

Original Article

## ANALYSIS AND CHEMICAL PROFILING OF HONEY USING <sup>1</sup>H-NMR SPECTROSCOPY, FTIR SPECTROSCOPY AND TLC USING VARIOUS CHROMOGENIC REAGENTS FOR DERIVATIZATION

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### ABSTRACT

**Objective:** Honey is a natural sweet substance known for various health benefits and is used in many traditional medicines and dietary supplements. It contains various bioactive constituents like sugars, amino acids, polyphenols, flavonoids and various minerals. Quality control of honey is an essential part for ensuring its health benefits and therapeutic usage. In the present study, honey was analyzed by using various spectroscopic approaches and physicochemical methods.

**Methods:** The samples of honey were analyzed by Thin-layer Chromatography (TLC) derivatization, ATR-FTIR, and <sup>1</sup>H-NMR fingerprint and the total phenolic content (TPC) and total flavonoid contents (TFC) were measured by Uv-Vis spectrophotometry and analysis were carried out for various physicochemical parameters of honey.

**Results:** All the physicochemical parameters of the honey were as per the desired quality. The UV-Vis analysis was successfully used in the determination of total phenolics and flavonoid contents in the samples of honey. TLC analysis showed the presence of flavonoids, phytosterols, phenolics, sugars and carbohydrates in honey. The Thin-Layer chromatography analysis showed good resolution for various components of honey on the TLC plates. The FTIR analysis showed the presence of various functional groups characteristic of amino acids, carbohydrates and sugars, which was further supported by <sup>1</sup>H NMR chemical profiling.

**Conclusion:** In the present work, the application of various spectroscopic techniques and physicochemical tests were found to be useful in analysis of honey.

**Keywords:** ATR-FTIR, Fingerprint, <sup>1</sup>H-NMR, Honey analysis, Quality control, TLC

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### INTRODUCTION

Honey, commonly known as *Madhu*, is a naturally occurring light yellow to yellowish-brown fluid with a pleasant flavour and sweet taste. It is produced by honeybees like (*Apis mellifera*, *Apis florea*, *Apis cerana indica*, *Apis dorsata*) through enzymatic transformation of floral nectar collected by them. Honey is widely used in many Ayurvedic formulations such as *Asava*, *Arishtam*, *Leham* and various herbal syrup formulations for its medicinal properties and natural sweetener [1]. Honey is traditionally used for gastric troubles, wound healing, skin burn and ulcers. Honey has been reported for various medicinal properties like anti-inflammatory, anti-viral, anti-bacterial, antiseptic, wound healing, antioxidant and immunomodulatory properties [2-7]. Modern pharmacological study has recommended the therapeutic application of honey in wound healing, bone health, metabolic diseases like obesity, diabetes mellitus, dyslipidaemia, and hypertension. Honey has been reported to work on various signalling pathways like inhibition of various enzymes such as COX, LOX, i-NOS, IL-6, TNF- $\alpha$  and NF- $\kappa$ B [8-11]. The composition, color, flavour, and aroma of the honey varies with kind of floral origin, geographical area and season [12]. In general, the honey consist of mainly 75 to 80% monosaccharide like glucose and fructose, 10 to 15% disaccharide sugars such as sucrose and maltose and a wide range of phenolic compounds like *p*-coumaric, gallic acid, caffeic and ferulic acids; compounds like apigenin, luteolin, naringenin, quercetin, kaempferol, chrysin; enzymes like-invertase, glucose oxidase, and diastase; minerals, vitamins like A, B1, B2, B3, B5, B6 and C, minerals and trace elements such as calcium, copper, iron, phosphorous, magnesium, sulphur, manganese, iodine, sodium and potassium. These substances provided various pharmacological activity and nutritive properties to honey [2, 13].

In recent years the demand of honey has increased as a dietary supplement or functional food because of increased concern and awareness about immune health and natural sweetener. The global

market of honey as per a 2021 was USD 8.52 Billion dollar and under rapid growth ([www.grandviewresearch.com](http://www.grandviewresearch.com)). The increase in the consumer demand of honey has led to an increase in price and adulteration of natural honey [14]. Therefore to ensure the authentic quality of honey, there is a need for standardization and stringent quality control to ensure high-quality and safe products to consumers. In the present study, physicochemical studies, <sup>1</sup>H-NMR profiling, FTIR and TLC fingerprinting, qualitative analysis using various chromogenic TLC reagents was carried out for honey samples.

### MATERIALS AND METHODS

The samples of honey were collected from a honey farmer in Tamil Nadu involved in apiculture. Total three batches of samples of honey were collected to study the batch-to-batch analysis. The samples were filtered through a 100-micron nylon filter to remove any extraneous particulate matter. All the chemical and reagent used in analysis were of analytical grades.

#### Physicochemical evaluation

Moisture content was calculated using loss on drying method as per the pharmacopeia procedure. The overall quality and shelf life of honey depend on the moisture content. Higher moisture led to spoilage due to fermentation caused by yeast. All three honey were having a moisture content less than 20%. The pH of honey was determined with a digital pH meter. The pH of honey affects the taste of honey and high pH leads to the formation of hydroxy-methyl-furfural in honey during long storage time and exposure to high heat. Total reducing sugar, sucrose and fructose was estimated using the titration method as mentioned in the pharmacopoeia. Hydroxymethyl furfural (HMF) content was measured by carrying out Fiehe's test. Presence of a high level of HMF is a result of the degradation of fructose by long storage time at high temperature. The test is based on reaction between pet ether extract

of honey and resorcinol solution. Appearance of cherry red colour indicates positive reaction and pink to yellow color indicates negative reaction. To check the presence of commercial invert sugar aniline chloride test was carried out. Appearance of dark red colour indicates positive reaction while the appearance of yellow color indicates negative reaction [15]. Preliminary phytochemical analysis of honey was performed for detection of various constituents like alkaloids, carbohydrate, sugar test, flavonoids test, and phytosterols [16-18].

#### UV spectroscopy analysis

The spectroscopic fingerprinting technique has been used as a novel technique for understanding the chemical composition of various herbal ingredients without the previous chromatography purification of chemical constituents. In the present study the UV/Vis spectra were recorded with a spectrophotometer (UV-1700 Shimadzu model). The spectra were recorded between 200 to 800 nm using an aqueous solution (100 mg/ml) of all honey samples. No reference cited for the method followed.

#### Total phenolic and flavonoid content

The amount of total phenolic compound contents were determined by the Folin-Ciocalteu method as described by Woisky and Salatino [19]. The honey sample (0.5 ml of 1 gm/ml of honey solution) were mixed with 4 ml of Folin-Ciocalteu reagent (Sigma-Aldrich) and 6 ml of 20% sodium bicarbonate solution and the volume was adjusted to 50 ml with distilled water. The absorbance was measured at 760 nm after 2 h of incubation at room temperature using a UV-Visible spectrophotometer. The results were expressed as gallic acid equivalents. Calibration curve was obtained from absorbance recorded from gallic acid solution at a concentration of 10 to 50 µg/ml dissolved in methanol.

Total flavonoids were estimated using the method of Woisky and Salatino [19]. To 1 ml of honey sample solution (1 gm/ml of honey solution) 1 ml of 5% AlCl<sub>3</sub> ethanol solution was added. After 1 h incubation at room temperature, the absorbance was measured at 425 nm. Total flavonoid contents were calculated as quercetin equivalents from a calibration curve. Calibration curve was obtained from absorbance recorded from quercetin solution at a concentration of 20 to 100 µg/ml dissolved in methanol.

#### Thin-layer chromatography analysis

Thin-layer chromatography is a popular method for analysis of complex herbal mixtures in natural products. It helps in speed analysis by separation and visualization of the individual components on the TLC plates [21, 22]. For each samples, 10 g of honey was dissolved in 100 ml of water. The sample was then subjected to liquid-liquid partitioning (3×10 ml) with *n*-butanol. The *n*-butanol soluble organic fraction was separated, pooled and dried over a water bath to get a brown-colored sticky extract. The resultant extract was then dissolved in 5 ml of methanol and used

for TLC analysis. The samples were applied on pre-coated silica gel 60F<sub>254</sub>TLC plates (Merck-Sigma Aldrich) of uniform thickness (0.2 mm). The chromatogram was developed in a pre-saturated twin-trough CAMAG TLC chamber with a mobile phase, toluene: ethyl acetate: formic acid (6:4:0.5). Visualization was done after spraying with anisaldehyde-sulphuric acid and vanillin-sulphuric acid chromogenic reagent. The TLC plate was observed under UV 254 and 366 nm in CAMAG TLC visualizer. Sugar analysis was carried out by dissolving 200 mg of honey in water: methanol (1:1) mixture. The solution was applied on TLC developed with a mobile phase consist of chloroform: methanol: water: formic acid (6:4:0.2:0.1) along with standards sugars of sucrose, glucose, fructose and maltose. Visualization was done after spraying with diphenylamine-aniline-phosphoric acid reagent followed by heating at 105 °C for 5 to 10 min till the colored zones are developed [20-25].

#### Fourier transform infrared attenuated total reflectance (FTIR-ATR) analysis

The FTIR spectra were recorded in ATR mode using Jasco-FTIR-4700 instrument paired with ATR-PRO-ONE accessory. The scanning was performed from 4000 to 500 cm<sup>-1</sup> and the data was analysed using JASCO software.

#### Proton-nuclear magnetic resonance (<sup>1</sup>H-NMR) analysis

In recent times the <sup>1</sup>H-NMR has been used for analysis of various food materials like fruit juice, wine and honey. The <sup>1</sup>H-NMR spectroscopy is a simple, rapid and reproducible technique to generate the fingerprint of honey in less than 30 min. Proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectra can be a holistic approach that can analyse several chemical constituents simultaneously [26]. As the <sup>1</sup>H-NMR profiling is unique for each sample, so can be used as a fingerprint. For <sup>1</sup>H-NMR analysis 50 mg of sample was dissolved in 900 ml of deuterium oxide (D<sub>2</sub>O); the <sup>1</sup>H-NMR spectra of honey was acquired in Bruker Avance III HD Nanobay 400 MHz FT-NMR spectrometer.

## RESULTS

#### Physicochemical parameter evaluation

All the parameters of honey were evaluated as per the official procedures (table 1). As per Codex Alimentarius Standard, 2001, the HMF level should not exceed more than 80 mg/kg. All three samples demonstrate pink color for Fiehe's test, indicating absence of HMF, inferring samples were within the desired specification. In general pH of honey is mildly acidic within accepted range of 3.50 to 5.50. All the tested honey had pH values within the above range. All the honey was having moisture not more than 20 % as stated by Codex Alimentarius Standard, 2001. The percentage of total reducing sugars was within the required specification (not less than 60g/100g) as recommended by CAS. The other sugar parameters, such as sucrose content and the fructose-glucose ratio, was within the CAS specifications [27].

Table 1: Physicochemical evaluation of honey

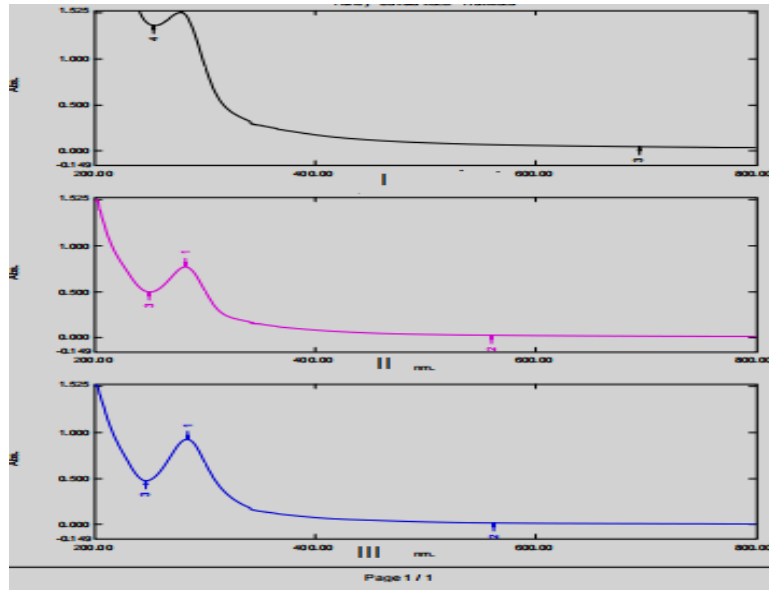
S. No.	Parameters	Sample-I	Sample-II	Sample-III
1	pH	3.52	3.57	3.89
2	Moisture value	14.66	13.29	15.21
3	Ash value	0.43	0.32	0.51
4	Specific gravity	1.4151	1.4214	1.3881
5	Refractive index	1.4740	1.4895	1.4870
6	Fiehe's test	Negative	Negative	Negative
7	Aniline chloride Test	Negative	Negative	Negative
8	Sucrose	3.82	3.87	3.78
9	Total reducing sugar	77.60	79.85	78.76
10	Fructose-glucose ratio	1.02	1.04	0.98

#### UV spectroscopy analysis of honey

The qualitative UV-Vis spectrum of honey showed bands at 284 nm, 282 nm and 278 nm with absorption of 0.930, 0.773, and 1.509. This characteristic pattern of the fingerprint of the honey can be used as a quality control tool for the analysis of honey and the detection of any adulteration.

#### Total phenolic and flavonoid content of honey

Total phenolic content was found to be 450.476, 815.556 and 844.127 µg per gram of honey samples I, II and III, respectively expressed as Gallic acid equivalent. Total flavonoid content was found to be 342.72, 147.27 and 142.72 µg per gram of samples I, II and III, respectively expressed as quercetin equivalent.

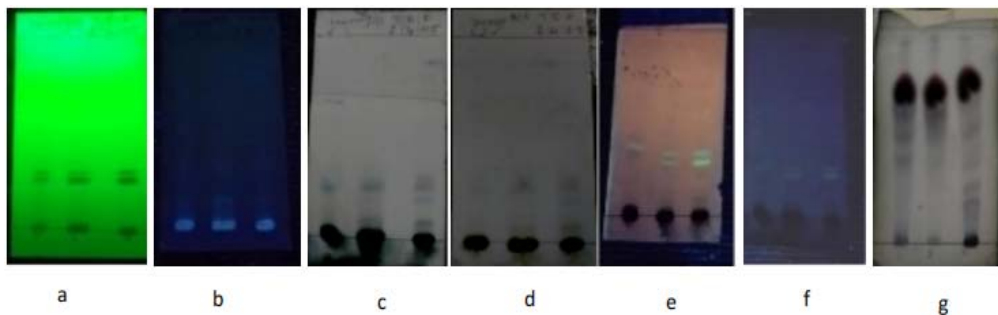


**Fig. 1: UV spectra of honey**

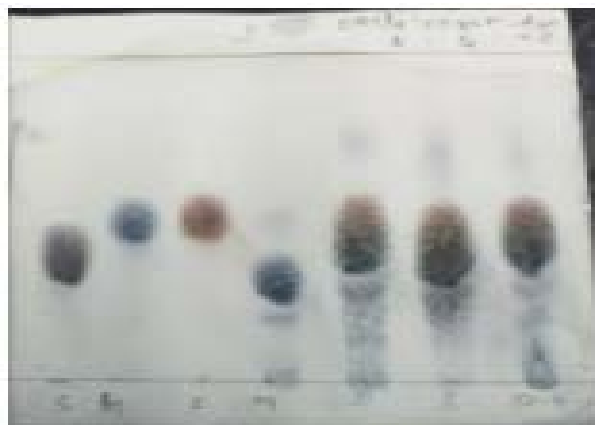
**Thin-layer chromatography analysis**

The TLC profile of all three honey samples (fig. 1) showed two distinct bands at  $R_f$  0.21 and 0.22. With derivatization with vanillin, sulphuric acid showed brown, pink and grey spots, while derivatization with anisaldehyde sulphuric acid showed green and pink spots. Derivatization with vanillin-sulphuric acids showed characteristic

yellowish fluorescent color at  $R_f$  0.24 and 0.25 under 366 post-derivatization. Sample-III showed a different profile few additional spots, only  $R_f$  0.11 and 0.18, indicating a different quality. Spraying with diphenylamine-aniline-phosphoric acid showed grey spots for sucrose, blue spots for glucose and maltose, and brown spots for fructose. All three samples of honey (fig. 3) confirm the presence of sucrose, glucose, fructose and maltose at  $R_f$  0.38, 0.48, 0.51 and 0.32.



**Fig. 2: Chromatographic profile by TLC of sample: a. 254 nm; b.366 nm; c. VS; d. AS; 1e. Under 366 after VS derivatization; 1f. Under 366 after AS derivatization**



**Fig. 3: Chromatographic profile by TLC for sugar analysis (Track-1: sucrose, Track-2: glucose, Track-3: fructose, Track-4:maltose, Track-5 Honey sample I, Track-6:Honey sample II and Track-7: Honey sample III)**

**Table 2: Phytochemicals in honey**

Constituents	Test	Sample-I	Sample-II	Sample-III
Alkaloids	Mayer's test	-ve	-ve	-ve
	Wagener test	-ve	-ve	-ve
	Dragendorf test	-ve	-ve	-ve
Carbohydrate	Molish test	+ve	+ve	+ve
	Benedicts test	+ve	+ve	+ve
Sugar test		++	++	++
Flavonoids test	AlCl <sub>3</sub> Test	+ve	+ve	+ve
Phytosterol Test	Liebermann-Burchard	+ve	+ve	+ve

**Phytochemical analysis of honey**

The preliminary phytochemical analysis of honey showed various constituents such as carbohydrates, sugar, flavonoids and phytosterols. etc. The details of the analysis are presented in table 2.

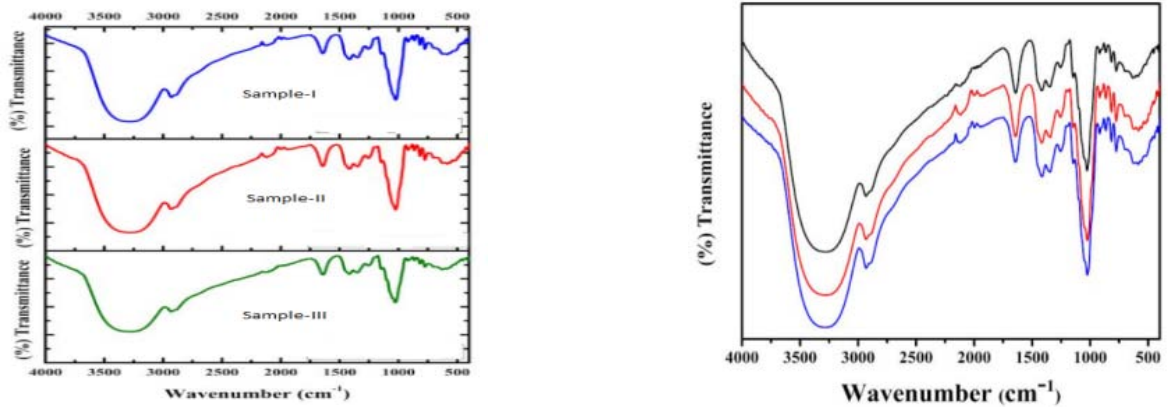
**FTIR-ATR analysis**

The FTIR analysis showed the peaks (fig. 4) at  $\nu_{max}$  3274.54, 2930.31, 1996.93, 1644.02, 1415.49, 1346.07, 1256.40, 1145.51, 1024.02, 917.95, 864.70, 816.70, 775.24  $cm^{-1}$ . All three honey sample were found to be having similar spectral fingerprints. It indicates the

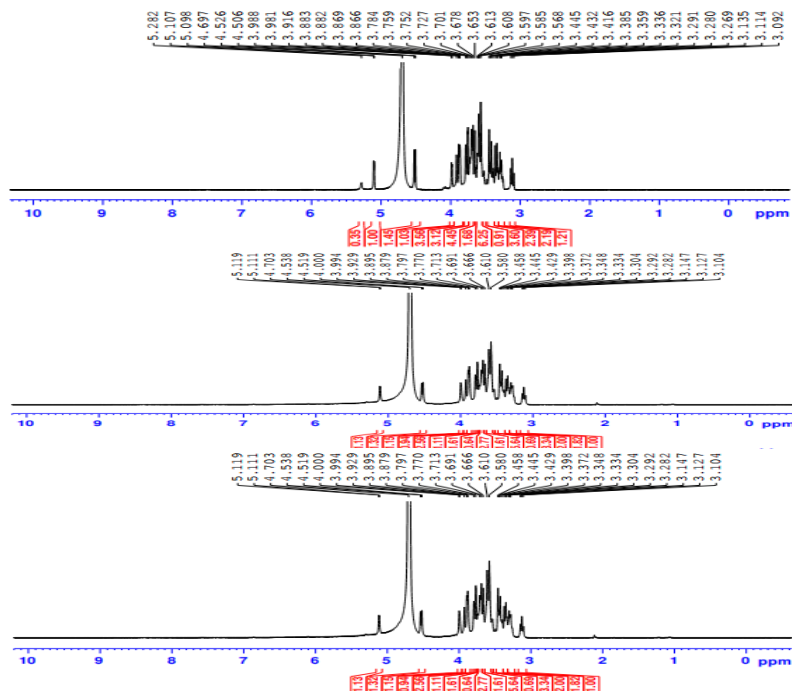
presence of O-H, C=O, C-O, C-C, C-O-C, C-OH, and O-H. On the basis of the reported literature presence of amino acids, carbohydrate and carboxylic acid-containing compounds were identified in honey by comparison with the literature data [28].

**<sup>1</sup>H-NMR spectral analysis**

The <sup>1</sup>H-NMR analysis is an advance technique to obtain holistic information about the complex sample in a single non-destructive experiment. It helps in the simultaneous analysis of multiples organic compounds present in a known sample. The <sup>1</sup>H-NMR spectral data (fig. 5) of the honey is summarized below.



**Fig. 4: ATR-FTIR spectra for the honey samples**



**Fig. 5: <sup>1</sup>H NMR spectra of honey samples in D<sub>2</sub>O**

**Sample I** (<sup>1</sup>H-NMR, 400MHz, D<sub>2</sub>O, δ<sub>H</sub> in ppm):

5.28, 5.10, 4.69, 4.52, 3.98, 3.91, 3.88, 3.86, 3.78, 3.75, 3.72, 3.67, 3.65, 3.61, 3.59, 3.56, 3.44, 3.41, 3.38, 3.35, 3.32, 3.29, 3.26, 3.13, 3.11.

**Sample-II** (<sup>1</sup>H-NMR, 400MHz, D<sub>2</sub>O, δ<sub>H</sub> in ppm):

5.11, 4.53, 3.99, 3.92, 3.87, 3.79, 3.77, 3.71, 3.69, 3.66, 3.58, 3.45, 3.42, 3.39, 3.37, 3.34, 3.30, 3.29, 3.28, 3.14, 3.12

**Sample-III** (<sup>1</sup>H-NMR, 400MHz, D<sub>2</sub>O, δ<sub>H</sub> in ppm):

5.10, 4.69, 4.52, 3.98, 3.91, 3.87, 3.78, 3.72, 3.69, 3.67, 3.61, 3.59, 3.56, 3.53, 3.47, 3.44, 3.41, 3.39, 3.35, 3.33, 3.31, 3.29, 3.27, 3.26, 3.13.

All the honey samples revealed similar spectral fingerprints, indicating the similarities in the major chemical compositions. The intense <sup>1</sup>H-NMR spectra shows dominant signals between 3.0 to 4.2 ppm representing the sugar region. The spectral region between 5.30 to 4.50 was due to the anomeric protons of sugars. The δ<sub>H</sub> values were comparable with that reported for monosaccharides like glucose, fructose and disaccharides like maltose and sucrose. The compounds were identified based on their spectroscopy data and on comparison with the published literature source [29-31].

**DISCUSSION**

The use of Thin-Layer Chromatography (TLC) coupled with use of various chromogenic reagents for derivatization have been found to be a useful approach in analysis of honey composition like sugars and polyphenolic compounds [23-25]. Three different derivatization system (anisaldehyde-sulphuric acid, vanillin-sulphuric acid and diphenylamine-aniline-phosphoric acid) were developed for analysis of low polar n-butanol fraction of honey and direct honey for its sugar components respectively. The derivatization with diphenylamine-aniline-phosphoric acid facilitates visualization of different types of sugar constituents present in honey. Further it has been observed honey on exposure to heat, undergoes degradation leading to the formation of various by-products of sugar like hydroxymethylfurfural [25]. The Fiech's test was found to be a simple and quick test for qualitative analysis of HMF in honey. The FTIR fingerprint analysis was found to be a simple, rapid and comparatively less expensive approach for checking the authenticity of honey. FTIR spectroscopic method has been found to be a robust and promising technique that helps in the identification of various functional groups present in the honey [28]. The FTIR-ATR overlay spectrum of all the honey showed identical fingerprints, ensuring consistency in the quality of honey samples and provides information about various functional groups present in the honey. The <sup>1</sup>H-NMR profiling of all the honey samples demonstrated various chemical shift regions, which can be used as a fingerprint for comparative analysis of various types of honey for identification, checking adulteration, and quality control purposes. The UV-Visible spectrum displayed a unique spectral pattern for the honey, which can be used for the identification of the honey samples for with respect to the quality analysis.

**CONCLUSION**

The physicochemical parameters such as TLC-based sugar analysis, fieh's test, aniline test, HMF test, ash value analysis and various spectroscopic interpretations were done for analysis of honey. The study of the chemical nature of honey was carried out by simultaneous using of <sup>1</sup>H-NMR profiling, FTIR-ATR fingerprint and UV/Vis spectroscopic analysis. The <sup>1</sup>H-NMR and FTIR-based metabolomics study provides a broad overview about the chemical composition and various functional groups of the honey in their native state without going for any prior chromatography purification. These techniques were reliable, rapid, and sophisticated technique for the analysis and simultaneous identification of various metabolites in the complex matrix. The above methods can be an efficient approach for analysis of various types of honey.

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Nil

**AUTHORS CONTRIBUTIONS**

Manas Ranjan Sahoo carried our analysis, interpreted the spectra, and write the manuscript. Dr. Ramesh R varrier provides overall guidance and provides critical planning and application of advance technological insights for the experiment. Mrs Anithakumari rajendran carried out the TLC and HPTLC analysis and interpret the spectral data.

**CONFLICT OF INTERESTS**

Authors declare no conflict of interest

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