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Research Article

# QUANTITATIVE EVALUATION OF POLLEN PROTEIN FROM *HELIANTHUS ANNUUS* FOR HONEY BEE NUTRITION

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**ABSTRACT**

**Objective:** The objective of our study is to estimate the protein content of pollen and bee pollen (collected from honey bees) from the flowers of *Helianthus annuus* and also determined its shape, size, and granularity.

**Methods:** For estimation of protein using nanodrop method and also quantified through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, both proteins exposed to hemolymph containing hemocytes separately pertaining to determine forward (shape and size) and side (granularity) scatter using flow cytometry.

**Results:** SDS-PAGE analysis represents bands of various protein sizes, i.e., pollen (25 KDa and 34 KDa) and bee pollen (25 KDa). In contrast, the data also indicate that proteins from bee pollen showed more enhancement in forward and side scatter as compared to pollen.

**Conclusion:** Overall, the data suggest that bee pollen could be a potent candidate for nutrition to honey bees.

**Keywords:** *Helianthus annuus*, Pollen, Protein, Hemocytes, Hemolymph, Flow cytometry.

## INTRODUCTION

Honey, one of the natural products is generally synthesized from honey bees using nectar or secretions of living parts of plants [1], where the bees collect, transform by combining with enzymes or with specific substances of its own, deposit, store, and quit from honeycombs to ripen or mature [2]. Ultimately, honey is manufactured or produced either from many flowers of different species called multifloral or from single variety flower called unifloral honey [3]. There are different varieties of honey and totally varied in color, flavor, and taste, depending on the botanical source from which nectars were collected. Honey is generally used for food and specifically used as sweetener [4]. Honey is a semi-liquid product (water: 15-18% approx.). Sugars are the main constituent in the honey mainly glucose and fructose, the total content of sugars that relies on the botanical origin of honey and varied between 60 and 85% [5]. Other sugar which is already present is in traces, depending on the floral origin [6]. In addition, number of amino acids, minerals, vitamins, enzymes, organic acid, pollen, wax, and pigments are also present [7]. However, the most recent research revealed that honey displayed various immunobiological functions, i.e., anti-inflammatory; antioxidant, and antibacterial properties [8]. In addition, the practice of apitherapy using bee products such as honey, pollen, propolis, royal jelly, and bee venom to obstruct illness and encourage healing process [8].

*Helianthus annuus* (sunflower; family Asteraceae) used by Indians as food, oil, and also used it for livestock feeding [9]. In contrast, sunflowers are self-sterile but it needs seeds to produce, so it will take pollen from other sunflower, and this phenomenon is known as cross breeding or cross-pollination [10]. Normally, seeds of sunflower mainly consist of oil (36-42%) and protein meal (38%). Most of the sunflowers produce abundant pollen that unfolds the surface on which a vase of sunflowers sits. Apart from flower nutrients, pollen is the ultimate ingredient of proteins, lipids, sterols, vitamins, minerals, etc., [9,10] and also involved in colony level for jelly production by young workers and is generally used to feed larvae, queen, drones, and older workers. Therefore, a direct outcome of nutritional shortage (pollen shortage) is a decrease in the colony population and likely a deficient health of honey bees. In this study, our group evaluated the protein from pollen and bee pollen and directly exposed on hemolymph for determining its effect related to nutrition and is determined through flow cytometric analysis.

## METHODS

### Sample collection

One frame of brood and 50 foragers adult honey bees were collected from 10 colonies from Apiary, VSBT, Baramati. Pollen and bee pollen samples of *H. annuus* were collected in the month of January 2016 (total 25 g of pollen in 2 days collections from single bee hive). Collected and bee pollen was then stored at −80°C in air tight bags to avoid any contamination.

### Extraction of protein

For extraction of protein from pollen and bee pollen using Tris-HCl (pH 7.2) and acetone to remove fat contents. In this study, pollen samples were weighed and crushed in mortar and pestle using liquid nitrogen (−196°C) to prepare fine powder and dissolved in Tris-HCl (pH 7.4) and then incubate it for 10 minutes at room temperature followed by centrifugation. Thereafter, acetone solution was added in the supernatant and incubated for 20 minutes [11]. Finally, the extract was centrifuging, and supernatant was discarded and proteins settled at the bottom. The pellet was filter sterilized in autoclaved membrane filters (0.22 µm) and determined the protein content using nanodrop (sunflower, 7.04 mg/ml; mustard, 5.21 mg/ml). In addition, mineral content in pollen (Ca, 0.423; Fe, 0.96; Mg, 7.612; Mn, 0.035; Cu, 0.071; Zn, 0.530 µg/g) and bee pollen (Ca, 0.154; Fe, 0.116; Mg, 7.032; Mn, 0.017; Cu, 0.003; Zn, 0.171 µg/g) is reported by VSBT and totally varied from one another.

### Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving (15%) and stacking (4%) gels were used for isolation of protein pollen bands of flowers and honey bees. About 20 µl of protein pollen sample of flowers and bee pollen was loaded into the wells and voltage (80 Volts) was required to run the gel. After the separation of protein bands of flowers and bee pollen through electrophoresis,

staining solution was utilized to stain the gel to make bands visible. Afterward, the gel was placed into a destaining solution for 24 hrs on shaker and was changed frequently until clear gel was obtained [12].

**Hemolymph extraction**

The hemolymph of honey bees was collected after feeding different diets of protein (pollen and bee pollen), the hemolymph was collected from 3rd dorsal tergite (small incision at this site) using microcapillary tubes. The hemolymph was individually stocked in microcapillary tubes at −20°C for determination of protein concentration through nanodrop method.

**Flow cytometric analysis**

To determine the hemocytes count in hemolymph samples after feeding with protein diets of pollen and bee pollen. Incubate hemolymph samples along with protein sample of pollen and bee pollen for 2 hrs at 37°C in carbon dioxide incubator. After incubation, add FACS lysing solution and incubated for 10 minutes. Afterward, centrifuge the protein sample at 2300 rpm for 7 minutes at 4°C and washed with phosphate buffered saline (PBS) and then analyzed through flow cytometer (FACSCalibur) for estimating forward (shape and size) and side scatter (granularity of the cell) gating applied for data acquisition of 10000 events [13] of cell populations representing different phenotypes analyzed using cell quest software.

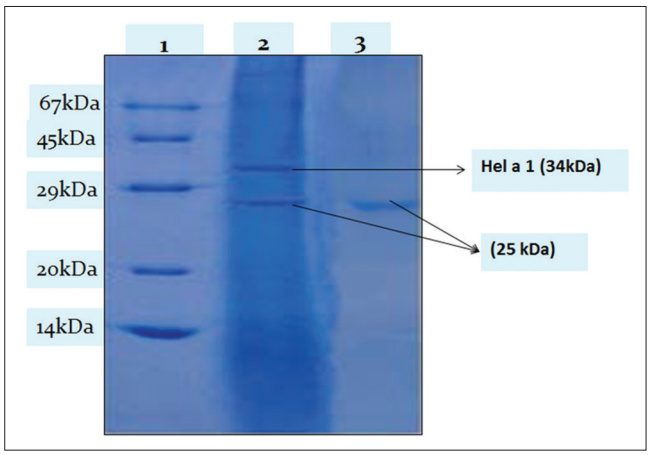


**Fig. 1: Protein profiles of pollen and bee pollen from *Helianthus annuus* and determined through sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lane 1: Marker protein solution. Lane 2: Flower pollen. Lane 3: Bee pollen**

## RESULTS

**Estimation of protein content**

For measuring the quantity of protein present in pollen, bee pollen and also determined in hemolymph after feeding with protein diets of pollen and bee pollen, using nanodrop. The quantity of protein in pollen and bee pollen was found to be 45 µg/ml and 10 µg/ml. In addition, pollen (10.48 µg/ml) and bee pollen (8.24 µg/ml) are also reported in hemolymph samples of honey bee.

**SDS-PAGE**

In SDS-PAGE, pollen proteins (Hel a1, 34 kDa, and 25 kDa) were observed, whereas, in bee pollen, only protein of 25 kDa was observed which might be due to the low protein content found in the same sample and already reported that <50 KDa that can be characterized as allergenic protein bands (Fig. 1).

**Flow cytometric analysis**

For hemocytes count in hemolymph samples of honey bees after feeding with proteins of pollen and bee pollen and then exposed to particular type of protein as shown in Fig. 2. The results showed that bee pollen proteins showed enhancement in forward and side scatter with respect to shape and size including granularity of the pollen as compared to pollen proteins (Fig. 2).

Hemocytes were incubated with proteins of pollen and bee pollen separately and then lysed and washed with PBS buffer and then finally analyzed through flow cytometer. Data acquisition of 10000 events and fraction or separation of cell populations representing forward and side scatter using cell quest software.

## DISCUSSION

The aim of this study was to evaluate the protein content from pollen and bee pollen of *H. annuus* and characterized these proteins through SDS-PAGE and determined its effect on hemolymph samples related to nutrition pertaining to measure its forward and side scatter using flow cytometry. The results of these studies showed that proteins from bee pollen support the nutritional value of honey bees and is responsible for enhancing its immunity through nutrition which is observed through exposure of these proteins from bee pollen on hemolymph

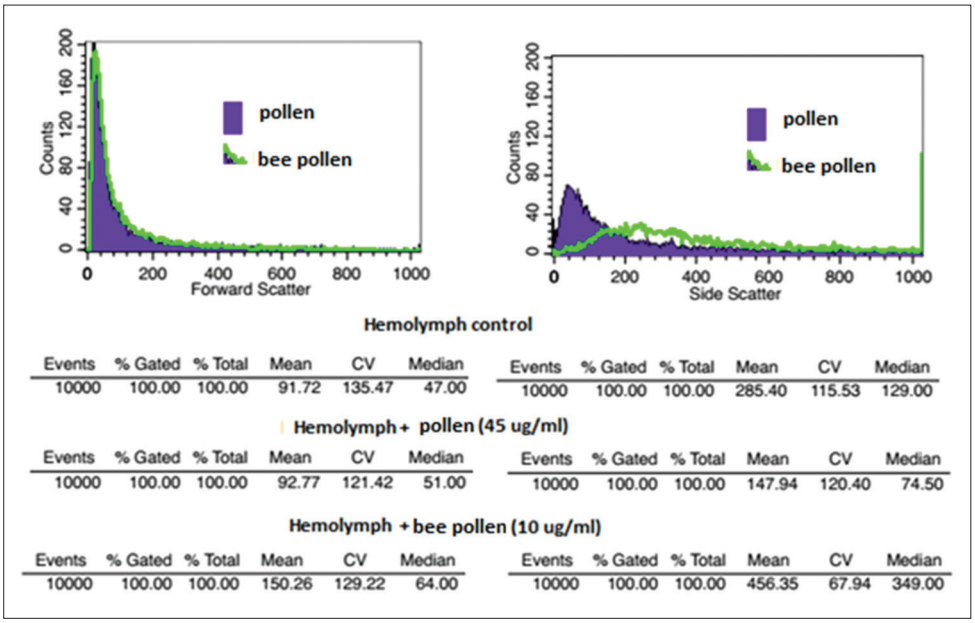


**Fig. 2: Flow cytometric analysis of protein from pollen (flower) and bee pollen using forward and side scatter**

samples (compared to proteins from flowers) and confirmed through flow cytometric analysis.

As per the literature, bees store lot of protein in their body and use it to make wings, muscles, and other body organs. Higher the amount of protein in their bee bodies, stronger the bees are and longer they can live. Hence, these proteins are vital requirement for honey bees and normally it will reduce during honey production, hot or cold weather, wax production, increase in breeding (during spring season). Indeed, if honey bees have low body protein content, they will survive only for a short period and suffered from various diseases, i.e., European brood disease, Nosema, etc., and could be poor honey producers. As per our results suggests that concentration of proteins from flowers pollen is much higher as compared to bee protein, but this bee protein enhances its immunity in the form of shape, size, and granularity of the cells which is confirmed through flow cytometry analysis. Analysis of bee protein and flowers proteins through SDS-PAGE and showed that bee pollen contained only 25 KDa that is responsible for enhancing its immunity, whereas flowers protein contained both 25 KDa and Hel 1 a(34 KDa).

The determination of protein concentration in the hemolymph of honey bees is an accurate method to evaluate the efficiency of protein diets (flower pollen and bee pollen). The data show a considerable variability in protein content in the hemolymph of bees feed on different diets (i.e., proteins extracted from flowers pollen and bee pollen) even though consumption was similar. This test is relatively inexpensive and required small quantities of inexpensive chemicals and kept only few hundred bees. In addition, identification of hemocytes present in a fluid after exposure of these proteins from pollen flower and bee pollen is measured through light scatter properties as it has been shown that there is a direct relationship between forward scattered light and cell volume and this has become common practice in flow cytometry [14,15]. The scattering of light, i.e., coherent light source (488 nm, blue) using forward scatter (small angle scattering between 0.5 and 5°C and measured its shape and size of the cell) and side scatter (large angle scattering between 15 and 150°C dark field and measured its complexity and granularity of the cell) [14,15]. In this study, the results showed that bee pollen protein showed enhancement in the level of forward and side scatter as compared to flowers protein.

In conclusion, our data show that measurements of protein content in the hemolymph of honey bees after feeding with proteins diet of flowers pollen and bee pollen and then again exposed to these proteins is a useful, rapid, practical, and accurate method for determining the protein diets for honey bees in the form of shape and size of pollen quantitatively using flow cytometry.

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