tris-glycine tank buffer was poured in PAGE apparatus until the plates sank in it. Comb was carefully removed from gel. 20 µl of each sample was loaded. Initially 60 V was given to electrophoresis, when sample run off the stacking gel the voltage was increased to 110 V. The process was allowed to run about 1-2.5 hours. When sample ran off the resolving gel, stopped the voltages and took out the gel casting plates with intact gel. Opened the gel casting plates with the help of an opener, and gel was separated out by shaking the gel in the fixative solution in a tray.

2.5.9. Staining and destaining of gel

Transferred the gel to another tray and Coomassie brilliant blue dye was added. Gel was dipped in it. Tray was placed on the moving plate shaker for overnight. After that excessive dye was removed with the help of a dropper. Destaining solution was added to the tray and again placed on the moving plate shaker at 40-50 rpm for 10 min. Destaining solution was replaced with the fresh one and left for overnight. After 24 hrs bands of protein were observed with white background. After destaining digital image of the gel was taken with help of gel doc. using white light. The apparent molecular mass of pollen proteins was determined by PageRuler Unstained Protein Ladder (Thermo Scientific).

RESULTS

Present study was conducted to understand and evaluate the harmful effects of pollen allergy caused by *Broussonetia papyrifera* in Islamabad during flowering period in spring season. Air born pollens are found to be the most important entities in relation to allergy disease and play an important role in provoking allergic response in susceptible persons. A series of experiment was performed including structure and protein studies of *Broussonetia papyrifera's* pollen as well as survey related to allergy patients. The detailed experiment as follows:

Part-A

3.1. Survey based Identification of different factors associated with pollen allergy disease in Islamabad

During the survey, 32 pollen allergy patients were interviewed in which 25 were female and 7 were male. In this way female patient with 78.125% was higher as compare to 21.875% of male patient. Age range of allergy affected patients were from 20-70 years but most of the patients were 30-35 years old. Patients were evaluated on the basis of symptoms related to allergic reactions. Patient's response was recorded and data was plotted using Microsoft word and excel. Detail of each part of survey is given below:

3.2. Clinical features

Responses regarding clinical features of disease were recorded. Rhinitis was found to be highly positive (28 patients) as compare to other clinical feature. It was followed by asthma, frequent sore throat and cough (Fig 3.1). 87.5% patients was with rhinitis symptoms while 81.25%, 78.125% and 78.25% shown asthma, frequent sore throat and cough symptoms respectively (Fig 3.2).

Mostly patients who were having rhinitis problem were also showing symptoms of watering eyes, inflammation of eyes, eye redness, skin irritation and fever during rhinitis. Patients included in the survey were residents of Islamabad and Rawalpindi areas. The symptoms of allergy in patients were existing from 3-22 years but most of them reported for 5-6 years.

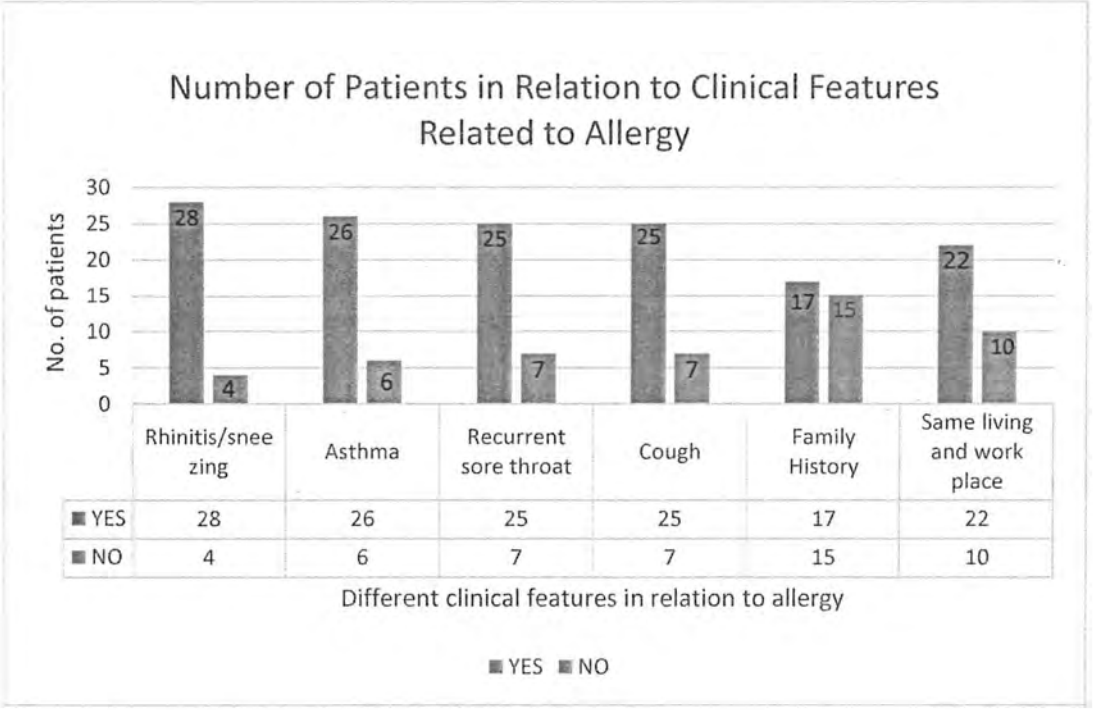

Fig 3.1: Distribution of clinical features of allergy in relation to no. of patients.

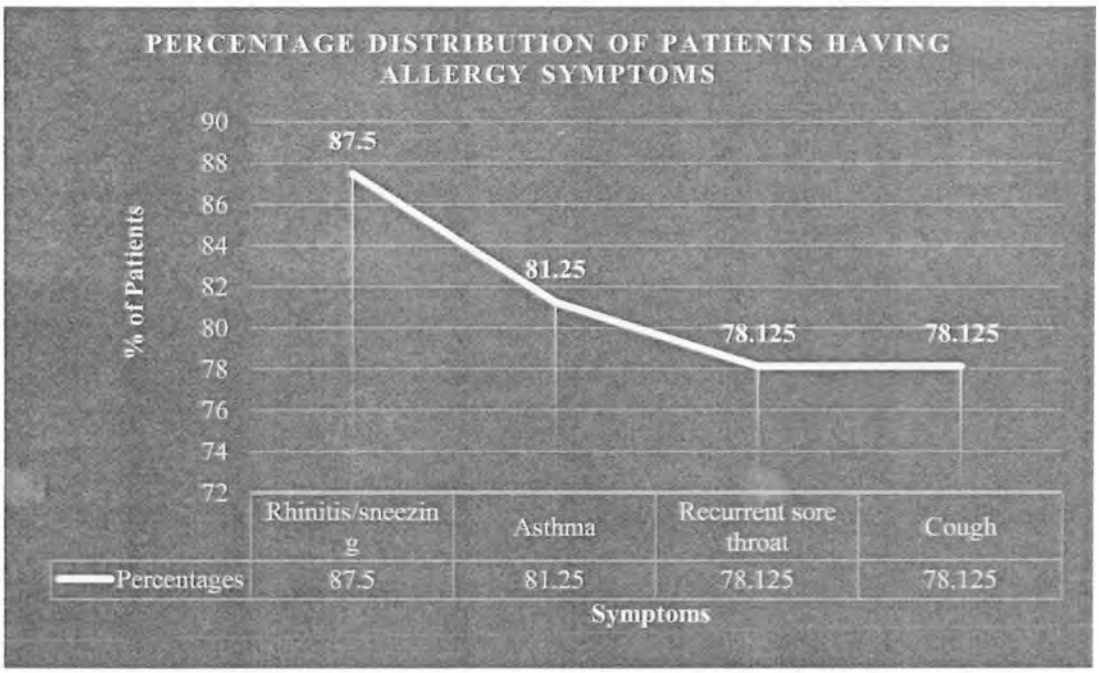

Fig 3.2: Percentage distribution of patients having allergy symptoms.

3.3. Genetics and environmental factors in relation to allergy disease

Role of genetic and environmental factors was evaluated based on family history of disease and living environment of patients. 17 patients were with family history of allergy disease while 22 patients were living and working at the same place (Fig 3.3). Among these factors percentage of environmental factors was as high as 68.75% compare to 53.125% of genetic factors (See fig 3.4).

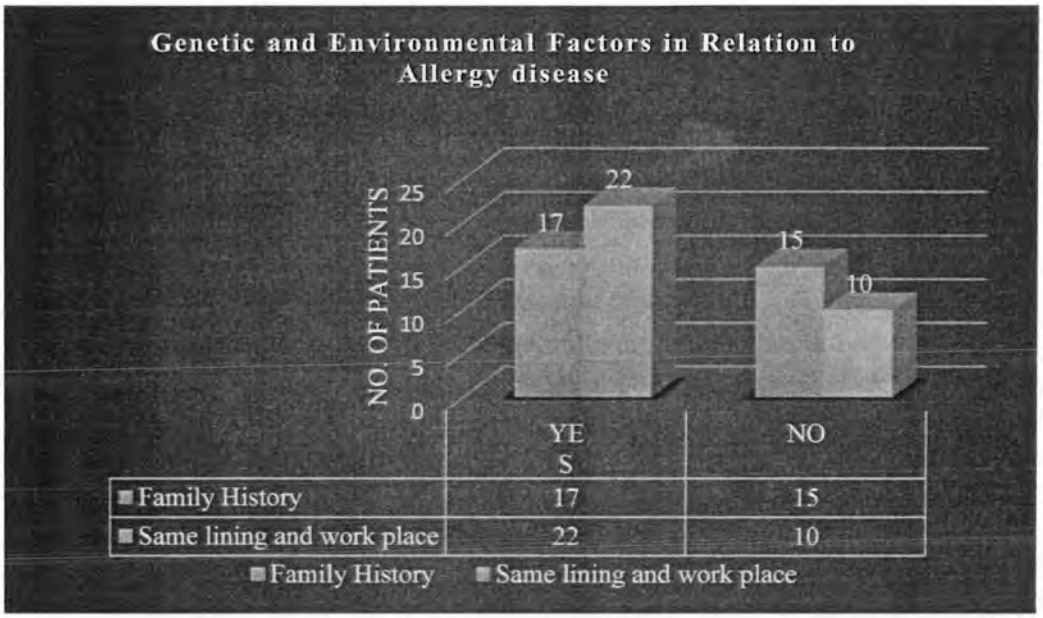

Fig 3.3: Genetic and environmental factors in relation to allergy.

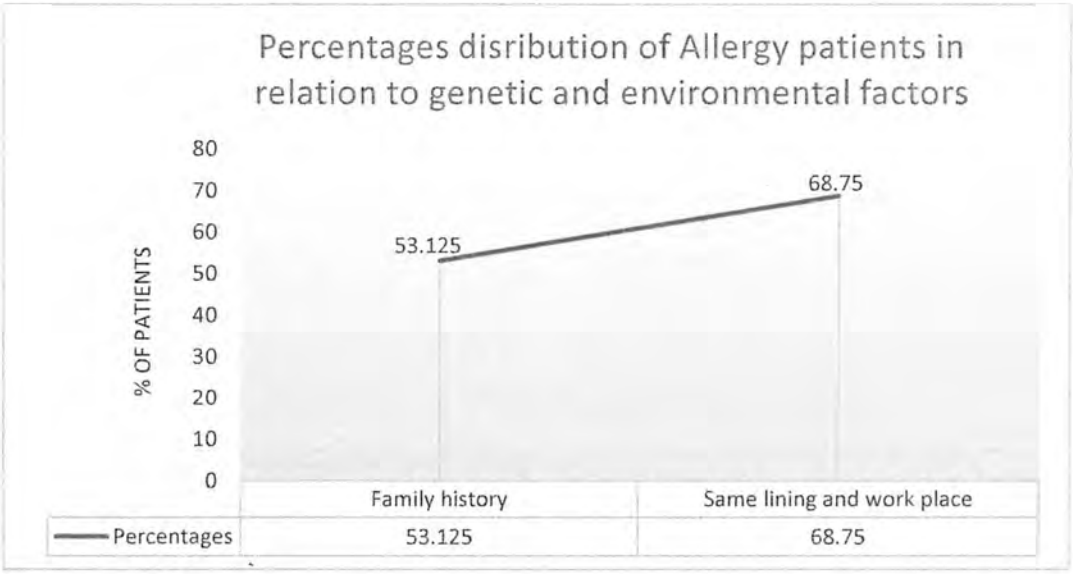

Fig 3.4: Percentage distribution of allergy patients in relation to genetic and environmental factors.

3.4. Rhinitis

Symptoms specific to rhinitis was evaluated and among all symptoms, sneezing was found to be highly positive as 28 patients out of 32 given positive response. After sneezing symptom of post nasal drip was fond to be highly associated with Allergy disease, 27 patients shown positive response for this. 26, 25 and 24 patients have shown positive response for frequent sore throat, running nose and blocked nose respectively (See fig 3.5).

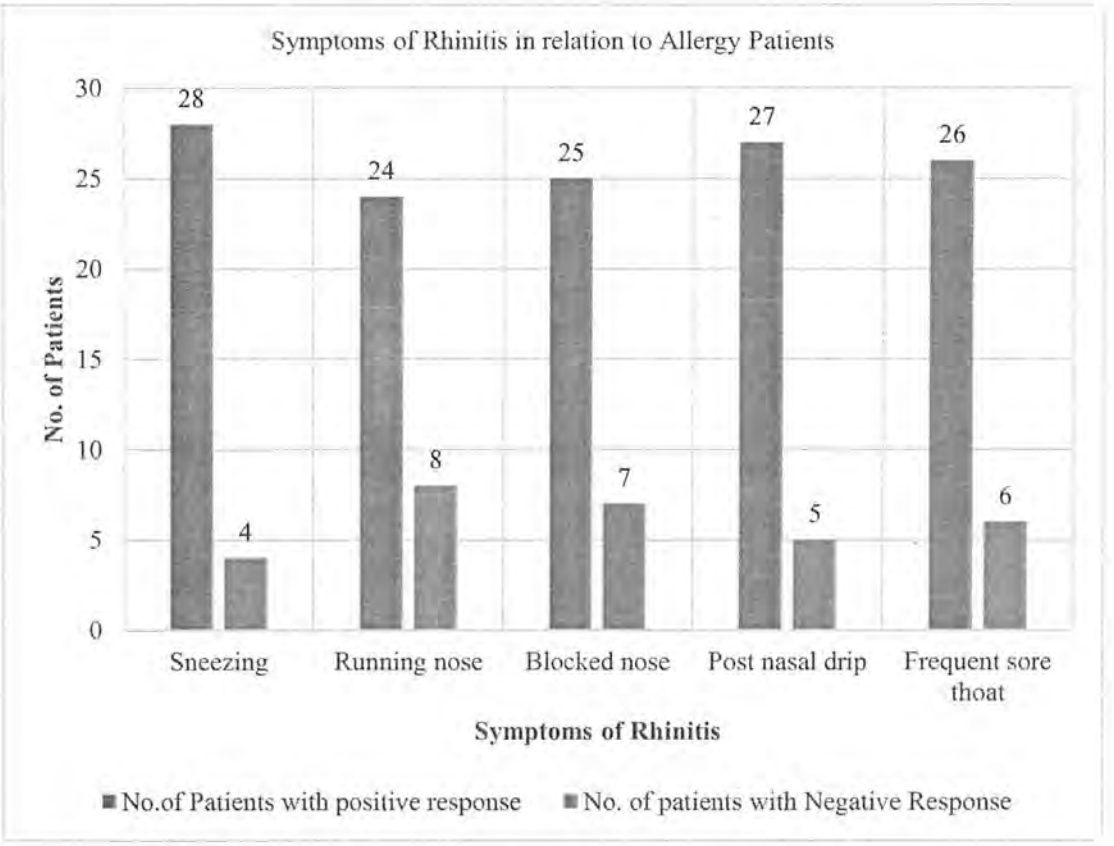

Fig 3.5: Distribution of rhinitis symptoms in relation to Allergy patients.

3.4.1. Rhinitis in Relation to seasonal variation

Relation of rhinitis symptoms with seasonal variation was evaluated. Among all the allergy patients 91% shown the seasonal variations in the rhinitis symptoms (See fig 3.6). Seasonal variation data indicated that 23 (71.4%) patients shown aggravated allergy symptoms from mid-March to mid-April. After that there is sharp decline in summer, leading to negligible cases afterwards (See fig 3.7).

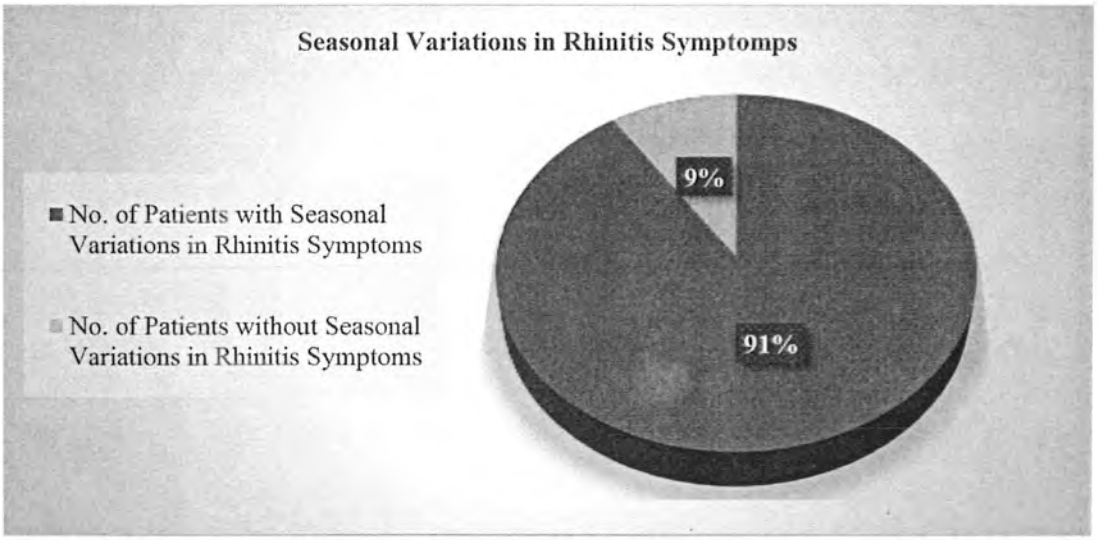

Fig 3.6: Rhinitis symptoms aggravation in relation to seasonal fluctuation.

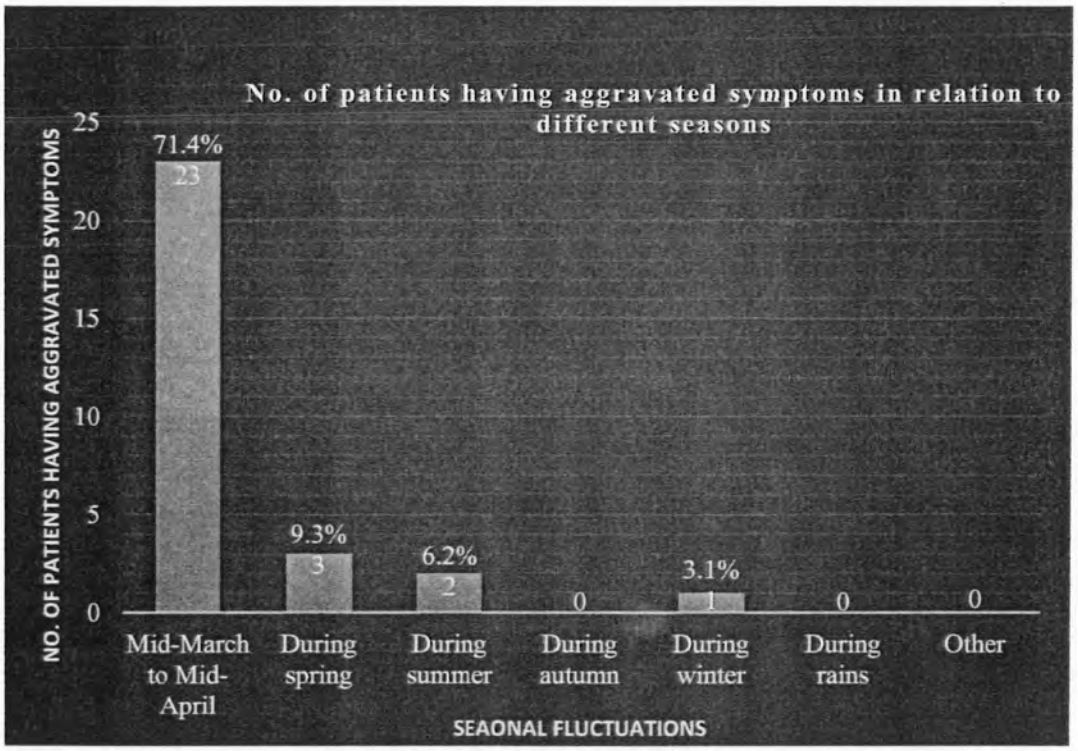

Fig 3.7: Distribution of patients having aggravated symptoms of allergy in different seasons.

3.4.2. Aggravation of rhinitis symptoms in correlation to particular time

Aggravation of allergy symptoms in association to particular time was evaluated and responses were compared. Morning and night time was found to be closely associated with aggravation of allergy symptoms. As 13 patients showed positive response for morning and 11 for night time. Response for evening time and on waking was not much high as 4 and 2 patients were having symptoms at these times respectively. Although there were 3 patients who were having aggravated allergy symptoms irrespective of time (Fig 3.8).

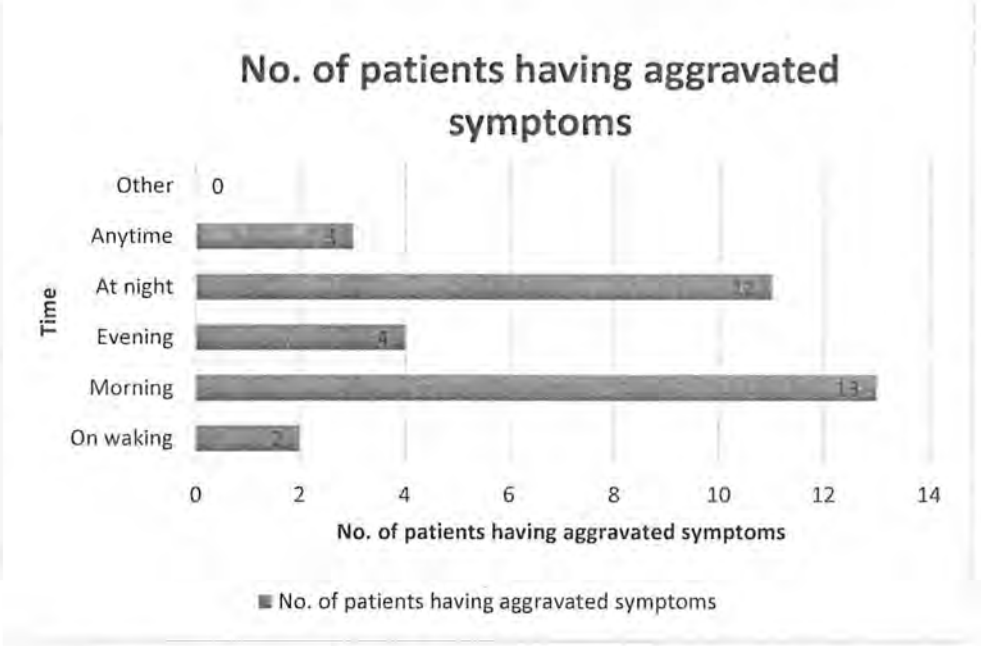

Fig 3.8: Aggravation of rhinitis symptoms in correlation to particular time.

3.4.3. Rhinitis symptoms in relation to other environmental factors

Effect of different environmental factors on the rhinitis was evaluated. House dust was found to have greater effect on the rhinitis symptoms as 25 patients indicated this. Cold was found to be least associated with rhinitis symptoms. 11 patients indicated the symptoms association with cold. Outdoor dust, smoke and strong smell found to have equal effect on rhinitis symptoms as for each 15 people showed positive response (See fig 3.9).

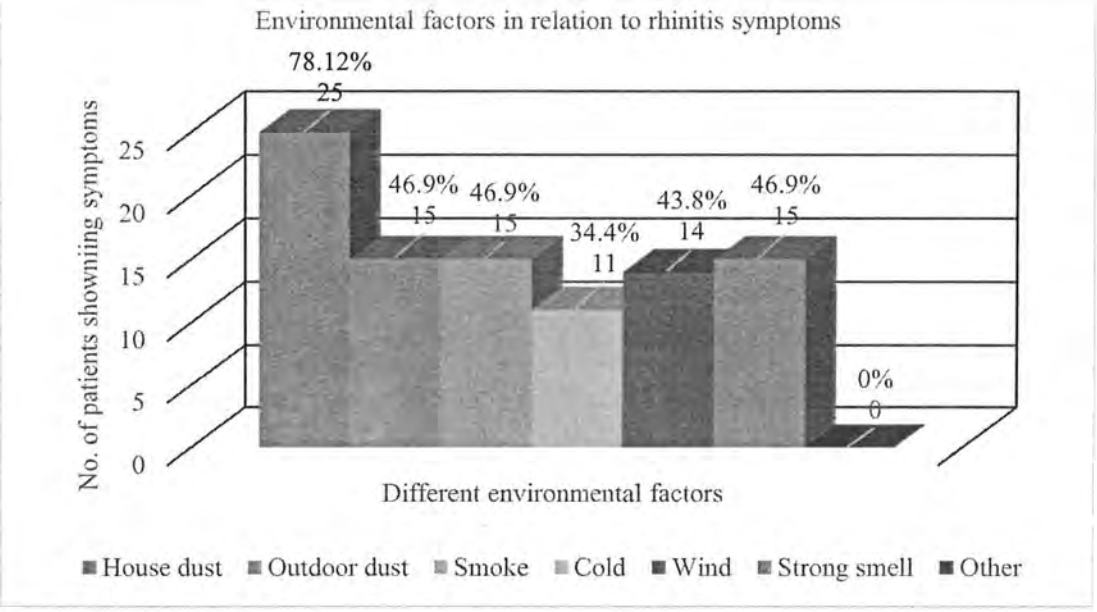

Fig 3.9: Rhinitis symptoms in relation to different environmental factors.

3.4.4. Illness relieved outside Islamabad

Data was also recorded for the illness relieved when there is change in the environment of the patient.it was found that more patients feel relieved from allergy symptoms when they move outside Islamabad. 72% patients indicated that when they change their environment and move outside Islamabad in the month of March and April there is significant decrease in the symptoms of Allergy. There were 28% patients who did not feel relieved with change in environment (See fig 3.10).

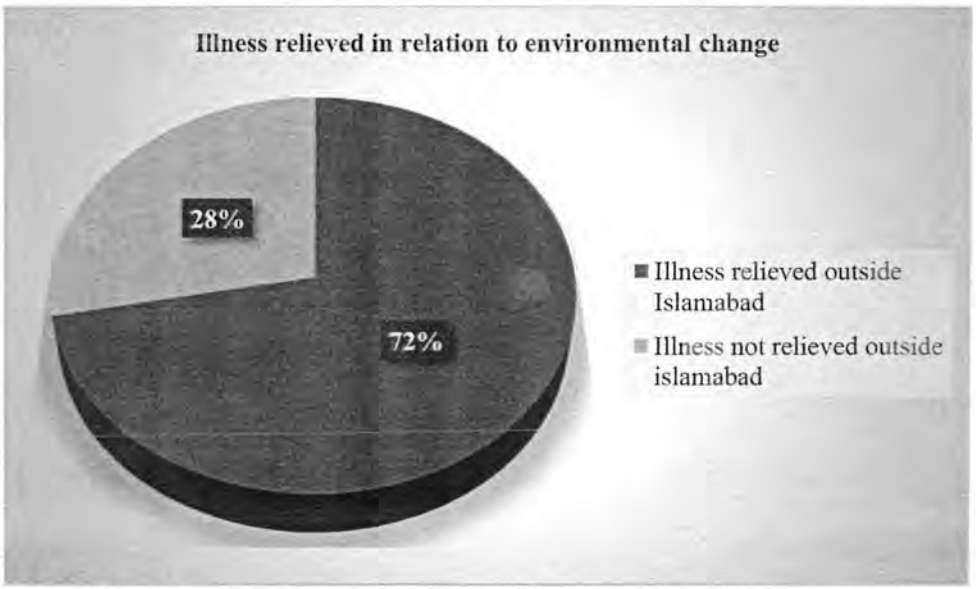

Fig.10: Percentage distribution of illness relieved in relation to change in environment.

3.5. Asthma

Different symptoms related to asthma was evaluated. Amongst all symptoms tightness in chest and difficulty in breathing were found to be present in high percentages. 29 patients showed positive response for these high percentage symptoms. While 28 patients indicated wheezing at any time as the symptoms in relation to their asthma problem. 27 and 22 patients shown positive feedback for cough and expectoration symptoms respectively (See fig 3.11).

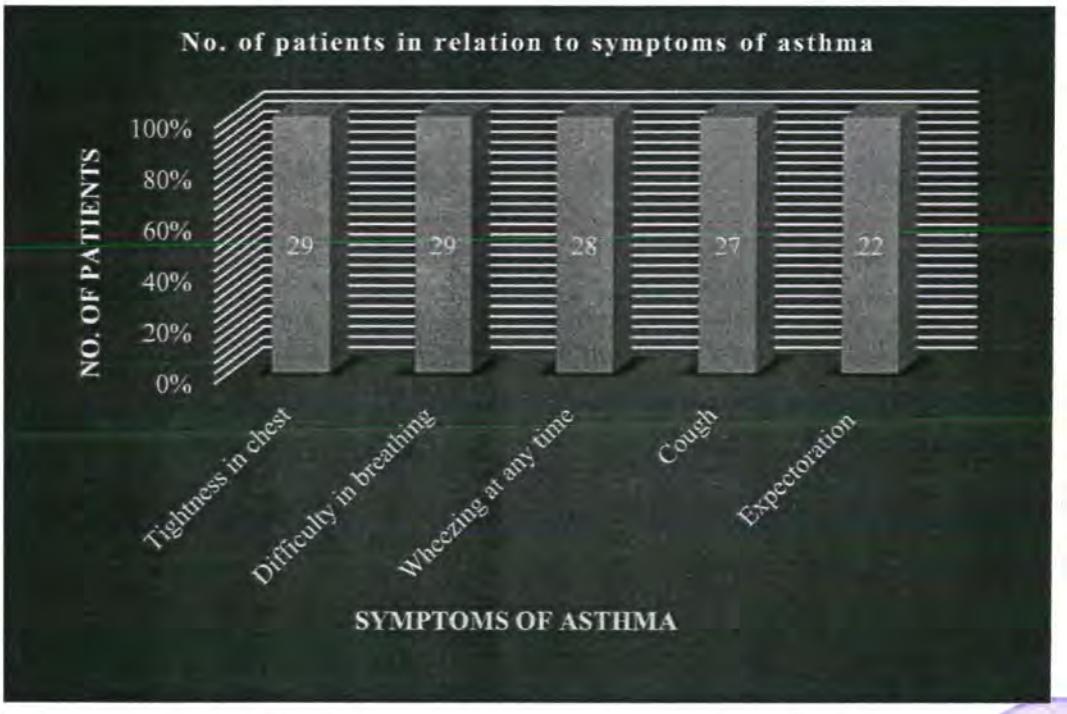

Fig 3.11: Distribution of patients in relation to symptoms of asthma.

3.6. Cough in Allergy patients

30 patients were observed with cough in which 17 patients were having dry cough while 13 were with productive cough. Response of 18 patients was found to be positive for cough associated with fever. While 21 patients have given positive response for cough after rhinitis (See Table 3.1)

Table 3.1: Cough and related symptoms in allergy patients

No. of patients with cough	No. of patients with dry cough	No. of patients with productive cough				No. of patients having cough associated with fever	No. of patients having cough after rhinitis
		White sputum	Yellow sputum	Green sputum	Blood stains		
30	17	3	7	1	2	18	21

PART-B

3.7. Palynological study of *Broussonetia papyrifera's* pollen

3.7.1. General pollen characters of the family Moraceae

Pollen grains of moraceae family are variable. In the studied taxa of moraceae pollens are di-stephano or peri-porate, spherical to oblate and their average diameter is about 30 µm. Scabrate can be regulated by exine sculpturing with small supratectal spinules. Tectate-granular with a bilayered basal layer was observed in pollen wall (only studied in *Dorslenia sp*). The tectum is occasionally traversed by minute channels. (Zavada and Dilcher, 1986).

3.7.2. Morphology of *Broussonetia papyrifera's* pollen

Different morphological character of *Broussonetia papyrifera* were studied. Studies were based on two different microscopic techniques in which one was light microscopy and the other was scanning electron microscopy. Fig 3.12 shows micrograph of polar and equatorial view of pollens using light microscopy (LM) while polar and equatorial views using scanning electron microscopy (SEM) are demonstrated in fig 3.13.

Pollen shape observed was spheroidal. Pollen was biporate. Aperture of pollen was dicoporate. Columella was weakly developed. Exine of the pollen was very thin. Exine sculpturing was scabrate with very prominent scabrae of less than 1µm length. Pori was with grannute opercula. Additionally plasma was subtle granular.

Maximum polar length observed was 10.52 µm and minimum polar length was about 7.89 µm. Mean polar length of *Broussonetia papyrifera* pollen was 9.86 µm. Aperture was 1.5 µm. Maximum polar diameter observed was 10.52 µm and minimum was about 7.89 µm while mean was 9.86 µm. P/E ratio of pollen measured was 100. Pore diameter was 2.63 µm (Table 3.2).

Table 3.2: Morphological character of *Broussonetia papyrifera's* pollen

Sr.	Pollen plant specie	Mean polar length	Aperture size	Mean polar diameter	Pore diameter	P/E ratio
1	*Broussonetia papyrifera*	9.86 µm	1.5 µm	9.86 µm	2.63 µm	100

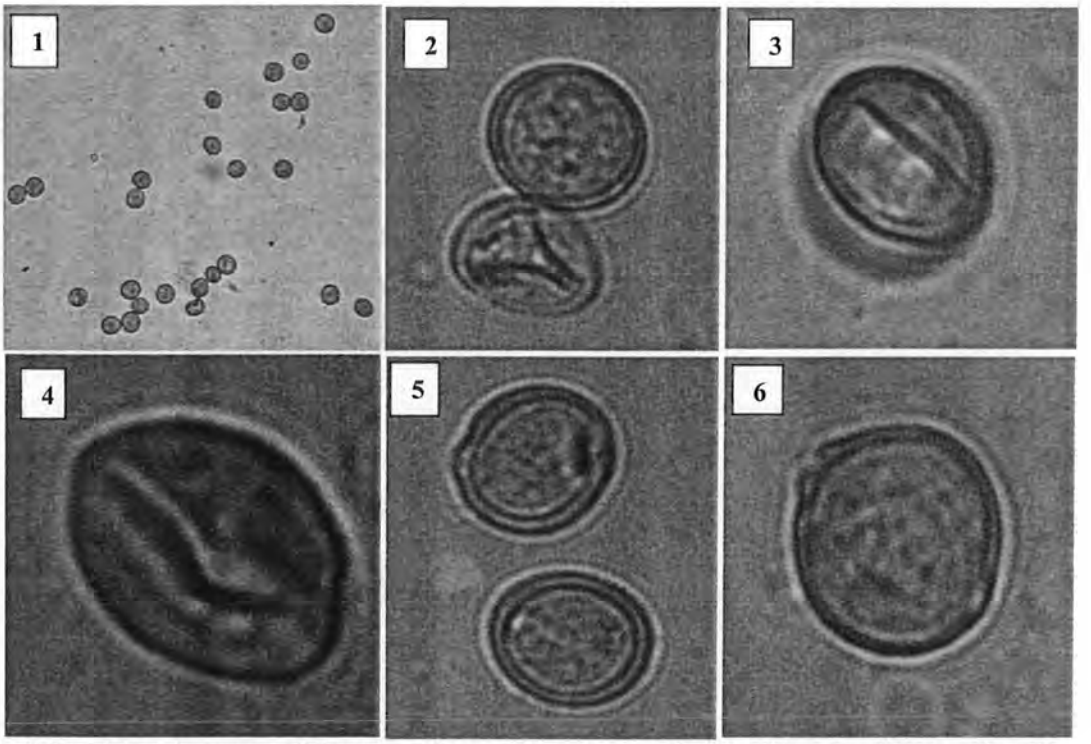

Fig 3.12: Light microscopy of *Broussonetia papyrifera*: 1, overall slide of *B. papyrifera's* pollens; 2, showing Polar & Equitorial view of *B. papyrifera's* pollen; 3 & 4, Equitorial view of *B. papyrifera's* pollen; 5 & 6, Polar view of *B. papyrifera's* pollen with prominent bipolar structure.

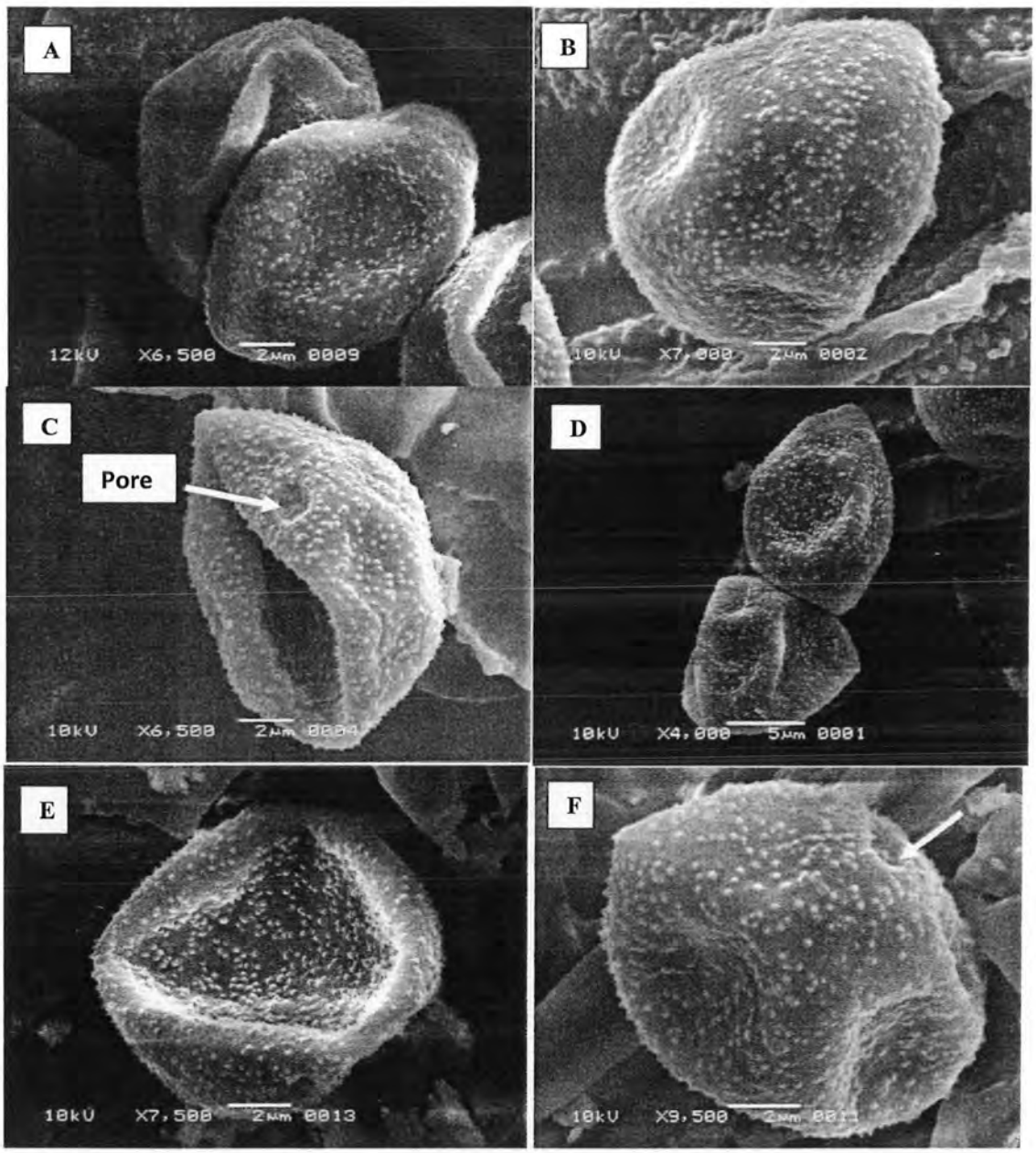

Fig 3.13: From A-F: Scanning Electron Micrographs (SEM) of pollen grains of *Broussonetia papyrifera's* pollen from Pakistan. A, SEM of *B. papyrifera* (scale bar = 2 µm); B, SEM of *B. papyrifera* showing exine sculpturing (scale bar = 2 µm); C, SEM of *B. papyrifera* showing exine sculpturing and pore (scale bar = 2 µm); D, SEM of *B.papyrifera* (scale bar = 5 µm); E, SEM of *B. papyrifera* showing Scabrate sculpturing of exine (scale bar = 2 µm); F, SEM of *B. papyrifera* showing a prominent pore and scabrate structure (scale bar = 2 µm).

3.8. Protein characterization using SDS-PAGE

3.8.1. Protein estimation of pollen samples

Lyophilized pollen samples weight 50 mg were dissolved in 50 µl of the respective buffer in which pollens were extracted before lyophilization while one sample was prepared with distilled water to compare the solubility with Coca's extract. Absorbance of each sample was taken at 595 nm. Protein in each sample was determined by using linear equation of BSA standard curve. Higher concentration of protein was observed in PBS buffer sample up to 2.407 µg. Coca's extract contained 1.838 µg of pollen protein while lowest concentration was observed in water dissolved sample as it contained 1.629 µg (See Table 3.3).

Table 3.3: Protein estimation of pollen samples by using BSA linear equation

Sr.	Pollen extract	OD at 595 nm	BSA linear equation	Protein in Pollen sample (x)
1	PBS buffer	0.076	x = 0.076+0.0044/0.0334	2.407 µg
2	Coca's extract	0.057	x = 0.057+0.0044/0.0334	1.838 µg
3	Distilled water	0.050	x = 0.050+0.0044/0.0334	1.629 µg

3.8.2. Protein pattern of *Broussonetia papyrifera's* pollen

Sodium Dodecyl Sulphate polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed using 5% stacking and 12% resolving gel. Protein pattern of *Broussonetia papyrifera's* pollen was obtained using two different extraction buffers namely phosphate buffer saline (PBS) and bicarbonate buffer saline (Coca's extract). Protein concentration in PBS was 2.407 µg/50 µl while in Coca's extract was 1.838 µg/50 µl. Water sample which was prepared to compare the solubility showed protein concentration up to 1.629 µg/50 µl. Protein pattern in all three sample was same.

A noticeable band of approximately 75 kDa band was observed in all three samples. From band intensity it was clear that protein was more soluble in Coca's extract as compare to water (Fig 3.14).

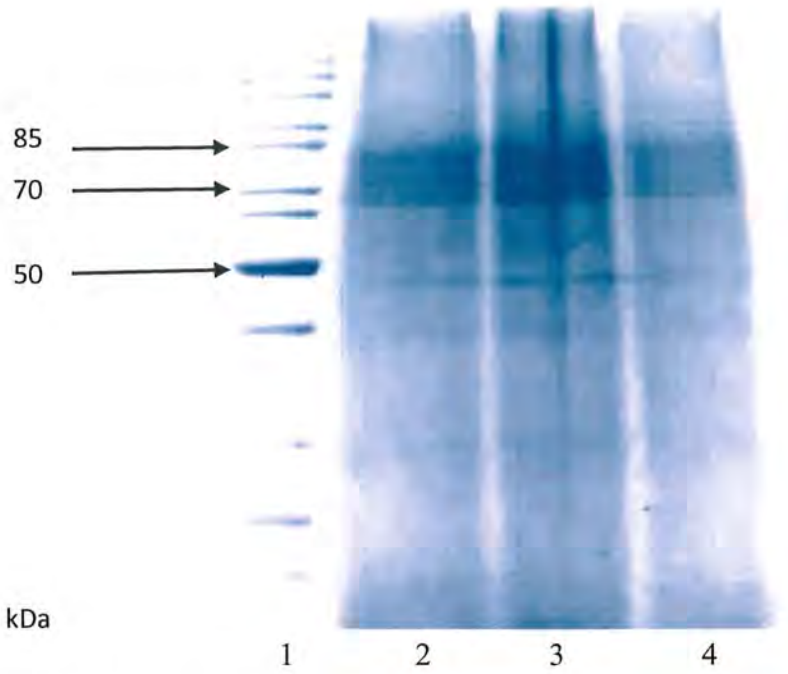

Fig 3.14: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12%) with Coomassie staining of crude *Broussonetia papyrifera* pollen extracts. Lane 1, molecular marker; Lane 2, phosphate-buffered saline extract (0.96 µg/lane); Lane 3, Coca's extract (0.74 µg/lane); Lane 4, water sample (0.65 µg/lane).

DISCUSSION

Present research work was conducted to explore the factors associated with the allergenicity of invasive *Broussonetia papyrifera* in Islamabad. The interest of research was to conduct pollen studies of *Broussonetia papyrifera*, As well as to study different patterns of allergy symptoms shown by allergy patients. Pollens from this invasive tree are frequently reported as a major cause of allergy in Islamabad. Especially during the spring season it is reported as a key factor to bring deaths to allergy patients (Abbas *et al.*, 2012). Therefore a survey based study was conducted to evaluate pollen allergy prevalence as well as conditions of allergy symptoms in order to develop deeper understanding of the issue.

During March, a comprehensive survey was developed under the guideline of allergy specialist. As survey was conducted in the allergy camp at Community Center Abpara, Islamabad. Therefore, mainly includes residents of Islamabad. Our survey showed that 78.125% of females and 21.875% of males are with seasonal allergy. Result of the survey indicates that there are high number of females as compare to males suffering from allergy disease. This is in accordance with the previous studies (Bennett *et al.*, 1997; Schatz, 2007). As Bennett *et al.*, (1997) found out that among all allergy patients female patients were 58% as compare to 42% of the male patient. Whereas, Schatz, (2007) found out female percentage was 60.9% and male percentage was 39.1%. This indicates that females are either more sensitive to allergens or they are more exposed to allergy eliciting aeroallergen. This could also lead to the idea of presence of other allergy contents like house dust and paints in houses. However, research is required at genetic level to further certify the reason behind the gender discriminating nature of this disease.

There is no specific age limit for allergy patients. As in our studies patients from 20-70 years of age are observed with allergic reactions. However, median age group of allergy patients was 30-50 years of age. This result was also in accordance to the findings of Bennett *et al.*, (1997). As it was shown in their studies that individuals in the age set of 40 years or above are at higher risk of developing allergy related problems.

Our studies showed that among all clinical features, rhinitis was highly positive with a percentage of 87.5%. However, features of asthma and sore throat were also not negligible as percentages of these were 81.25% and 78.125% respectively. These findings are supported by the recent studies of Chen *et al.*, (2013). As they found out that most of the allergy patients were suffering from both rhinitis and asthma (Chen *et al.*, 2013). These results can be related to the concern shown in the studies of Shabbir and Bajwa, (2006). They concluded that a long exposure to aeroallergens can gradually lead to the development of asthma and bronchitis. Our survey results have clearly demonstrated the explanation given by Papadopoulos *et al.*, (2012) that allergies related to respiratory tract, mainly rhinitis and asthma, frequently coexists in the same patient. Therefore, currently these are recognized as part of a common disorder. "Chronic respiratory allergy" along with different terms have been proposed for this common syndrome. In our study patients with rhinitis were observed with clinical features of watering eyes, inflammation of eyes, eye redness, skin irritation and fever. These are the common symptoms related to respiratory ailments as indicated by several studies (Bennett *et al.*, 1997 Canonica *et al.*, 2007).

Moreover, in current survey genetic and environmental factors in association to allergy are also investigated. Which showed that percentage of environmental factors in relation to allergy is high up to 68.75% as compare to 53.12 % of the genetic factors. This shows that although percentage for environmental factors is high but concern regarding genetic factors cannot be neglected. It is reported that inherited susceptibility to various allergy aliments depends on several genes (Puc, 2003). These genes are involve in the enhanced capacity building of allergy activation cells, immunoglobulin E production, cytokines production and regulation of histamine release with the hypersensitivity of allergy affected organs (Negrini, 1992). According to previous judgements there are 50-80% chances of an allergic child to parents having same pollinotic symptoms (Munshi, 2000). Even chances remains up to 30% with one allergic parent (Brostoff and Hall, 1993). This outcome may offer an explanation for the inherited allergies in Pakistan, where marriage between first and second cousins is a normal tradition and a common practice in the society.

While higher contribution of environmental factor has been reported by many previous studies (Bennett *et al.,* 1997). Therefore, allergies in Islamabad can be related to the presences of invasive flora in the region. This fact can be supported by the presences of higher number of pollinosis patients in the urban areas where artificial green areas are mostly aggregated with highly allergenic plants (Von Hertzen and Haahtela, 2004; Khan *et al.*, 2010). Pollens from these invasive plants are also reported as allergenic in nature which can also play a vital role in eliciting allergic reactions. According to Mandal *et al.*, (2008) number of airborne pollen has a significant role in pollinosis. Moreover, many plant species are found to be associated with the pollinosis problem of Islamabad. Among these species *Broussonetia papyrifera* is frequently reported as a marked cause of pollen allergy related diseases (Malik and Husain, 2007; Khan *et al.*, 2011). During the month of February until April proximity, *Broussonetia papyrifera* contributes to severe pollen allergy. Especially, in Islamabad pollen count of *Broussonetia papyrifera* goes as high as 55000/ m^3 and causes severe allergy problems for the residents of Islamabad (PMD, 2012).

Rhinitis symptomology in our study showed that sneezing and post nasal drip symptoms were highly positive among all allergy patients. Percentage for sneezing was 87.5% while for post nasal drip was 84.38%. Similar findings were reported by Zaidi *et al.*, (2012) and Canonica *et al.*, (2007). Where all allergic rhinitis patients showed high percentage of sneezing during disease state. Other than sneezing, symptoms related to nasal secretions were found to be equally positive. These are the symptoms mostly related to Type 1 hypersensitivity reactions (Buczylko, 1996). Which is basically involve in the pollionsis disease (Puc, 2003).

Present survey confirmed the presence of seasonal allergic rhinitis. Symptoms showed high percent of variations in relation to seasonal changes. With a very high value of 91% patients indicated the relation of rhinitis symptoms to specific season. More importantly out of 91% patients with seasonal variations 71.4% reported aggravation of rhinitis symptoms in the month of March. Same was found in the previous studies by Bennett *et al.*, (1997) and Ghufran *et al.*, (2013). Seasonal variation of disease symptoms clearly indicated the presence of specific allergenic content in the atmosphere during certain period of the year. These results relates to the pollen count studies of *Broussonetia papyrifera* in the month of March for the city of Islamabad. As

studies of Abbas *et al.*, (2012) showed that during 2005-2007, *Broussonetia papyrifera* pollen was highest amongst all the pollen trapped. Moreover, Ghufran *et al.*, (2013) compared allergic patients with monthly pollen count during the study period of 2009-2010. They have found that in 2009 and 2010 highest number of allergy patients were recorded in the month of March. Notably, 1642 allergy patients were registered in the March, 2009 when the pollen of *Broussonetia papyrifera* was showing its peak value of 4143.21 pollens/ m^2. Similarly in March, 2010 when pollen count for *Broussonetia papyrifera* was 4431.5 pollens/ m^2 the highest number of 1724 allergy patients were recorded. Eventually, it can be concluded that during March *Broussonetia papyrifera* is the main contributor towards the allergy issue of Islamabad.

Survey result for aggravation of rhinitis symptoms in relation to particular time showed that mainly morning and night time was the painful time for the allergy patients. Similar was reported by Rashid *et al.*, (2014) that after Fajr and between Asar till night pollen counts of *Broussonetia papyrifera* were highest. They also reported that suspended pollens of *Broussonetia papyrifera* were regarded as a main cause of increasing agony of the pollen allergy patients.

Relation of other environmental factors with rhinitis was also evaluated in our survey which indicated that house dust is the most dominant environmental factor related to rhinitis problem. Mostly patients who were having allergic reactions in March were also showing hypersensitivity to house dust and outdoor dust. Similar, results were shown by Abbas *et al.*, (2012). Their studies revealed that patients with positive skin prick test (SPTs) for *Broussonetia papyrifera* have also highly positive SPTs for house dust. This shows that allergy patients are hypersensitive to multiple allergens. Present results can be related to Cox and Wathes, (1995) description that airborne pollen can interact with different air suspended substances. These could be deposits of microorganism on the surface of pollen grain. According to previous studies, these interactions can not only change the properties of pollen but can also enhance the allergic symptoms by acting as an adjuvant molecule (Schappi, 1996; Spiewak, 1996). Similar kind of interactions could be present among pollen and dust particles. However, further studies at molecular level are required to investigate the type of interactions.

Allergy condition in relation to Islamabad was evaluated, which clearly indicated that allergy was specific to the capital city. As 72% patients indicated relive from allergy symptoms outside Islamabad. Bennett *et al.*, (1997) reported same response in their survey that in the spring season allergy patients left Islamabad due to reoccurrence of disease in each year. According to allergy patients this was the only choice they had to avoid spring allergies. In present survey allergy patients with asthma have shown higher level of breathing problems, tightness in chest and persistent cough. Same was reported by Bennett *et al.*, (1997) that among sever cases of pollen allergy patients up to 51% were suffering from asthma, breathing problems and congestion in the chest. Modifications of pollen and other aeroallergens due to climatic changes are playing significant role in increasing the incidence of asthma and allergies (Shea *et al.*, 2008). According to recent studies of Rashid *et al.*, (2014), features of pollen allergy are changing due to sudden rise in pollen contents. Thus, patients are showing the symptoms of severe persistent cough with difficulty in breathing and wheezing at their early stage of disease.

For the purpose of understanding, different morphological features related to *Broussonetia papyrifera* pollen were explored. The findings of light microscopy and scanning electron microscopy were merged to clearly define the structure of *Broussonetia papyrifera* pollen. Firstly in our research it was observed that pollen size of *Broussonetia papyrifera* was very small. Mean pollen diameter observed was 9.86 µm and pore diameter was 2.63 µm. However, values of pollen diameter and pore diameter given by Kim and Zavada, (1993) were 13.8 µm and 0.9 µm respectively. Other morphological studies regarding shape, sculpturing and pore number of pollen was similar to Kim and Zavada, (1993). As pollen was spheroidal, biporate and sculpturing was scabrate with very prominent scabrae. From the present studies it can be concluded that over the period of time with the climatic changes structure of pollen is also showing variations. According to D'Amato, (2000) pollen grain of size 15-40 µm cannot get access to the lower thoracic region of the respiratory tract while smaller size allergens can enter and induce the hay asthma. In our study pollen pore size observed was significantly higher as compare to previously report. Allergenic pollens contain granular plasma. According to Nigrini, (1992) granular structures in pollen grains contain protein, starch and lipids when such kind of pollen come in contact with

the mucous membranes it discharge these granular proteins which can induce hypersensitivity reactions. Moreover, Motta *et al.*, (2006) reported that these allergenic granules can increase the bioavailability of pollen allergens. We can concluded that due to large pore size of pollen it can easily and more hastily release allergens in the air.

In addition, protein studies related to *Broussonetia papyrifera* were performed. Pollen was physically purified and protein was extracted using PBS buffer and Coca's extract. Due to crude pollen and manual purification techniques protein content was very low as maximum value with PBS was up to 2.1 µg per 50 µl. Similar was reported by Chen *et al.*, (2013) that purification of *Broussonetia papyrifera* pollen was difficult due to which only 300µg/ ml of protein concentration was achieved.

Moreover, it is known that natural environmental allergens are normally proteins. Molecular weight of these natural allergenic proteins are mostly higher than 10 kDa. To exhibit complete antigenic properties binding of such proteins with organismic proteins is essential (Puc, 2003). In order to determine the size of protein present in *Broussonetia papyrifera* pollen, sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. As a result a 75 kDa protein was observed. Resolution of bands on gel map was not very clear which was also reported in the case of Chen *et al.*, (2013) when protein concentrations were very low. In the previous studies pollen protein allergens from 10-70 kDa were reported (Belin, 1972; Esch and Klapper, 1989; Knox and Suphioglu, 1996; D'Amato, 2000). However, Micheal *et al.*, (2013) proved the presences of wide range of protein from 6-100 kDa in the pollen extract of *Broussonetia papyrifera*. A presences of such higher molecular weight of protein indicates proteolytic stability of pollen extracts. Moreover, Chen *et al*, (2013) has identified a protein of 72 kDa as an allergen from the extract of *Broussonetia papyrifera* pollen. Therefore chances are high that even more than 70 kDa protein can also act as pollen allergen. However, further studies are required to completely certify this point.

Conclusion

From the above discussion, finally we can conclude that due to sever and highly allergenic nature, pollen allergen of *Broussonetia papyrifera* is getting importance day by day. Present studies have proven that there is a strong link between pollen of *Broussonetia papyrifera* with the spring allergies of Islamabad. As pollen of *Broussonetia papyrifera* has various structural modifications and contains highly stable protein, which adds to its allergenicity. Today there is a great need to overcome the exponentially increasing pollen allergy issue. Therefore, extensive and unified studies are required to find a pragmatic and permanent solution for these spring allergies. Further purification of active protein with higher concentrations and Immuno-screening of a cDNA expression library using patient sera can be effective strategies toward the diagnosis and vaccination against *Broussonetia papyrifera*. Moreover, a complete characterization of pollen proteins can lead to handy and effective diagnostic tools.

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