

# Anti-inflammatory Activity of Sting Protein from *Apis mellifera*

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Received: 13 January 2017/Revised: 31 January 2017/Accepted: 10 February 2017

**ABSTRACT-** The objective of our study is to determine its anti-inflammatory potential of protein extracted from the stings of honey bee (*Apis mellifera*). In this study, protein extracted from the stings of *A. mellifera* using Tris HCl/ice cold acetone and determined through Nano drop method and then determined its Da protein using SDS-PAGE. In addition, indirect ELISA was performed using the rubella vaccine as coating antigen and determined its antibody titre using variable concentration of sting protein (15.62-250 µg) and also determined its activity on human whole blood for determining total cellular content and proliferation against rubella vaccine antigen. The results showed, protein from stings of *A. mellifera* showed drastically declined in antibody titre at higher doses, but there was slightly enhanced in antibody titre, total cellular content and proliferations at lower concentration as compared to control and rubella vaccine (standard). Overall, this study suggested that stings protein by *A. mellifera* showed anti-inflammatory potential against rubella vaccine antigen.

**Key-words-** Anti-inflammatory, *Apis mellifera*, ELISA, Nanodrop, Rubella vaccine, Stings

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## INTRODUCTION

Apitherapy is considered to be one of the most important practiced in many cultures whereas bee venom therapy is used in the form of live bee stings in order to treat various diseases e.g. arthritis, multiple sclerosis, diseases of the central and peripheral nervous system, heart and blood system, skin diseases and other diseases [1-3]. In general, honeybee venom is generally produced by two glands which is associated with sting apparatus of worker and queen bees. The production of bee venom increased during first two weeks of life and reached up to maximum when they become involved in hive defence and foraging [1,4,5]. In contrast, honey bee venom showed complex variety of numerous peptides including proteins that showed strong neurotoxic and immunogenic effects.

As per the literature, most active component that is present in major amount of bee venom i.e., mellitin which showed antimicrobial activity [6,7].

Among many species of insects belongs to the Phylum *Arthropoda*, only few of them have the ability to defend themselves with a sting or venom injection during stinging [3,8]. Most of these insects that can sting which represents the members of the order *Hymenoptera* (includes ants, wasps and bees). Normally, stings are always present at the abdominal end of *Apis mellifera* and pain inflicted by *Apis mellifera*, defending its colony, is not caused by a bite, as is frequently said, but by a sting [9]. In the present study, we focused on protein extracted from the stings of *A. mellifera* and determined its immunological studies against specific protein antigen i.e. rubella vaccine.

## MATERIALS AND METHODS

### Collection of samples

Twenty five *A. mellifera* (females, worker) were collected in the month of January, 2017 from Apairy, VSBT bee hive. *A. mellifera*, workers were pressed manually using forceps and placed on glass slide. Then *A. mellifera* begin to release viscous liquid from stings and were collected in glass slide and kept in a sterile

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DOI: 10.21276/ijlssr.2017.3.2.7

condition in order to avoid contamination. Thereafter, viscous liquid was dried at room temperature for estimating protein content using Nano drop method.

### Estimation of protein content

In this study, dried sample of viscous fluid from stings was taken in eppendorf tube and then added extraction buffer (i.e. 20 mM Tris HCl) dissolved in PBS, pH 7.4. Incubate sample along with extraction buffer for 5-8 minutes (room temperature) and centrifuged (6000 rpm; 10 minutes at 4°C). The supernatant was collected and then added equal volume of ice cold acetone. Incubate the samples for 10–15 minutes at room temperature and then centrifuged. Collect the pellet and washed with PBS. Finally, protein concentration was determined by using the Nano drop method<sup>[10]</sup>.

### SDS-PAGE

In this study, we used to resolve (15%) and stacking (4%) gels for determining the sting protein bands of *A. mellifera*. For SDS-PAGE, using protein sample extracted from stings of *A. mellifera* was loaded into the wells (voltage, 50 Volts was required) and run it for 5 h. After loading the samples, separation of protein bands of sting isolated from *Apis mellifera* through electrophoresis, staining solution was required to stain the gel of sting protein in order to make bands visible. Afterwards the gel was placed into a de-staining solution for 24 h on shaker and was changed frequently until clear gel was obtained.

### ELISA

For determination of antibody (IgG) titre against Rubella vaccine (Serum Institute of India Limited, India) using variable doses of protein extracted from stings of *A. mellifera*. In this study, Elisa plates were coated overnight with Rubella vaccine (1:500 dilutions, 100 µl) in high binding 96 well plates (Himedia, India). Washed the plate with PBS and then add 1% BSA (bovine serum albumin; 100 µl) in 96 well plates. Incubated the plate for 1 h at room temperature and then again washed the plate with PBS. Thereafter, added variable concentration of protein (15.62–250 µg) and incubate it another 4 h at carbon dioxide incubator. After incubation, wash again with PBS and then added horse anti-serum (1:1000 dilutions; 100 µl) used as secondary antibody. Incubate the plate for another 1 h at carbon dioxide incubator. After incubation, trimethyl benzidine (TMB, 100 µl) substrates were added and keep it in dark for 15 minutes and then add stop solution (1N H<sub>2</sub>SO<sub>4</sub>). The optical density was measured at 450 nm<sup>[11]</sup>.

### Total cellular content

Virally infected whole blood samples (n=5) of human were collected from the Mangal pathology lab, Baramati, District Pune, Maharashtra. For the determination of total cellular content using human whole blood (100 µl) was taken in each falcon tube along with rubella vaccine (1:1000 dilution; 10 µl) and variable concentration of protein (15.62–250 µg) and incubated the blood samples containing protein and rubella vaccine for 2 h at room temperature. Thereafter, lysis (red cell lysis buffer), and washing these samples once with PBS (pH 7.4). Finally, the samples were again dissolved in PBS and centrifuged at 10,000 rpm (4°C, 10 minutes) and the supernatant was collected for estimating total cellular content using Nanodrop method<sup>[12]</sup>.

### Proliferation assay

This study was conducted by human whole blood samples (n=5; 100 µl) and exposed to rubella vaccine (1:1000 dilution, 10 µl) along with variable concentrations of protein (15.62-250 µg). Incubated these samples related to protein in 96 well plates for 24 h incubated in carbon dioxide incubator. After incubation, added MTT solution (5 mg/ml, 10 µl) and then incubated it for another 4 h at carbon dioxide incubator. Finally, centrifuging the samples after incubation, fresh formazan crystals were settled at the bottom and these crystals were dissolved in dimethyl sulphoxide (DMSO) in a final volume of 0.2 ml. The optical density (OD) was measured at 570 nm<sup>[13]</sup>.

### Statistical analysis

The difference between control and variable doses of protein extracted from viscous fluid of stings from *A. mellifera* was determined through one way ANOVA test (Bonferroni multiple comparison test).

## RESULTS

### Estimation of protein

Immunobiological studies of protein extracted from the stings of *A. mellifera* shows protein content (10 µl, 0.978 mg/ml), which was determined through Nano Drop as shown in Fig. 1 and its protein in the form of Dalton (Da), which was confirmed through SDS-PAGE and its range between 2050–2810.

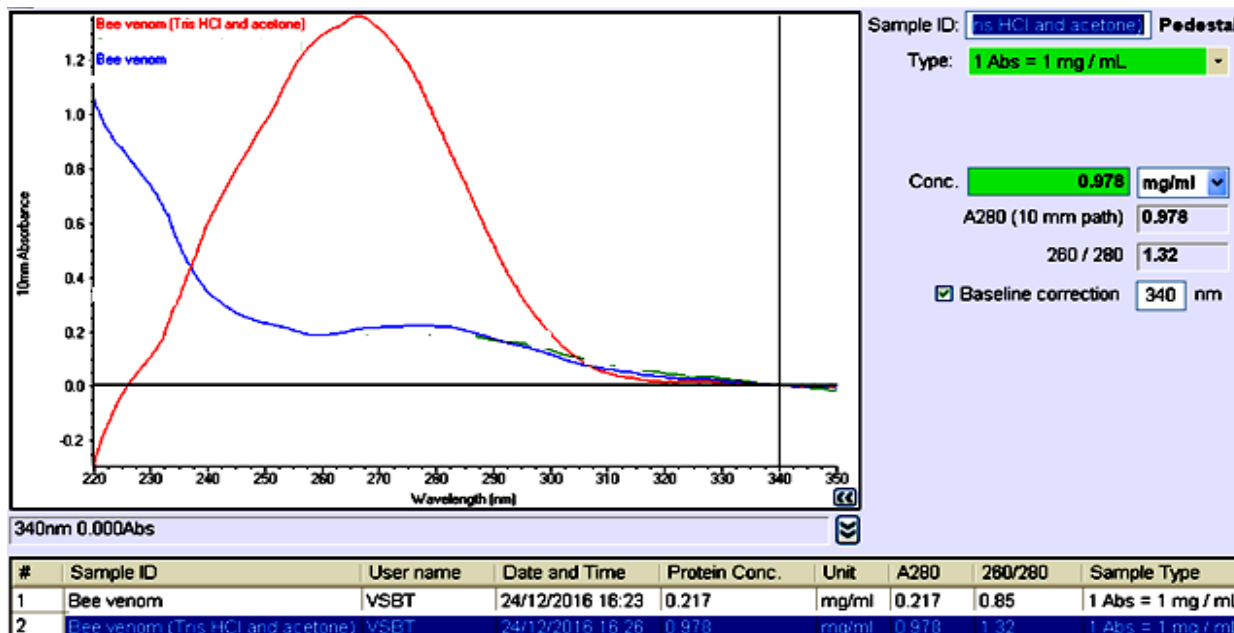


Fig. 1: Estimation of protein content from viscous fluid (Bee venom) of stings from *Apis mellifera*

**ELISA**

The results of these studies related to anti-rubella antibody titre using variable concentration of protein (15.62–250 µg) as shown in Fig. 2. The results showed that protein declined

in antibody production at higher doses as compared to rubella vaccine control. In other words, protein from stings could be a potent anti-inflammatory agent.

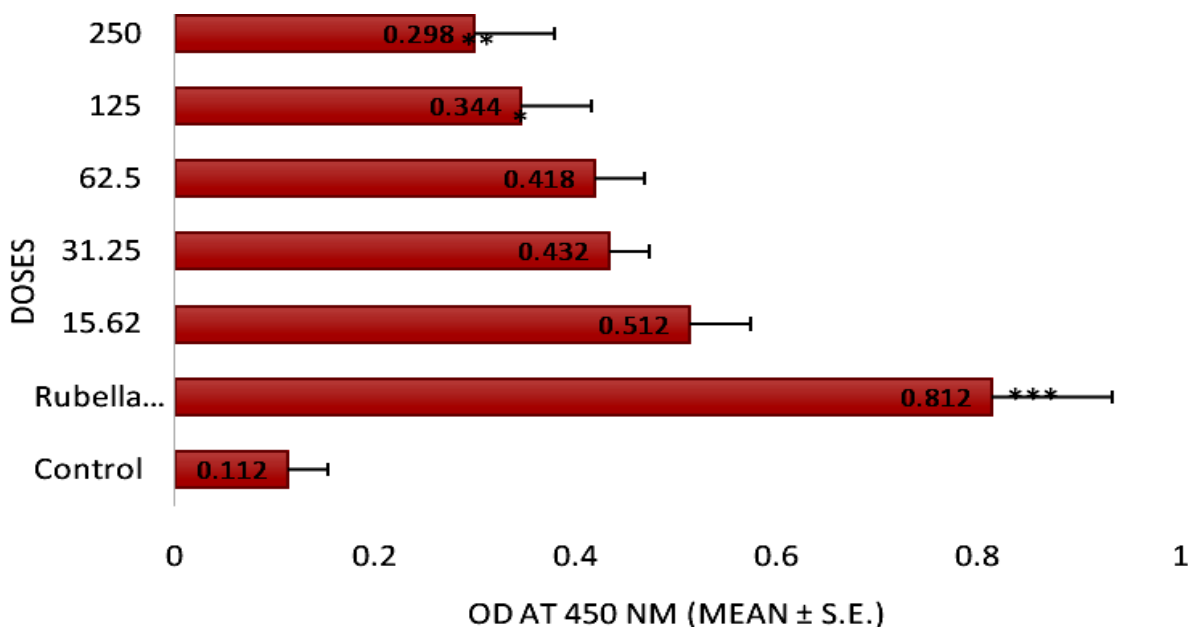
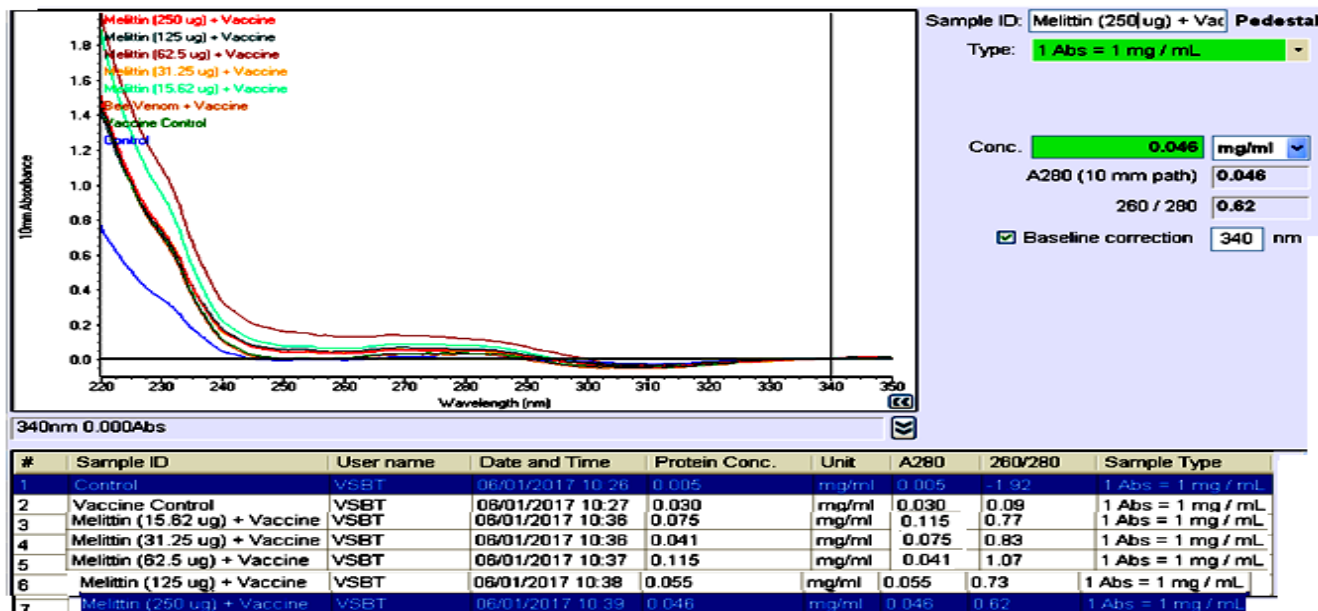


Fig. 2: ELISA assay. Indirect ELISA was assayed using rubella vaccine as coating antigen using variable doses of sting protein for determining antibody titre. Horse anti-serum used as secondary antibody. The difference between control and variable doses of protein determined through one way ANOVA test (Bonferroni multiple comparison test) \*p< 0.05; \*\*p< 0.01 and \*\*\*p< 0.001

### Total cellular content

Immunobiological studies were conducted in order to estimate total cellular content in lysed human whole blood using variable concentration of protein (15.62–250 µg) in

presence of rubella vaccine as shown in Fig. 3. The results showed that protein showed declined in total cellular content at higher doses as compared to rubella vaccine control.

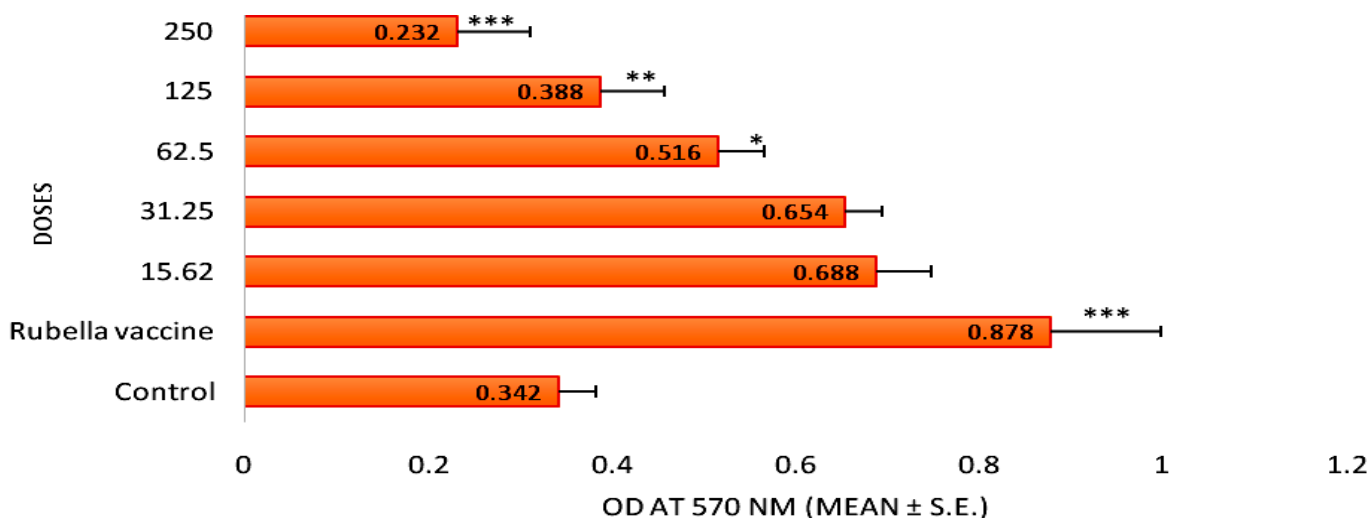


**Fig. 3:** Estimation of total cellular content in human whole blood. Lysed human whole blood was cultured with variable concentration of sting protein (melittin) in presence of rubella vaccine. After incubation, lysis and washing (PBS, pH 7.4) the samples after centrifugation at speed (10000 rpm) for estimating total cellular content using Nanodrop method

### Proliferation assay

The effect of variable concentration of protein (15.62–250 µg) extracted from the stings of *Apis mellifera* stimulated proliferative response in lysed human whole blood along with rubella vaccine as shown in Fig. 4. The results show

that there was dose dependent decline in proliferation as compared to rubella vaccine control. Overall, the data indicates that protein from stings of *A. mellifera* inhibits T cell proliferation.



**Fig. 4:** Proliferation assay. Lysed human whole blood was cultured with variable concentration of sting protein in presence of rubella vaccine. After incubation, centrifuge the samples and add MTT solution (5 mg/ml, 10 µl). Fresh formazan crystals were appeared and settled at the bottom and then finally dissolved in dimethyl sulphoxide (DMSO) in a final volume of 0.2 ml. The optical density (OD) was measured at 570 nm.

The difference between control and variable doses of protein determined through one way ANOVA test (Bonferroni multiple comparison test) \*p< 0.05; \*\*p< 0.01, and \*\*\*p< 0.001

## DISCUSSION

Inflammation (means tissue injury or complex cascade of nonspecific events) is generally triggered by innate and adaptive immune systems in order to maintain homeostasis. The term inflammation is usually classified or described in the form of acute and chronic inflammation. These two types of inflammation are usually classified on the basis of cell types that take part in the inflammatory response. One of the major causes of acute inflammation i.e. microbial infections, hypersensitivity reactions, chemicals etc. in contrast, if some agent causing acute inflammation and is not properly removed or treated, it may progress to the chronic stage of inflammation. In other words, the physiological response to tissue injury (infection, surgery, radiation, etc.) involves both local and systemic reactions. Normally, inflammatory responses are responsible to maintain homeostasis condition and allows for tissue healing, whereas chronic inflammation leads to multiple organ dysfunctions. So, there is an intimate relationship between the mechanism of inflammation and the immune system response [14,15].

As per the literature, bee venom extracted from the stings of *A. mellifera* has been widely used medicinally in Europe for the treatment of rheumatic diseases. During the ancient period, healers have practiced apitherapy with respect to honeybee products that are used for various curative purposes [2-6]. As per the literature, researchers focused or explored the potential of bee venom extracted from stings for treating a wide variety of conditions from acute tendonitis to chronic back pain to rheumatoid arthritis [1]. In the present study, we focused on protein extracted from stings and determined its immunological studies against specific protein antigen i.e. rubella vaccine.

In the present study, we determined its immunological effect of protein from the stings on rubella vaccine using human whole blood and showed that protein at higher doses showed anti-inflammatory effect against rubella vaccine antigen. This vaccine is used as standard for these studies for determining T cell activation or proliferation using variable concentration of sting protein. As per the literature, this protein i.e. melittin has the capability to reduce the inflammation rate at higher doses and also showed anti-inflammatory effect at lower doses [16]. Similar types of results were also observed and shown anti-inflammatory effect. A lot of research work was related to melittin protein and showed several anti-inflammatory mechanisms in different types of disease models. Similarly, bee venom and its melittin with respect to anti-arthritis mechanism which directly targets in order to inactivate NF- $\kappa$ B through direct binding of p50 subunit [17].

As per the literature, *in vitro* assays were already performed and revealed the potential of melittin as an effective agent for the prevention of various neurodegenerative diseases. In addition, melittin also showed potent immunosuppressive effect with respect to pro-inflammatory responses of BV2 microglia and claimed that melittin may have potential to treat various neurodegenerative diseases, which was

accompanied with microglial activation [18]. Lot of research work related to melittin protein had already done and considered as allergenic peptide (major constituent of apitoxin) which is responsible for cell lysis including cell death [19].

Studies were also conducted in order to determine its proliferation assay and total cellular content of human whole blood using rubella vaccine. The results showed that sting protein showed drastic decline in rubella vaccine proliferation and total cellular content at higher doses as compared to control. In other words, sting protein showed cytotoxic effect against rubella vaccine using human whole blood in a dose-dependent manner. Overall, the data showed that protein from stings showed anti-inflammatory effect against rubella vaccine antigen.

## CONCLUSIONS

In this study, our result showed that sting protein should be possibly used for anti-inflammatory agents only if careful provisions or supervision are taken in order to avoid adverse effects. In our next study, we tried to determine or explore or synthesized protein derivatives for developing novel pharmaceutical agents.

The future therapeutic application of melittin on inflammatory disorders will be depended on new study protocols to validate the efficiency and safety of sting protein.

## AUTHORS CONTRIBUTION

This work was carried out in the collaboration between five authors. Amit Gupta designed the study, wrote the protocol and interpreted the data where SS anchored the field study, gathered the initial data related to his M.Sc Microbiology dissertation work under Amit Gupta guidance and performed preliminary data analysis. Amit Gupta, Sumesh Shah, Shweta P Karne, Sanjay Kamble, and Bharat Shinde managed the literature searches whereas AG and SS produced the initial draft. The final manuscript has been read and approved by all authors.

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**How to cite this article:**

Shah S, Gupta A, Karne SP, Kamble S, Shinde B: Anti-inflammatory Activity of Sting Protein from *Apis mellifera*. *Int. J. Life Sci. Scienti. Res.*, 2017; 3(2): 914-919. DOI:10.21276/ijlssr.2017.3.2.7

**Source of Financial Support:** Nil, **Conflict of interest:** Nil