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Comparison of proteins in two honey samples from *Apis* and stingless bee

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Abstract
Proteins are present in honey at low concentrations. The protein content varies depending on the plant species in which bees consume nectar and the species that produce it. Here the paper reports the comparative analysis of proteins in undiluted and diluted honey produced by *Apis* and stingless bees. A total of two and three proteins were produced after SDS- PAGE separation from both *Apis* and stingless bee honey. Protein bands with a molecular weight of 53.2 kDa and 65.5 k Da were common in both samples of honey.

Keywords: *Apis cerana*, *Apis* honey, Stingless bee honey, acetone

1. Introduction
Honey is one of the oldest traditional medicines and continues to be used in the treatment of several human ailments [1, 2]. It is a natural sweet substance produced by honey bees from nectar of plants. Honey is renowned for its wound healing properties since ancient times [3]. It acts as antibacterial, anti-viral, anti-inflammatory, anti-cancer, anti-diabetic and anti-oxidant agent. Honey is also used as laxative, blood purifier and it reduces the risk of heart disease and protects the liver against oxidative damage. This property of honey is due to the bioactive substances it contains [4]. Reports showed that honey from *Apis* species contains approximately 200 substances. Mainly honey consists of simple sugars such as fructose and glucose [5]. Minor components include proteins, amino acids, organic acids, vitamins, minerals and phenolic compounds. The composition of honey varies depending upon factors like honeybee species, floral origins, climate and environmental conditions, processing time and storage temperature [6].
Other than *Apis*, honey produced by stingless bees is of high medicinal value. The demands for stingless bee honey compared to *Apis* honey is increasing nowadays because of their antimicrobial, anti-cancer, anti-inflammatory and wound healing properties [6]. Both types of honey are different in terms of its color, viscosity and taste [7]. The quality of honey is a necessary criterion for using honey as medicine. It can be characterized by different parameters like moisture content, acidity, diastase number, amount of hydroxymethylfurfural, sugar profile and protein content. Proteins are the minor components of honey on which limited studies are available i.e., typically 0.1% to 0.5% [8]. *Apis mellifera* honey contains 0.2%- 1.6% proteins and *Apis cerana* honey contains 0.1% to 3.3% proteins [9]. White [2] states that honey contains 0.2% proteins which include enzymes and 18 free amino acids [10]. Bogdanov [11] reported that concentration of proteins in honey varies depending on the botanical, geographical origin and storage time. It ranges from 0.2 to 0.4 mg/100 g. Saravana and Mandal [12] found 0.5 to 1% protein in honey. Lee *et al*, [13] and Hermosin *et al*, [14] claimed that proteins in honey belongs to plant or bee origin. Some proteins are in the form of enzymes which are introduced by honeybees from their hypopharyngeal and salivary gland during honey processing [15]. Several proteins are also in the form of enzymes contained in pollen and nectar origin. One of the major proteins, MRJP1 with molecular weight of 55 kDa originates from hypopharyngeal gland [16].
Extraction of proteins from honey is difficult because of a sugary rich environment and the presence of pollen. Honey diluted with water is better for isolating the proteins present than undiluted honey because of its viscous nature. Several other physical and chemical methods are used for separating proteins. Centrifugation is the most commonly used physical method of protein separation. It involves the use of mechanical forces to separate proteins from honey and dialysis is used to remove small molecular weight compounds such as sugars. Chemical extraction involves the use of phosphotungstic acid, ammonium sulphate [17], alcohol [18] and TCA [19]. Both physical and chemical precipitation methods can also applied to determine the protein content of honey [20]. Kathireswari *et al*., [21] reported the precipitation of proteins using acetone and ethanol and examined the effects of antibacterial properties of honey proteins.
The current study compared the proteins in honey from *Apis* and stingless bee in both diluted and undiluted form by using acetone as precipitating agent.

2. Materials and Methods
2.1 Sample collection
Honey samples from *Apis* and stingless bee were collected from an Apiary at Trivandrum. It was harvested during the months of January to March, the period of honey production in Kerala. All the samples were stored at room temperature in air-tight glass bottles until analysis.

2.2 Protein Profiling

Sample preparation

Diluted and undiluted honey of both *Apis* and stingless bee were used for extracting protein. For the preparation of diluted samples, the honey was mixed with equal volume of distilled water to get a uniform suspension.

2.3 Protein precipitation

Proteins in honey were precipitated using acetone. One volume of honey was taken in a centrifuge tube and mixed with 4 mL of ice-cold acetone. The mixture was vortexed thoroughly and incubated in -20 ˚C deep freezer for 2 h. The tubes were centrifuged at 8,000 rpm at 4 ˚C for 10 min. The pellet obtained was air dried for 30 min and re-solubilised in 0.2 M phosphate buffer and kept at 4 ˚C.

2.4 Protein quantification

The protein concentration of all the samples after precipitation was determined according to the method of Lowry [22]. BSA was used as the standard and the absorbance was read at 620 nm in a UV/VIS spectrophotometer.

SDS-Page

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was preformed according to the method of Laemmli [23]. Honey proteins were resolved on 12% separation gel with 5% stacking gel. When the bromophenol blue in the loading sample reached the lower end of the gel, it was removed and transferred to staining tray with Coomassie brilliant blue. The gel was kept in the staining tray overnight. The stained gel was destained for about 1-2 h and the process was repeated until the bands in the gel were clear, photographed and analyzed in a transilluminator.

3. Results and Discussion

3.1 Protein profiling

The honey samples from *Apis* and stingless bee were used in both diluted and undiluted form for determining its protein content. Acetone was used for precipitating proteins from both the samples. The samples obtained after precipitation were quantified by Lowry's estimation. Protein content of pure undiluted and diluted *Apis* honey was 0.76 and 0.9 mg/ml respectively and that of stingless honey were 1.9 and 2.3 mg/ml. This indicates that diluted sample is good for better yield of proteins than undiluted samples. But after precipitation the protein yield of the undiluted and diluted honey samples from *Apis* honey were found to be of 7.1 and 10.9 mg/ml respectively and that of honey of stingless bee were 8.0 mg/mL and 11.5 mg/mL respectively. The present study indicates that acetone was more effective in extracting the protein from diluted sample than from undiluted sample of *Apis* and stingless bee. When compared with *Apis* honey, stingless bee honey showed higher protein content.

SDS-Page

The protein precipitates obtained after precipitation were analysed by SDS PAGE (Figure 1). In *Apis* honey, a protein band of molecular weight 66 kDa and 53.2 kDa was observed in diluted sample (Lane III) and a band of molecular weight 53.2 kDa (Lane I), was observed in undiluted sample. The protein band of molecular weight 53.2 kDa was found to be common in both diluted and undiluted samples of *Apis* honey. In stingless bee honey, one protein band was observed from undiluted honey (Lane II) with a molecular weight of 65.5 kDa. However the molecular weight of proteins from diluted stingless bee honey (Lane IV) was found to be 65.5 kDa, 93.5 kDa and 53.2 kDa. A protein band of molecular weight 65.5 kDa appeared in both diluted and undiluted samples of stingless honey. In the protein profile of both the *Apis* and Stingless samples, two bands were common with molecular weights of 53.2 kDa and 65.5 kDa.

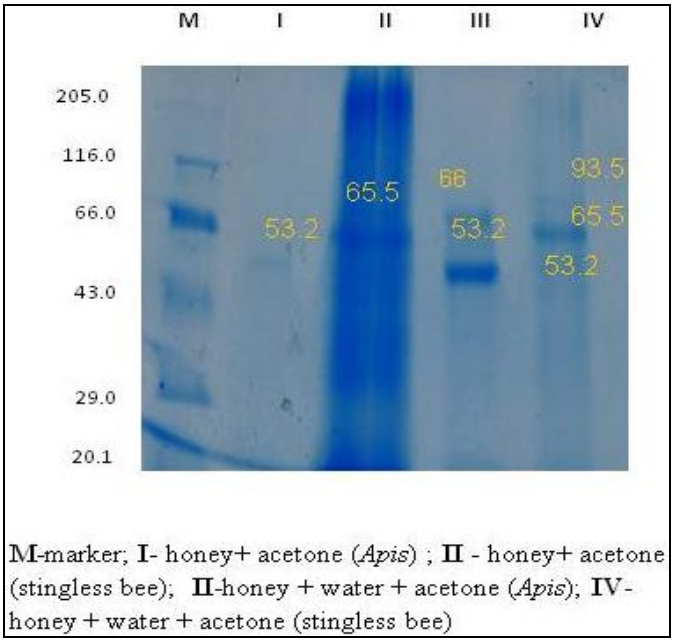

Fig 1: protein profile of *Apis* and stingless bee honey sample precipitated using acetone

Proteins of honey are honeybee specific and do not seems to be replaced by other components or ingredients. Previous work suggested that these proteins originate from plant on which the bees forage or honeybees themselves and the amount depend on the species of honeybees as well as environmental factors [24]. The present results showed that the yield and number of proteins obtained were higher when the samples were diluted with water. This is because of the uniform distribution of its components and decrease in viscosity of the honey when diluted with water. Undiluted samples did not show protein precipitation that much probably because of sugar rich environment. In conclusion samples diluted with water become precipitate with high yield of protein using acetone as precipitation agent.

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