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Quantification of Aflatoxins in Raw and Processed *Apis* Honey

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Abstract: ***Honey is a very interesting, attractive, valuable and nutritive product. Honey can be considered to be predominantly made of sugars, accounting for 95-97% of the dry matter content. The important components of honey are also proteins, organic acids, vitamins and amino acids. The honey composition varies depending on local flora, it may be characterized by source or kind. Honey, which can be a focus of the clinical trials studying the treatment of variety of diseases is a functional food. This study deals with the significance of honey quality standards and in vitro evaluation of biological properties of honey specifically aspects of further problems related to thermal treatments or improper storage conditions – the formation of so called "after treatments" – the aflatoxins. In this investigation, raw and processed (total 45) Apis honey samples were taken from Coorg, Karnataka for the determination of concentration of aflatoxins by HPLC-fluorescence detection method. Raw honey samples investigated were found to be contaminated with aflatoxins more than processed honey samples.***

Keywords: Apis honey, raw, processed, aflatoxins

1. Introduction

Plants, microorganisms and insects are traditional sources of natural products containing pharmacologically active compounds, particularly novel antimicrobial drugs, as they are still needed to tackle antimicrobial resistance problem (Atanasov et al., 2021). Honey is a complex mixture formed by honeybees in which a wide variety of valuable nutrients can be found, such as carbohydrates (mainly fructose and glucose), proteins, minerals, vitamins, organic acids, enzymes, polyphenols and flavonoids which account for the antioxidant, anti-inflammatory and antimicrobial activity of honey (Rodríguez et al. 2012). Honey is a highly valued food product by consumers and also has definite organoleptic properties and nutritional and medicinal values which are used in confectionary, pharmaceutical and cosmetic industries. Since ancient times, honeybee products have been used in traditional/folk medicine for remedies. Honey the most thoroughly investigated and applied product of bees, was not only explored as a sweetener but also as an appealing functional food (Berenbaum & Calla, 2021). Numerous clinical studies have been conducted on its health benefits in preventing and treating different diseases (Majtan et al., 2021). The composition of the honeys depends on the flora of the region in which the bees are active, and allows for the characterization of the honey according to the source or type (Machado et al., 2020). Pollution of honey by environmental pollution is a difficult issue that should be well addressed as it impacts quality and causes deterioration that may have potential health hazards (Al-Waili et al., 2012). Honey may be contaminated either from primary or secondary sources, i.e. from pollen, floral nectar, dust and during extraction or processing. The microorganisms, bacteria, yeasts and fungi counts have been accepted as an important indicator of the hygienic conditions for the quality of the honey, many cases of infant botulism have been related to contaminated honey (Ananias et al., 2013; Herrero-Latorre et al., 2017, Syraji, et al., 2025). The observed variability is due to the ambient environmental conditions, pre- and post-processing of natural products and their storage conditions. Synergic and poly-pharmacological effects of active ingredients is one of the major benefits of natural products. But it is hard to demonstrate these experimentally (David et al., 2015). Conversely, one of the main drawbacks to the therapeutic evaluation of natural products is the low and inconsistent levels of bioactive compounds they contain. Moreover, the lack of quality measurement parameters in correlation with its pharmacological activity, often results in a limited availability of natural product based medicinal products. Functional foods, which are industrially processed or raw natural foods with potentially positive health effects, thus form the largest group of natural foods (Granato et al., 2020). Food functionality is a concept that is dependent on the activity of food ingredients with specific biological properties, such as anti-oxidant, anti-inflammatory and anti-bacterial activities. These properties can be easily measured by in vitro test, however, the complexity of the physiological and biochemical process in the human body is not taken into consideration (Alongi et al., 2021). Therefore, preliminary in vitro screening of the biological activities of functional foods should be accompanied by in vivo testing in animal model and thus characterize the metabolic pathways driven by bioactive molecules in certain diseases.

The initial critical point in the pre-clinical and clinical development process of functional foods is to assess their in vitro bio-functionalities associated with a particular disease. Certain of the bioactive compounds present in functional foods like polyphenols and proteins/enzymes are prone to degradation and loss of bioactivity due to poor stability and sensitivity to heat and light (Cao et al., 2021). Functionality of bioactive molecules could be negatively affected by industrial processing and extended storage of functional foods.

Honey was used in a considerable number of pre-clinical and clinical trials as a topical application on several types of wounds. Due to the good clinical results obtained with the topically applied honey, so-called 'medical-grade honey' was developed. Medical honeys are produced from certain types of honey (medical grade) that are sterilized by gamma irradiation as medical devices (Hermanns et al., 2020). For medical-grade honey, strict requirements have to be fulfilled to ensure its safety and quality, and a thorough testing process has to be performed (Hermanns et al., 2020). But the vast amount of Global Honey is table honey, which does not require to be medical grade honey and does not require to possess strict criteria. At present, the quality criteria for honey are based on international standards (European Honey Directive, EU Directive, 2002 and Codex Alimentarius, 2001). However, other legislative parameters do not take into account the bioactive properties of honey that make it a functional food (Majtan et al., 2021). A recent survey conducted in the Balkans and Western European countries with honey consumers showed that the primary motivation for honey consumption is the health benefits (Kleisiari et. al., 2023). Despite the assertions that honey is a functional food, the clinical study findings are somewhat inconsistent and controversial; and it can be challenging to come to broad conclusions. Previously carried out clinical trials have several drawbacks, and one of the most neglected factors is the quality and functioning of the honey tested (Deglovic et al., 2022).

The present investigations aim to provide perspectives and scientific opinions on honey as a therapeutic agent and to stimulate discussion about the quality issue with honey used in clinical studies. Moreover, general recommendations for the conditions of honey production and storage are offered, where the biological function of honey is preserved during storage.

2. Materials and Methods

Collection and Preparation of honey samples

Forty-five Apis honey samples were collected from the beekeepers and honey hunters of Coorg, Karnataka during 2025-26, were designated as raw honey. The raw sample honey was filtered by using single thickness fine cloth which removed suspended particles including debris, beeswax etc. The raw honey, indirectly heated for the period of 1-3 hrs at the temperature of 60º C-70º C was considered as processed honey. Raw and processed honey samples were placed in sterilized polythene bottles, and stored in the dark at room temperature (Mukti et al., 2019).

The physicochemical parameters of honey were measured using established methods as follows: Optical Density (OD):

- Measured according to EI Sohaimy *et al.* (2015) method.
- Viscosity: Determined using Ostwald's viscometer based on the Akoh (1991) method. pH: Measured using a digital pH meter by Manam *et al.* (2021) method.
- Moisture Content: Assessed using a refractometer by modified method of Ajitha Nath et al. (2019). Ash Content: Determined using the Ivanov and Cherrenakova (1984) method.
- Aflatoxins: Estimation by HPLC Fluorescence detection method Nilüfer and Boyaciog˘ lu (2002) and Environmental Toxicology and Chemistry (2020).

3. Results and Discussions

Table 1: Physico-chemical Characteristics of Raw Apis Honey Samples of Coorg (Mean ± SD)

Types of Honey	Optical Density	Viscosity (Ns/m²)	pH	Moisture (%)	Total Ash (%)
Apis florea	0.048±0.002	8.9±0.2	3.7±0.2	12.7±0.1	0.22±0.01
Apis mellifera	0.19±0.005	9.4±0.3	4.9±0.3	14.9±0.2	0.38±0.03
Apis cerana	0.37±0.001	11.6±0.9	5.1±0.1	16.3±0.3	0.43±0.06
Apis dorsata	0.41±0.004	12.4±0.6	5.7±0.1	15.9±0.2	0.59±0.01

The figure 1& 2 represents the physicochemical characteristics of raw and processed *Apis* honey samples from Coorg. These characteristics include pH levels and optical density, which are indicative of honey's acidity and color respectively.

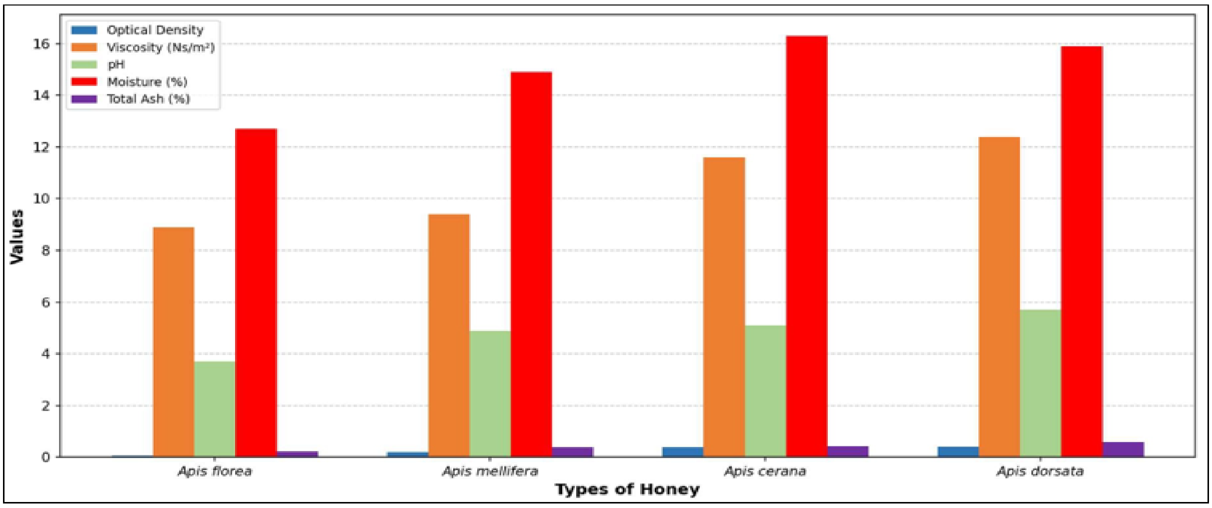


Figure 1: Physico- Chemical Characteristics of Raw Apis Honey (Coorg)

Table 2: Physico-chemical Characteristics of Processed Apis Honey Samples of Coorg (Mean ± SD)

Types of Honey	Optical Density	Viscosity (Ns/m^2)	pH	Moisture (%)	Total Ash (%)
Apis florea	0.053±0.005	9.4±0.8	4.6±0.7	10.2±0.02	0.26±0.04
Apis mellifera	0.28±0.003	10.6±0.2	5.1±0.7	11.5±0.07	0.39±0.03
Apis cerana	0.46±0.006	12.3±0.4	5.7±0.2	12.4±0.01	0.49±0.04
Apis dorsata	0.59±0.001	14.1±0.1	5.9±0.3	13.4±0.06	0.61±0.08

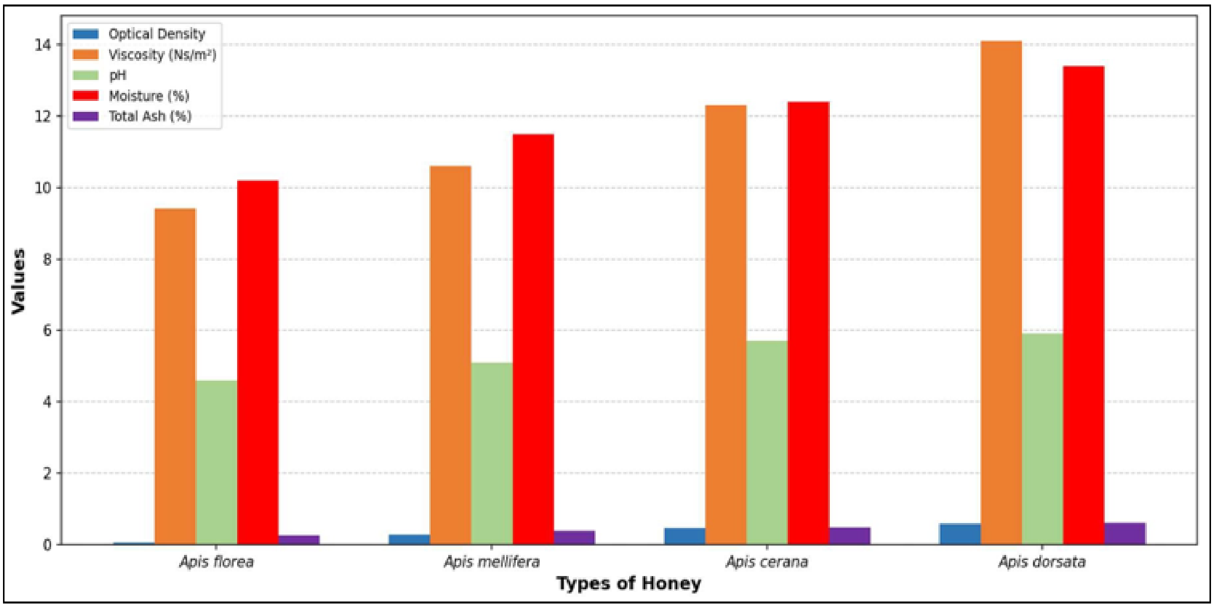


Figure 2: Physico- Chemical Characteristics of Processed Apis Honey (Coorg)

The optical density (OD at an absorbance at 560nm) of raw *A. dorsata* honey was 0.41±0.004, and *A. florea* honey was 0.048±0.02 (Fig.1). In processed honey, the range found was 0.053±0.005 and 0.59±0.001 for *A. florea* and *A. dorsata* samples (Fig. 2). The ANOVA for OD level of the honey produced by Coorg species of Honeybee was significant at 0.5%. The obtained colour of honey in this analysis was in the range of White and Domer (1980) defined as the colour of honey. Samples of lighter and fresh honey have a lower OD than those of stored and dark samples of honey. In general, consumers prefer lighter kinds of honey over darker jars of honey (Wakhle, 1997). The rise of OD values might be due to the crystallisation and granulation of saccharides during storage.

The viscosity values of the raw *Apis* honey studied in this work varied between 8.9±0.2 to 12.4±0.6 Ns/m2 (Fig. 1). The range of processed honey was 9.4±0.8 and 14.1±0.1 Ns/m2 for *A. florea* and *A. dorsata*, respectively (Fig. 2). Costa et al. (2013) reported similar results in terms of viscosity range with no more significant difference. Moreti et al. (2009) found the lowest deviation in the central region of Rio Grande do Norte, Brazil of 0.49 to 6.65 Pa.s for honey samples harvested. A viscosity between 0.36-6.77 Pa.s was recorded by Sodré et al. (2011). Honey viscosity depends on the types of nectar. Some of the main reasons for the viscosity of honey are its high sugar content (White et al., 1988). Viscosity is the measurement of a fluid's resistance to flow that is caused by the internal friction of the fluid, which means that the liquid flows at different rates in various parts of it. Honey samples are less dense at high temperatures. Samples of summer honey were more dense than those of autumn honey. The higher the viscosity of the honey the greater the problems during storage, processing and straining.

The pH of the collected raw honeys from Coorg (Karnataka) region was found to be different. The honey collected from *Apis dorsata* had the highest pH of 5.8±0.3 while the honey collected from *Apis florea* had the lowest pH of 3.5±0.1. For the processed honey, the pH of *Apis dorsata* was found to be 5.9±0.3 and the lowest pH was 4.6±0.7 for *Apis florea* honey. The low pH value of *Apis* honey has an inhibitory effect on the growth of microorganisms and contributes to the texture and stability of the honey and extends the shelf life. Besides, low pH is another factor that gives honey antimicrobial activity, making it a good substitute for other antimicrobial agents (Abou Kubaa et al., 2020; Brudzynski, 2021; Mărgăoan et al., 2021; Martinello & Mutinelli, 2021).

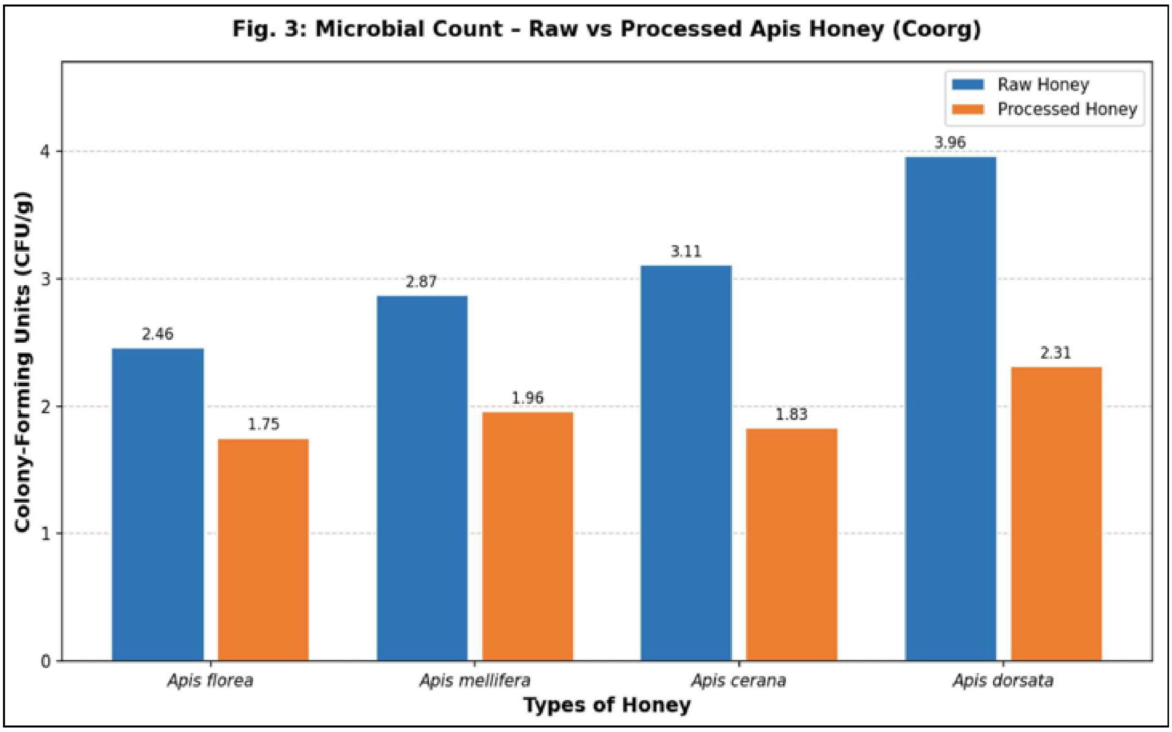
The percentage of moisture content of raw *Apis* honey samples recorded was from 12.7±0.1 to 16.3±0.3%. *Apis florea* samples showed the lowest range (12.7±0.1 %) while *Apis cerana* showed the highest range (16.3±0.3 %) (Fig. 1). The range of processed honey samples of *A. florea* and *A. dorsata* was 10.2±0.02 and 13.4±0.06 percent respectively. Because of its hygroscopic properties, when the surrounding has water, the honey will quickly absorb it. Thus, direct contact with the air will cause honey to be more moisturized. Honey's high moisture content can cause fermentation and hasten the honey's deterioration. According to the FAO (2021) the honey moisture content should be <22 % whereas SI (International Standards) allow the moisture content of honey to be <19 % (Brudzynski, 2021; Mărgăoan et al., 2021; Martinello & Mutinelli, 2021).

The average moisture level of the honey samples examined was 12–13%. It was found that raw and fresh honey samples collected from the beekeepers of various regions of Kashmir Valley had moisture contents of 18.60 and 18.20 percent, respectively, while the moisture content in pine honeydew, multifloral honey and *Acacia* honey was 19.11 percent (Nayik & Nanda, 2015; Meena et al., 2022). The moisture content of the honeydew, floral, and fir honeys was 15.20, 17.12 g/100 g (Jafar et al., 2017). From 2010 to 2013, honey samples were collected from local beekeepers in 4 different locations of Greece and when analyzed, the moisture content was found to be 15.40 - 18.59 g/100 g (Karabagias et al., 2017). Later, in 2018, there was a report of honey of different moisture levels (16.00-21.80 percent) harvested from different parts of the Southwest of Kef, Tunisia containing Medicago, Salvia, Rosmarinus, Calluna, Lavandula, Robinia, and Citrus (Jilani et al., 2018). Their low moisture also was confirmed by the absence of sugar granules in the samples during the different analyses. Although there were differences in values, all "honey samples from the studied areas were within the range of the internationally acceptable values (Mărgăoan et al., 2021).

There was qualitative relationship between the different botanical regions of the honey and the variability of total ash content. A fundamental need is to be able to recognize the botanical origin of the honey possibly. The quantity of ash in the honey after carbonization will be directly correlated to the quantity of inorganic residues in the honey. In the study areas where the honeys are produced, the difference in total ash content of the honey samples was attributed to the different types of flowers from where they were collected (Vit et al., 1998). All the honey samples analyzed in this study were within the range recommended by Bogdanov (<0.65%) (2008). The range of raw honey was 0.22±0.01 % (*Apis florea)* to 0.59±0.01 (*Apis dorsata*). It showed that the processed honey of the different *Apis* species ranged from 0.26±0.04 (*Apis florea)* to 0.61±0.08 (*Apis dorsata*) percent (Fig. 1). Ash contents were found to be similar to those of Kahraman et al. (2010) and Adenekan et al. (2010). A similar approximate value was found by Great Iruoghene Edo et al., (2022) where 0.58 ± 0.15% was obtained in fresh honey samples with an average value of 0.17percent. The honey samples' ash content ranged from 0.014 to 0.31g (Tesfaye et al., 2016). The ash content of all the honeys examined ranged from 0.01 to 1.2% according to the Quality and Standards Authority of Ethiopia (QSAE) (2005).

Table 3: Microbial Count – Raw vs Processed Apis Honey (Colony-forming units per gram ±SD)

Types of Honey	Raw Honey (CFU/g ±SD)	Processed Honey (CFU/g ± SD)
Apis florea	2.46±0.032	1.75±0.012
Apis mellifera	2.87±0.033	1.96±0.011
Apis cerana	3.11±0.092	1.83±0.002
Apis dorsata	3.96±0.005	2.31±0.151



As a result of the testing of honey samples for fungal contamination, the presence of aflatoxins is predicted. Table.3 and Fig.3 show the data relating to various levels of total aflatoxins present in the Apis honey samples of processed and raw. Data on aflatoxin concentration (CFU/g) is shown as Mean ±SD. Multiple-comparisons one-way analyses of variance (where $p<0.05$ was statistically significant) were performed using Duncan's test and the IBM SPSS Statistics 23 software program to analyze the data.

Raw and processed raw honey samples revealed the results of aflatoxins in raw honey samples were contaminated with these aflatoxins. Apis dorsata raw honey samples had the highest value of 3.96±0.005 (CFU/ g±SD) while Apis florea processed honey samples had the least value of 1.75±0.012(CFU/ g±SD). The results here confirm the important importance of honey quality and safety assessment prior to approval of use for honey market. With this in mind, it can be noted that the antioxidant activity of the honey samples can, at least to a certain extent, be a remedy for the toxicity of aflatoxins as environmental poison (Sereia et al., 2017; Odebode and Adekunle 2019; Jia et al., 2020; Revainera et al., 2020, Stroka et al., 2000; Syraji, et al., 2025).

4. Conclusions

In recent decades the use of honey has grown in popularity because of its potential therapeutic and pharmacological activities. Microorganisms can also be present in honey as a result of its contact with plant material or from the environment, soil, beekeeping and equipment that the bees utilize. These microorganisms have the potential to multiply, modify the product in an undesirable way, and compromise the safety of the honey, therefore it is imperative to control the physicochemical and microbiological parameters of honey. However, the components of the honey (carbohydrate, hydrogen peroxide, polyphenols, etc.) have antimicrobial properties and honey itself is safe from microbiological point of view. A detailed analysis of the chemical composition of the honey makes it possible to estimate the concentrations of the antibacterial components of the honey, which act synergistically.

Raw or minimally processed honey is a high-quality honey offering several nutritional and health preventative benefits but could be microbiologically contaminated due to yeasts, some anaerobic bacteria, *Clostridium botulinum*, and molds with their toxin products. To prevent fermentation, delay crystallization and remove impurities from raw honey, it is necessary to heat, filter and remove moisture. It is difficult to preserve its nutritional values and health properties and enhance the products attractiveness during processing.

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