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Research Article

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Innate immunological activity of immunoglobulin Y towards *Parthenium hysterophorus* leaf proteins

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ABSTRACT

Considering the augmented importance of immunoglobulin's role in allergic reactions and the underlying mechanisms of IgY in allergic conditions, the objective of our study is to purify proteins from Parthenium hysterophorus leaves, IgY antibodies from chicken eggs and to check the immunological activity of the antibodies with Parthenium hysterophorus allergens. Phytochemical analysis of the constituents in leaves of Parthenium hysterophorus plant revealed the presence of glycosides and proteins. To elucidate which proteins have been extracted and thus could contribute to immunological activity, they were subjected to SDS-PAGE analysis by various staining methods. Later, the leaf proteins were screened for their activity against antibodies for allergic responses by using ELISA technique, which proved that they are capable of triggering immunological responses by IgY.

Keywords: *Parthenium hysterophorus*, IgY, ELISA, Ponceau S, Normal egg, Double yolk egg.

INTRODUCTION

Parthenium hysterophorus L. belonging to Asteraceae family is an insidious weed commonly known throughout the world as congress grass, bitter weed, white top, star weed, altamisa, carrot grass and the wild feverfew [1]. Chemical analysis of *Parthenium hysterophorus* has indicated that all its parts including pollen contain toxins known as sesquiterpene lactones [2]. Sesquiterpene lactones when exposed due to direct contact with the plant, with its pollen leads to allergy [3]. Phytochemical constituents play a key role in the quest to elucidate the pharmacological functions of any plant, *Parthenium hysterophorus* in particular has emerged as a key traditional plant for all its parts exhibiting antimicrobial, antihemolytic, antioxidant, cytotoxic activities [4]. *Parthenium* dermatitis is a major problem in urban and rural India where patients suffer with severe allergic rhinitis due to exposure. Control of *Parthenium* has been tried by various methods, but no single management option would be adequate to manage *parthenium*, and there is a need to integrate various management options [5]. Future distribution of the species may be limited seasonally by the inability of seed to germinate in soils of low water potential and by the inability of seedlings to establish and grow at low light intensities [6]. Studies were also conducted on symbiotic enhancement

of leaf extracts activity of *Parthenium hysterophorus* against leukemic cell lines of humans. This outcome needs to be proven *in vivo* which may then results in production of cancer vaccines for the treatment of various cancer diseases [7]. Pollens of *Parthenium hysterophorus* are reported to have *Parthenium* specific IgE and IgG antibodies [8]. Antibodies and its overall effect on health and development of resistance to the disease is therefore an important area of research. IgE [9] and IgG [10] antibodies specific for *Parthenium* pollen allergens were demonstrable in the sera of *Parthenium*-sensitive rhinitis patients. The specificity of these antibodies to *Parthenium hysterophorus* allergens was established by ELISA [11]. Because allergens in the extract must undergo frequent analysis, efforts should be made to reduce the volume of the sample for testing. One way to accomplish this is to increase the measurement sensitivity for particular target proteins. Thus, an ultrasensitive ELISA assay [12] has been carried out to check the immunological responses of plant extract towards antibodies. This study is aimed at exploring the possibility of the fresh leaf of *Parthenium* weed being allergic [13] or not to the society in addition to its characterization of extracts for antigenicity nature [14] and also its use in the development of products from *Parthenium hysterophorus* for biomedical based industries [15]. We hypothesized that immunological responses triggered by IgY are responsible for the observed differences in allergic activity. Depending on the allergen concentration, the interaction of allergen with IgY was either supported or inhibited.

EXPERIMENTAL SECTION

Leaf protein extraction

Leaf proteins were extracted by grinding leaves of the plant with a pestle and mortar in the presence of liquid nitrogen and or made into smoothie [16]. The material was transferred to a vial and 500µl of 2x IP lysis buffer (Pierce) was added and the sample was vortexed. Later, the suspension was mixed for one hour at 37^0C and filtered. The filtrate was spin at 14,000 rpm (Thermo, MicroCL 21 Microcentrifuge) in cold conditions for 12 min and supernatant was removed and stored at 4^0C until further analysis.

Phytochemical analysis of leaf smoothie

Phytochemical analysis [17] was carried out for the leaf extract for detection of alkaloids, flavonoids, phenols, carbohydrates, glycosides, terpenoids, saponins, proteins and tannins using standard procedures to assess the behaviour of *Parthenium hysterophorus* with different chemical reagents.

Extraction of IgY from chicken eggs

Antibodies were isolated from normal egg yolk and double yolk egg of chicken eggs with the use of varying concentrations of polyethylene glycol [18]. This method consists of two successive precipitations in PEG, by using 3.5% PEG to remove fatty substances, and then 12% PEG to precipitate the IgY and the samples were run on desalting column (Merck Biosciences) to remove salts and or stored at -20^oC until further use.

DEAE purification of IgY

Sample from the desalting column was further purified by using DEAE cellulose ion exchange column (BioRad, USA). Column was equilibrated with 25mM phosphate buffer pH 8.0. Sample was loaded, washed with phosphate buffer and eluted with increasing sodium chloride (0-2M) buffer. Elutions were collected with purity and analysed on 12% SDS-PAGE [19]. Protein concentrations were determined using Bio-Rad protein assay (BioRad, USA) using bovine serum albumin as a standard.

SDS-PAGE analysis of proteins

Purified proteins were suspended in a sample buffer (4% SDS, 150mMTris HCl (pH 6.7), 20% glycerol, 0.1% bromophenol blue, 1% beta-mercaptoethanol) and subjected to 12% polyacrylamide gel elctrophoresis (PAGE). All fractions (washes, elution) collected before, during and after protein elution were diluted 1:1 times with milliQ water before SDS-PAGE analysis. Puregene molecular weight markers were used according to the manufacturers' instructions. Protein samples of ~10ug/ml were loaded per lane and migrated with constant volatage at 100V for two hours. Gel bands were visualized after staining with Ezee blue direct stainer (Merck Biosciences) for one hour at room temperature [20].

Ponceau S staining

As per manufacturer’s instructions, after gel electrophoresis the nitrocellulose membrane (iBlot, Thermo) was applied upon the gel and the blotting was mediated at 50 V for one hour. Ponceau S Staining Solution (HiMedia)

was incubated with the nitrocellulose membrane for ~30 seconds and then removed. After detection the stain was removed by a number of washes in 1x TBS [21].

Determination of antigen binding towards IgY by ELISA

The 96 well plates (affymetrix eBioscience) were coated with 100µl of plant extract dilution with a concentration of 10µg/ml. Then the plate was incubated for two hours at 37°C. After washing three times with washing buffer 200µl blocking buffer were loaded into each well. The plate was incubated for one hour at room temperature. IgY extracted from column method was used as Primary antibody and HRP conjugated mouse anti-chicken-IgE antibody (Abcam, USA) was added. Subsequently, 100µl of the substrate (TMB, eBiosciences) were loaded into each well and analysed in ELISA reader at 450nm [22].

RESULTS AND DISCUSSION

Phytochemical analysis of *Parthenium hysterophorus* leaves

As indicated in the Table 1, tests were found positive for proteins and glycosides. Also, it is clear that the leaf proteins contain glycosidic bonds as the test was positive for glycosides**.** The results have shown a remarkable variation on glycosides compared to proteins. The presence of the constituents was also found to be similar to those reported for most medicinal plants.

Table 1: Phytochemical constituents in *Parthenium hysterophorus* leaves

Alkaloids	Flavonoids	Carbohydrates	Glycosides	Terpenoids	Saponins	Proteins	Tannins
-	-	-	+	-	-	+	-

SDS-PAGE analysis of leaf proteins

SDS-PAGE analysis revealed four major bands of *Parthenium* leaf extract (200ug/ml) with molecular weight 33KDa, 23KDa, 20KDa and 18KDa (Figure 1) against 5ul of protein marker (0.1mg/ml). Various fractions (flow-through, wash, elution) collected before, during, and after protein elution were diluted 1/2 with milliQ ultrapure water before SDS-PAGE and protein determination. It was noteworthy that all the four bands were less than 40 KDa which can be characterized as allergenic protein bands.

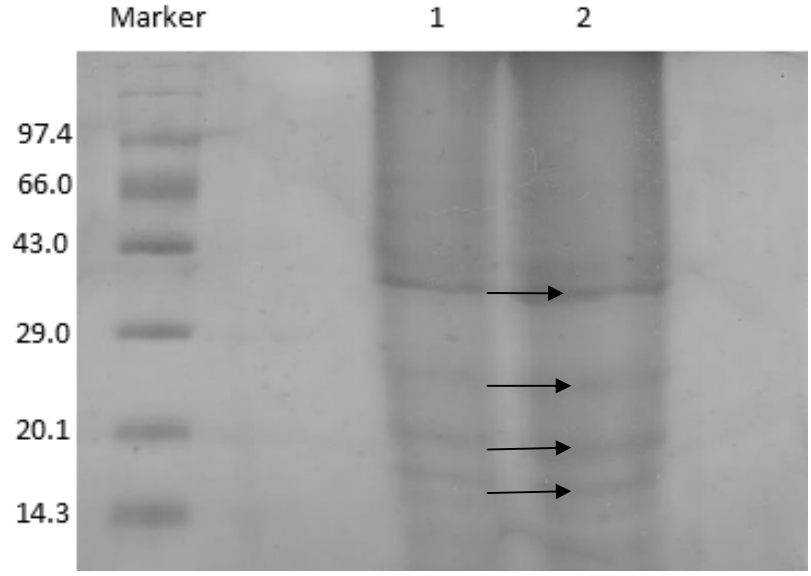

Figure 1: Analysis of the leaf proteins using SDS-PAGE: Protein marker (Puregene) along with leaf extract (lane 1 and 2) were loaded on 12% gel indicating the molecular weight of allergens as 33KDa, 23KDa, 20KDa and 18KDa respectively

SDS-PAGE analysis of IgY

SDS-PAGE analysis showed two major bands indicating the heavy chain and light chain for the purified IgY from both normal egg and double yolk egg. The bands were observed at molecular weights of ~68KDa and ~25KDa (Figure 2) indicating both heavy and light chains respectively. The control IgY (Genei, Bangalore) and the purified IgY is found similar with respect to the molecular weights of the heavy chain and light chain.

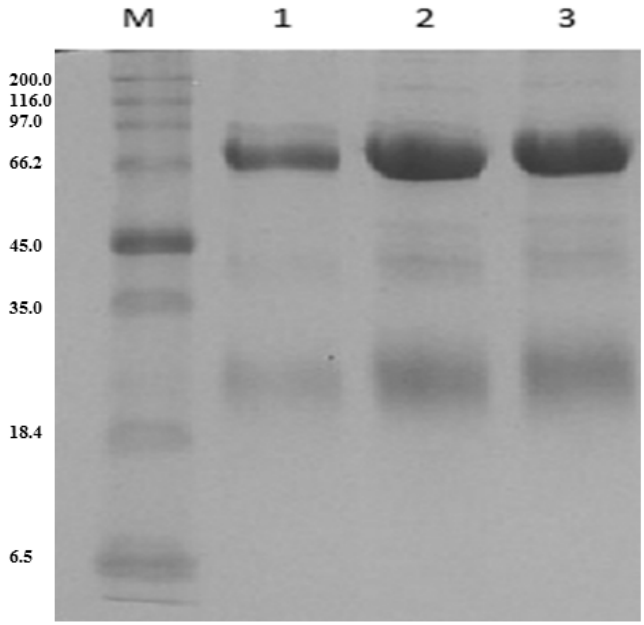

Figure 2: SDS-PAGE analysis of purified immunoglobulin Y on 12% gel after PEG purification. Samples: M — Protein marker, 1 — Control IgY (Genei); 2 — IgY extracted from normal egg; 3 — IgY extracted from double yolk egg

ELISA for IgY against antigen

ELISA experiment was performed using increasing concentration of antibodies with a constant concentration of antigen. The concentration of antibody increases from 1µg/ml to 96µg/ml and antigen at constant concentration of 10µg/ml. A complete 96 well ELISA plate was performed and at 450 nm filter the readings were noted. It has been clearly observed that IgY has maximum concentration at 50ng/ml for normal egg antibodies and 60ng/ml for double yolk antibodies. The binding assay results clearly show that protein in extract showed specific direct binding to IgY. Similarly, PBS used as a negative control showed no affinity for IgY (Figure 3).

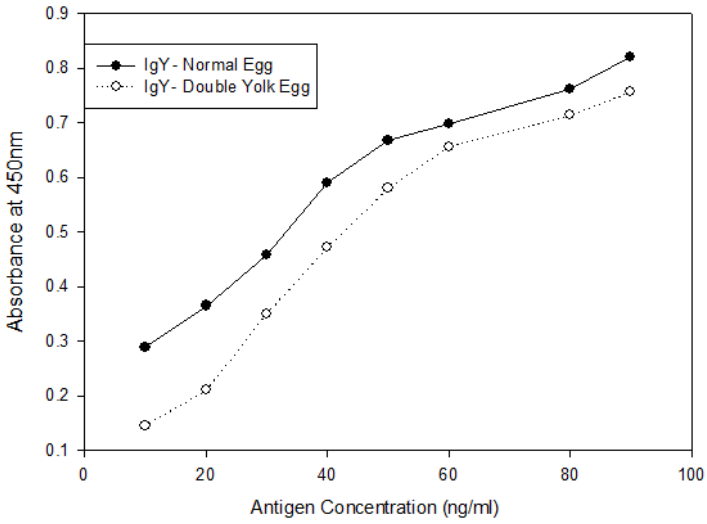

Figure 3: Activity of leaf proteins towards IgY by ELISA

Ponceau Stain of Nitrocellulose Membrane

Briefly, proteins from SDS-PAGE gel were transferred onto nitrocellulose membrane at 50V for 2hours and then stained with Ponceau red (HiMedia, Mumbai). This staining is reversible and rapid method of detecting proteins with a clear background and red protein bands. By increasing the contrast of the surface of interest (LunaPic Photo Editor), the 23KDa, 20KDa and 18KDa bands clearly appeared on the membrane in contrast to Ezee Blue stainer (Figure 4).

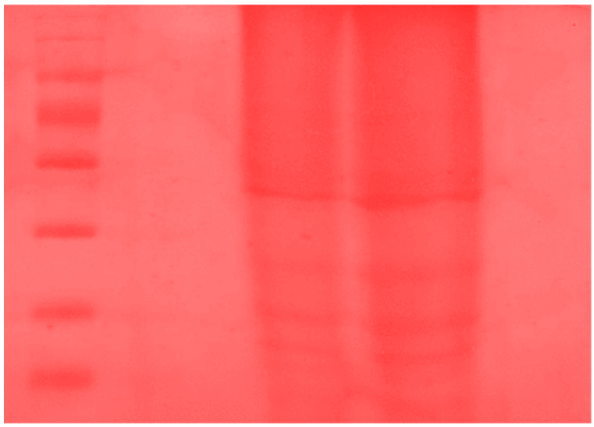

Figure 4: Ponceau S Stain of the nitrocellulose membrane

CONCLUSION

Although research has identified the *Parthenium hysterophorus* contain several important chemical constituents as anticancer [23], antioxidant [24], anti-HIV agents [25], its utility as food and fodder [26], in future it may find an important place in medicine. We have investigated the role of its allergenicity to decrease the dose exposure and the risk of developing allergic sensitization and disease. One of the major components of the plant is its leaf proteins that act as pollen and spread over to damage ecosystem and induce type I hypersensitivity. Evidence has shown that the incorporation of *Parthenium hysterophorus* plants to the soil affect the growth and yield of succeeding crops. Despite increasing evidence that *Parthenium hysterophorus* provide psychosocial and other possible health benefits or damages to the crops or society, further studies, mechanistic and epidemiological, are necessary to address the gaps in the research.

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