Section A-Research paper
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Comparative analysis of Physiochemical, Antioxidant, Antibacterial, NMR and Rheological properties of honey from different locations of Himachal Pradesh, India (A*pis mellifera*)

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Declaration of competing interest

The authors declare no conflict of interest.

Authors contribution

MS and AS helped in designing the study. SR performed the experiment. SR, MS and FB interpretated and analysed the data. The manuscript was designed and approved for final submission by FB and MS.

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Abstract

The current research was conducted to analyse the multifloral honey samples from different districts of Himachal Pradesh for their proximate antioxidant, antimicrobial, structural characteristics and rheological characteristics of *Apis mellifera* honey obtained from different districts of Himachal Pradesh. Physical characteristics such as pH (3.497±0.009 to 4.533±0.028), moisture content (17.493±0.006 to 23.055±0.00289), electrical conductivity (0.137±0.003 to 1.923±0.015), acidity (25.67±1.15 to 30.67±1.5), specific gravity (1.353±0.003 to 1.407±0.009), total ash (0.2±0 to 0.451±0.001), total carbohydrates (76.24±0.631 to 82.303±0), total reducing sugar (67.227±0 to 73.04±0.042), fructose (34.433±0.01 to 39.043 ±0.011), sucrose (0.873±0.009 to 1.197±0.003), glucose (32.797±0.007 to 37.67±0.086), fructose/glucose ratio (0.941±0.001 to 1.153±colour analysis (25.333±0.333 to 125.293±3.979) were analyzed. To determine the antioxidant properties tests such as FRAP (2.563±0.003 to 3.48±0.015), DPPH (15.707±0.093 to 18.817±0.005) and ABTS (0.187±0.003 to 0.847±0.03) assays were performed and procured honey showed highest antioxidant activities. Several strong positive and negative correlation was observed among physiochemical and antioxidant parameters. A significant difference ($p<0.001$ and $p<0.005$) was observed in

physiochemical properties and antioxidant properties whereas all studied honey samples showed greater zone of inhibition which ranged from 7 to 30 mm towards bacterial growth. Further study of honey is done on structural characteristics of honey where three major sugars were identified in all the samples (α-D-glucose, β- D-glucose and β-D-xylose). In addition, honey's viscosity under the temperature ranges from 0–50°C. All studied samples showed Newtonian behaviour in the whole temperature ranges in slopes and intercepts of loss modulus (G″) versus angular frequency (ω). In general, all study honey samples had a good level of quality and the findings are in line with FSSAI (2018) and international standards.

Keywords: *Apis mellifera,* honey, physiochemical, antioxidant, antibacterial, NMR, Rheology, Himachal Pradesh

Introduction

Honey, the worldwide known natural herbal sweetener, is an economic apiary product produced by *Apis* species obtained from plant flower nectar (Sohaimy *et al*., 2015; Aljohar *et al*., 2018; Azonwade *et al*., 2018). It is gathered, converted, and stored in honey combs to ripen (FSSAI, 2017) and used for consumption all over the world since ancient times (Almeida-Muradian *et al*., 2013). At present, four honey bee species are well known for honey production and pollination i.e *Apis florae, Apis dorsata, Apis mellifera*, and *Apis cerana. Apis mellifera* which were introduced in India during 1960 (Atwal and Goyal, 1973).

In different parts of world, honey bee is domesticated for crop pollination and honey production. In India, honey consumption for medicinal purpose was accelerated physical action by inhibiting fatigue, inflamation and stress (Minhas *et al*., 2016). It has many therapeutic properties used to heal wounds, digestive and respiratory orders and many more diseases like skin ulcer (Nweze *et al*., 2019). *Helicobacter pylori* bacteria which cause gastritis and peptic ulcers is strongly inhibited by honey (Ali *et al*., 1991; Al-Somal *et al*., 1994; Osato *et al*., 1999). Paul *et al*. (2007) have reported that honey is more effective at treating coughs than chemical cough syrup. 59,999 MT of natural honey is exported by India worth 96.77 million USD during 2020-2021, with the US taking major shares at 44,881 MT. Himachal Pradesh has great deal of floral diversity because of different agro-climatic conditions, while natural honey with minimum pollutants harvested from low to high altitude increases its marketing value (Thakur *et al*., 2021). According to Devi et al. (2015) investigation in Kangra adjacent areas revealed a total of 219 plant species as nectar and pollen sources. Of these, 49 were the most important, 39 were intermediate, and 55 were the least important pollen sources. Further investigation revealed that pollen grains of both entomophilous and anemophilous species were present in Kangra and adjacent areas of Himachal Pradesh. The anemophilous types were Psidium guajava Sudher and Bundla, Pinus sp. in Kangra, and Poaceous members in Jwalamukhi during the summer season. All other morpho-species, such as *Eucalyptus* sp. *Brassica* sp., *Cedrella* sp., *Taraxacum* sp. *Trifolium* sp., which was detected in different honey samples, belongs to the entomophilous type.

Further, honey's antioxidant properties includes both non-enzymatic as well as enzymatic substances (Al Mamary *et al*., 2002; Khalil *et al*., 2012). Plant polyphenols act as antioxidants and also have many functions such as reducing agent, singlet oxygen quenchers and metal chelators. Several studies reported that antioxidant property of honey correlates

with presence of total phenolics (Viuda- Martos *et al*., 2008; Khalil *et al* 2012; Socha *et al*., 2016).

Honey has been known to have antibacterial properties and is used to treat and prevent wound infection (Paulus *et al*., 2012). In 1892, it was first reported to have antibacterial properties and also found that it is against 60 species of bacteria such as *Escherichia coli, Vibrio cholera, Salmonella, Shigella,* and *Staphylococcus aureus* including anaerobes and aerobes, gram-negative and gram-positive (Jeffrey *et al*., 1996; Pooya *et al*., 2003; Waili *et al*., 2004). A number of studies on honey's antibacterial properties have been carried out till date. Honey's antimicrobial and antioxidant properties are due to phenolic, glucose oxidase, proline, ascorbic acid, vitamins, catalase and α-tocopherol. Several authors also studied the correlations between colour, it's antioxidant and antibacterial activities with content of the bioactive compounds of honey (Dustman 1979; Brunet *et al*., 2014; Oryan *et al*., 2016).

One-dimensional (1D) NMR experiments are also referred to as NMR at ^{1}H or ^{13}C. Using one-dimensional 1H NMR spectra, the saccharides of honey from various countries were profiled. NMR is a powerful tool for extracting structural information and understand structure of compounds present in complex form like food. ^{1}H NMR spectroscopy is another tool for fingerprinting (Bertram *et al*., 2005). Labelling and verification of monofloral and multifloral honey was possible, ^{1}H NMR profiling combined with chemometric (Consonni *et al*., 2008; Donarski*et al*., 2008; Schievano *et al*., 2010) and shear rate plots in non-Newtonian fluids are not linear and do not start at the origin.

The determination of rheological features is one of the indirect methods (Louveaux *et al*., 1966; Oses *et al*., 2017; Pridal *et al*., 2021). The fact that some honeys have different rheological behavior can be used as an indicator of particular area. Rheology is deformation and flow of material under given external factors or pressure and have significant role in the food industry (Bambang *et al*., 2019; Faustino *et al*., 2021). The ease of flow of a fluid is directly inversely correlated with its viscosity. Honey's viscosity mostly depends upon water content, composition and temperature (Sopade *et al*., 2002; Ahmed *et al*., 2007). Honey was classified into Newtonian and non-Newtonian fluids based on its rheological properties. Honey's fluid behaviour is botanically derived feature that contributes to food's structural organisation and fluid heat transmission. In Newtonian fluids, shear rate is proportional to the shear stress and the plot starts at the origin. Gomez *et al.* (2009) found that the maximum monofloral honeys have Newtonian behaviour and viscosity is highly influenced by temperature. In non-Newtonian fluids shear stress and shear rate plots are not linear and do not start at the origin.

As per previous studies and literature, Parihar *et al*., 2020 evaluated the physiochemical parameters of honey extracted from *Apis mellifera* and *Apis cerana*. The honey samples were collected from different districts of Himachal Pradesh (Kangra, Mandi, Bilaspur, Solan, Hamirpur and Chamba. Presently, there were no reports on the study of NMR and rheological properties of honey samples extracted from *Apis mellifera* from various districts of Himachal Pradesh. The present study aim to assess the physiochemical, antioxidant, antibacterial, NMR and rheology of honey collected from various districts of Himachal Pradesh.

2. MATERIALS AND METHODS

The present investigation on honey samples has been executed in the Department of Zoology at Sri Sai University, Palampur with the help of Dove Research and Analytics, Panchkula, Haryana, IIT Mandi (department of chemistry) and microbiology laboratory of Kehloor Biosciences and Researh Centre, Ghumarwin. The methodology used for performing the research is detailed below:

The methodology used for performing the research is detailed below:

2.1 Collection of samples:

Composite multifloral honey samples of *Apis mellifera* were collected from six districts viz. Kangra (D1); Mandi (D2); Bilaspur (D3); Solan (D4); Hamirpur (D5); Chamba (D6) of Himachal Pradesh and were purchased directly from local distributors of honey. Each sample was collected in fresh, clean bottle containers and stored at room temperature (22–24 °C) in air-tight plastic containers until analysis. All the tests were performed in triplicates.

2.2 Physical Parameters:

2.2.1 Colour analysis

The color analysis of honey was performed using the Pfund scale at absorbance of 560 nm as described by Marchini et al. (2004) and Biochrom (2013).

2.2.2 Moisture content and Refractive index:

The refractive index of the sample at 20 ^{0} C was used to calculate the moisture content of honey using a table procured from FAO (1984). As the honey sample's total solid content increases, the refractive index also increases (Bogdanov *et al*., 1997 and AOAC, 2012).

2.2.3 Specific gravity:

A specific gravity bottle was used for the determination of specific gravity which was cleaned, dried and weighed with water maintained at 27^{0} C. The water was removed and the bottle was dried once more. After this, it was filled with honey samples maintained at an identical temperature. The bottle was weighed 2-3 times to calculate the weight (International Honey Commission, 2009).

2.2.4 Ash content:

The empty silica crucible was dried completely in the oven at 105 °C for 10-15 minutes, then placed in the desiccator to cool. In the crucible, around 1.0-5.0 gm of sample was weighed and heated over a low flame with the lid half covered. The crucible containing the sample was placed in the Muffle furnace and heated to 550 °C for 3 hours. After ashing, the dish was placed in the dessicator with the partially closed lid to cool.

2.2.5 Electrical Conductivity (EC):

In 20 ml of distilled water, 5 g of honey sample was dissolved and the final volume was made by adding 50 ml of distilled water. The calibrated digital conductivity meter was used to measure the solution’s electrical conductance (Sharma at al., 2021).

2.2.6 pH:

pH of honey was measured with the help of pH meter (Eutech instruments PC700) and 10% (w/v) solution of honey prepared in ultra-pure water (Bio-age DuraQ series purification system Mohali India) with slight modification as mentioned in Bogdanov, (2009).

2.2.7 Determination of Acidity:

In an appropriate titration flask, 10 gm of the sample was mixed thoroughly in 75 ml of carbon dioxide-free water. Using 4-6 drops of phenolphthalein indicator titrate against standard sodium hydroxide solution until pink colour remains for 10 seconds. The volume of sodium hydroxide solution was correctly used by determining the blank on water and the indicator. The calculations were made using the formula:

$$\text{Acidity as formic acid (\%) by weight} = \frac{0.23 \times 0.05\ \text{N Sodium hydroxide}}{\text{sample weight (g)}}$$

2.3 Biochemical parameters:

2.3.1 Total carbohydrates: Analysis of sugar content was done by HPLC with RI Detector.

Standard Preparation: -

The final volume was made with mobile phase by properly weighing 50 mg of glucose, fructose and sucrose in a 50 ml volumetric flask. The individual concentration of mixeded sugar solution was found to be 1000ppm.

Sample Preparation: - 1 gm to 5 gm honey sample was weighed in 100 ml volumetric shakes and the sample solution was sonicated for 30 min before adding 75 ml acetonitrile. The material has filtered the material using a syringe filter (0.45 m) after shaking and sonicating it.

$$\textbf{Chromatographic condition}: \frac{\text{Standard Area} \times \text{Standard dilution} \times \text{purity}}{\text{Sample Area x Sample dilution}} = \text{Result \%}$$

2.3.2 Determination of sugar analysis:

Determination of sugar (sucrose, glucose, fructose and glucose-fructose ratio) in honey was done according to Alijohar *et al*. (2018) using HPLC. Standard and working solution was prepared in distilled water.

2.3.3 Determination of total reducing sugar:

Determination of total reducing sugar was done by using the Layne- Enyon method as described in (Codex Alimentarius Standard, 1969).

2.4 Antioxidant activity:

2.4.1 Free Radical Scavenging Activity (DPPH):

DPPH (1, 1-diphenyl-2-picrylhydrazyl) test reported by Isla *et al*. (2011), was used to measure the free radical scavenging activity of procured honey samples.

2.4.2 Ferric Reducing/Antioxidant Power Assay (FRAP):

Reducing power of honey samples was determined by using Benzie and Strain's (1996) technique with slight modifications.

2.4.3ABTS assay:
The experiment was carried out according to Re *et al*. (1999) with slight modifications.

2.5 Anti-bacterial activity:
The evaluation of antimicrobial activity of six honey samples was performed using methodology described by Sharma et. al. (2012) with slight modifications. The standard reference microorganism viz. *Eschericha coli (E. coli)*, *Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa),*and *Salmonella typhi (S. typhi)* were used. The standard antibiotic discs (tetracycline) were used as positive control. The diameters of the growth inhibition zones were measured in mm. Three replicates were carried out for each experiment to validate the results.

2.5.1 NMR analyses:
1g of honey sample was diluted in 100 ml of ultrapure demineralized water after dissolution. An aliquots of 20 ml each was transferred into glass plates and freeze- dried until complete removal of water. Each film obtained from freeze-drying was further dissolved in 0.6 mL of deuterium oxide (D_2O) and transferred to Wilmad NMR tubes for NMR analysis. NMR experiments were recorded on Nuclear Magnetic Resonance spectrometer. Due to high solvent capacity with respect to the principal component of honey, D_2O was selected as NMR solvent.

2.5.2 Rheology:
The Modular Compact Rheometer (MCR-102, M/s) was used to determine the dynamic rheological parameters of honey samples. (Anton Paar, Austria), with a 50 mm diameter parallel plate system with a 0.5 mm gap at varying temperatures (0, 5, 10, 20, and 30 °C). Frequency sweeps in the range of 0.63–63 rad/s at 3 percent strain were used to determine the rheological data. The honey samples were cooked in a water bath to 50°C about 1 hour to dissolve crystals, then cooled to 30°C to eliminate air bubbles (Yoo, 2004). The experimental data was obtained using Rheoplus data analysis software (32 V3.40.) to determine storage modulus (G′) and loss modulus (G″). The results were calculated by averaging two measurements.

Statistical Analysis:
The results of all experiments were expressed as mean ± SD of triplicate measurements. The significant differences were represented by one-way ANNOVA and Pearson correlation (r) were calculated using Microsoft office excel 2010 and SPSS variants 22 (TBM, corporation New York, USA). NMR was analyzed by using Mnova software.

Results
3.1 Correlation between physiochemical and antioxidant properties
The result of Pearson correlation (r) coefficient between physical parameters of *Apis mellifera* honey. Ash content and pH ($r= 0.884$; $p<0.001$) showed strong positive correlation.

However, strong negative correlation between moisture and colour (r= -0.764; p<0.001) was observed on other hand, moderate negative correlation (r= -0.642) between total solid content and moisture was reported whereas correlation between physiochemical parameters of *Apis mellifera* was shown in table 4.5. Strong positive correlation between carbohydrates and total solid content (r=0.793**; P<0.001) was observed whereas strong negative correlation was observed between glucose and fructose/ glucose ratio (-0.668**; p<0.001) as shown in table 3.1.

Correlation result of physiochemical parameter and antioxidant properties. The positive correlation between FRAP to total solid content (r= 0.932; p<0.01) followed by ABTS to electrical conductivity (0.924; p<0.01) and FRAP to glucose (0.766; p<0.01) was observed. However, strong negative correlation was recorded between DPPH and fructose (-0.889; p<0.01). On other hand, there was moderate negative correlation between DPPH and ash content (-0.797; p<0.01) as shown in table 3.1.

Table 3.1: Correlations (Pearson's correlation coefficients – r) between physiochemical and antioxidant parameters

S0.NO	Parameters	colour	pH	Acidity	Moisture	SG	RI	AC	EC	T.S.C	Reducing sugar	Carbohydrates	sucrose	Glucose	Fructose	Fru/glu	DPPH	FRAP	ABTS
1	**Colour**	1.000	0.658**	0.757**	-0.764**	0.799**	0.005	0.445	0.583*	0.574*	0.111	0.215	0.44	0.782**	-0.161	-.752**	-0.107	0.591**	0.507*
2	**pH**		1.000	0.492*	-0.262	0.663**	0.226	0.884**	0.424	0.357	-0.374	-0.205	0.089	0.305	0.299	-0.127	-0.557*	0.502*	0.646**
3	**Acidity**			1.000	-0.252	0.575**	-0.107	0.459	0.408	0.239	0.528*	-0.049	0.755**	0.734**	0.396	-0.305	-0.483*	0.368	0.376
4	**Moisture**				1.000	-0.590**	-0.143	0.091	-0.366	-0.642**	0.101	-0.563*	-0.177	-0.576*	0.681**	.932**	-0.505*	-0.484*	-0.162
5	**Specific gravity**					1.000	-0.121	0.468	0.609**	0.425	0.013	0.182	0.211	0.651**	-0.126	-.557*	-0.114	0.532*	0.609**
6	**Refractive Index**						1.000	-0.031	-0.586*	0.394	0.43	0.256	0.304	-0.144	0.124	0.165	-0.075	0.238	-0.488*
7	**Ash content**							1.000	0.37	0.205	-0.196	-0.362	-0.042	0.237	0.470*	0.097	-0.797**	0.438	0.654**
8	**EC**								1.000	-0.202	-0.06	-0.407	-0.123	0.236	-0.341	-.478*	0.062	-0.099	0.924**
9	**T.S.C**									1	0.039	0.793**	0.228	0.690**	0.111	-.574*	-0.039	0.932**	-0.2
10	**Reducing sugar**		1	0.264	0.571*	0.596**	0.356	-0.125	-0.148	0.139	-0.218								
11	**Carbohydrates**			1	0.181	0.570*	-0.293	-.536*	0.378	0.675**	-0.543*								
12	**Sucrose**				1	0.537**	0.408	-0.124	-0.261	0.206	-0.229								
13	**Glucose**					1	0.057	-.668**	-0.143	0.766**	0.132								
14	**Fructose**						1	.681**	-0.889**	0.111	-0.099								
15	**Fru/glu**							1	-0.490*	-0.454	-0.23								
16	**DPPH**								1	-0.281	-0.231								
17	**FRAP**									1	-0.016								
18	**ABTS**										1								

Note: Mean ± Standard Error Mean; Mean bearing **- p<0.001 highly *- p<0.005 statistically significant; Where, SG= Specific gravity, RI= refractive index, AC= ash content, EC= electricalCconductivity, TSC= total solid content

3.2 Colour analysis

The color analysis of procured honey samples as shown in table 3.1 were measured by Lovibond Tintometer after conversion of the absorbance values. The highly statistically significant difference was observed ($p<0.001$) as shown in table 3.1. The colour of obtained data ranged from (white) 25.33±0.00 to (dark amber) 125.29±3.97 mm Pfund. Statistically, highest colour range (Dark amber) of 125.29±3.97 mm Pfund was observed for honey from Kangra followed by Chamba honey (121.16±0.46 mm Pfund) which was at the par with Solan (114.7±0.05 mm Pfund), while light Amber colour was observed for Hamirpur honey 113.66±0.33 mm Pfund. The lowest range (white colour) was observed for honey from Mandi (25.33±0.33) statistically at par with Bilaspur (25.6±0.11 mm Pfund) honey.

3.3 pH value

pH values of procured honey samples were recorded where the highest pH value of 4.53±0.02 was observed for honey sample obtained from Solan which was statistically at par with Kangra (4.43±0.02) honey, and further followed by (3.91±0.015) whereas lowest pH of 3.49±0.00 was observed for honey of Mandi district, though significantly at par with Hamirpur (3.56±0.00) and Bilapur (3.53±0.00) honey as shown in table 3.1.

3.4 Acidity

The acidity of the *A. mellifera* honey from different districts ranged from 0.170±0.001 to 0.107±0.003 % as shown in table 3.1. Statistically, highest acidity of 0.170±0.001 % was recorded for honey from Chamba followed by Solan (0.170±0.001 %), Kangra (0.168%) and Hamirpur (0.167 %) honey, whereas lowest acidity was recorded for honey from Bilaspur (0.107 %) followed by Mandi (0.157%) honey.

3.5 Moisture

All the procured honey samples were analyzed for moisture content and obtained results were shown in table 3.1. The moisture content ranged from 17.49±0 to 23.05±0%. Statistically, highest moisture content of (23.05±0%) was observed for honey from Mandi followed by Bilaspur (20.05 ±0.0 %), which was at par with Solan (19.84± 0 %) honey, whereas lowest moisture content of 17.49±0% was recorded for honey from Hamirpur which was in line with Kangra (18.7±0.1%) and Chamba (18.84±0.03%) honey.

3.6 Specific gravity

The highest value of specific gravity was observed in honey samples procured from Chamba (1.407±0.009 gm^{1}) followed by Kangra (1.399±0.002 gm^{-1}) which were statistically at the par with Solan (1.397±0.002 gm^{-1}) and (1.387±0.003 gm^{-1}) whereas lowest range value for specific gravity was observed in Mandi (1.353±0.003 gm^{-1}) statistically at the par with 1.36±0 gm^{-1} Bilaspur honey as shown in (table 3.1).

3.7 Refractive index

The values of sample produced by *Apis mellifera* ranged from 1.459±0.003 to 1.495±0 .003 as shown in table 3.1. The highest value was observed in honey from Solan (1.495±0.003) followed by Hamirpur (1.493±0.003) honey was though statistically at the par with Kangra 1.487±0.001 and Bilaspur (1.486±0) honey whereas lowest refractive index (1.459±0.003) was recorded for Chamba honey, though it was statistically at the par with Mandi honey sample (1.4783±0). The highly statistically significant differences ($p<0.001$) were observed in present honey samples.

3.8 Ash content

The highest value was observed in honey samples procured from Solan (0.451±0.001) followed by Kangra (0.372±0.002) though statistically at the par with Chamba (0.350±0.001) whereas lowest range of ash content was observed for honey of Hamirpur (0.02±0) followed by Mandi (0.29±0) honey which was in line with Bilaspur (0.247±0.003) honey as shown in table 3.1.

3.9 Conductivity

All the procured samples were analyzed for electrical conductivity varied from 0.137±0.00 mS^{-1} to 1.92±0.00 mS^{-1} as observed in table 3.1. Highest electrical conductivity was observed for honey of Chamba (1.92±0.0 mS^{-1}), which was statistically at par with Kangra (1.88±0.04 mS^{-1}), lowest range value was observed for Bilaspur (0.137±0.00 mS^{-1}) honey statistically at the par with (0.187±0.00 mS^{-1}) in line with Solan (0.195 mS^{-1}) and Hamirpur (0.197 mS^{-1}) honey.

3.10 Total solid content (T.S.C)

All the procured samples were analyzed for TSC and obtained result were shown in table 3.1. Total solid content *Apis mellifera* honey samples ranged from 76.937±0.003 % to 83.48±0.0 %. Highest range of TSS was showed by Solan (83.48±0.063 %) which were statistically at the par with Hamirpur (82.433±0.067 %) and Chamba (80.543±0.063 %) honey sample. Lowest range was observed in Mandi (76.937±0.003 %) and followed by Bilaspur (79.943±0 %) at the par with Kangra (78.897±0.107%) honey.

3.11 Total reducing sugar

In present study, highest levels of reducing sugar were observed in Chamba (73.04±0.042 gm) as shown in table 3.1, which were statistically at the par with Mandi (72.87±0 gm) and Hamirpur (72.747±0.003 gm) honey, while lowest range of total reducing sugar was observed in Bilaspur (67.227±0) which was statistically at the par with Kangra (67.497±0.024 gm) in the line with Solan (70.017±0.019 gm).

3.12 Carbohydrates

In present study, total carbohydrates of the examined honey samples ranged from 82.30 gm to 76.24 gm as shown in table 3.1. Highest range value was observed for honey of Hamirpur (82.30 gm) followed by Solan honey (79.97 gm), though statistically at the Par with Bilaspur (79.68 gm) and Chamba 79.36 gm honey, whereas lowest range of carbohydrates was observed for honey from Kangra (76.24 gm), statistically at the par with Mandi (76.64 gm).

3.13 Glucose

In present investigation, the glucose of *A. mellifera* honey samples were analysed in which highest value of glucose was observed for honey from Chamba (37.67 gm) as shown in table 3.1 followed by Hamirpur (37.79 gm) honey, though statistically at the par with Solan (36.94 gm) honey, lowest value was observed for Bilaspur (32.79 gm) honey, though statistically at par with Mandi (33.83 gm) and Kangra (34.53 gm) honey.

3.14 Sucrose

In case of sucrose analysis honey samples collected from *A. mellifera* showed highest value for Hamirpur honey sample of 1.19 gm, followed by Mandi (1.10 gm) honey which was statistically at par with Solan (1.087gm) and Kangra (1.063 gm) honey, lowest sucrose

content of 0.873 gm was recorded for Bilaspur honey, though statistically at par with Chamba (1.00 gm) honey as shown in table 3.1.

3.15 Fructose

Sugar analysis of honey samples collected from *A. mellifera* showed that Mandi (39.04 gm) honey samples has highest fructose content as shown in table 3.1, though statistically at the par with Solan (38.62 gm) honey. Lowest value was showed by Bilaspur (34.43 gm) honey, followed by (35.32 gm) of Hamirpur honey, though statistically at the par with Chamba (35.48 gm) and Kangra (35.56 gm) honey.

3.16 Fructose: Glucose

In studied honey samples F: G ratio of 1.153±0 gm honey from Mandi was observed as shown in (table 3.1), though it was statistically at par with Bilaspur (1.05±0), Solan (1.04±0.003 gm) and Kangra (1.01±0.02 gm) honey, whereas lowest F: G ratio of (0.941±0.001 gm) was recorded for Chamba honey, which was statistically at par with Hamirpur (0.945±0 gm) honey.

Table 3.2: Physical parameters of honey

S.No.	Parameters	Mandi	Bilaspur	Hamirpur	Kangra	Chamba	Solan
1	**Colour analysis (P fund)**	25.33±0.33	25.6±0.116	113.667±0.33	125.293±3.97	121.16±0.465	114.7±0.052**
2	**pH value**	3.49±0.009	3.53±0.003	3.56±0.003	4.43±0.12	3.91±0.015	4.53±0.028**
3	**Acidity (%)**	0.157±0.003	0.107±0.003	0.167±0.003	0.168±0.001	0.17±0.001	0.17±0.001**
4	**Moisture content (%)**	23.055 ± 0.002	20.05±0.007	17.493±0.006	18.7±0.1	18.84±0.033	19.84±0.008**
5	**Specific gravity (gm^{-1})**	1.353±0.003	1.36±0	1.387±0.003	1.399± 0.02	1.407±0.009	1.397±0.002*
6	**Refractive index**	1.4783±0	1.486±0	1.493±0	1.487±0.001	1.459±0.003	1.495±0.003**
7	**Ash content (%)**	0.29±0	0.247±0.003	0.2±0	0.372±0.002	0.35±0.001	0.451±0.001**
8	**Electrical conductivity (mS/cm)**	0.187±0.003	0.137±0.003	0.197±0.003	1.883±0.044	1.923±0.015	0.195±0.002**
9	**Total solid solvent (%)**	76.937±0.003	79.943±0.00	82.433±0.067	78.897±0.107	80.543±0.063	83.48±0.00**
10	**Total reducing sugar (gm)**	72.87±0	67.227±0	72.747±0	67.497±0.02	73.04±0.04	70.017±0.01**
11	**Carbohydrates (gm)**	76.643±0.00	79.687±0	82.303±0	76.24±0.63	79.36±0.20	79.973±0.04**
12	**Sucrose (gm)**	1.1±0	0.873±0.	1.197±0	1.063±0.01	1.003±0.01	1.087±0.00**
13	**Glucose (gm)**	33.837±0.00	32.797±0	37.407±0.0	34.533±0.33	37.67±0.08	36.94±0.03**
14	**Fructose (gm)**	39.043±0.01	34.433±0.01	35.327±0.01	35.567±0.37	35.487±0.04	38.62±0.01**
15	**Fructose: Glucose (gm)**	1.153±0	1.05±0	0.945±0	1.01±0.02	0.941±0.00	1.047±0.00**

Note: Mean ± Standard Error Mean; Mean bearing **- $p<0.001$ highly statistically significant; *- $p<0.005$ statistically significant

3.17 Determination of total antioxidant content by ferric ion reducing antioxidant power (FRAP) assay:

The highest FRAP value was observed in Solan (3.48±0.015 µ mol kg^{-1}) honey, which was statistically at the par with Hamirpur (3.107±0.003 µ mol kg^{-1}) and Chamba (3.097±0.029 µ mol kg^{-1}) honey confirming its highest antioxidant properties whereas lowest FRAP value was observed in Mandi (2.563±0.003 µ mol kg^{-1}) which was in line with Kangra (2.74±0.078 µ mol kg^{-1}) and Bilaspur (2.81±0 µ mol kg^{-1}) honey (Table 3.2).

3.18 α, α-diphenyl-β-picrylhydrazy (DPPH) free radical scavenging activity:

The highest DPPH was recorded in honey of Bilaspur (18.817±0.005%), which was at the par with Hamirpur (18.757±0%) which was in line with Chamba (17.71±0.122%) honey and Kangra (17.583±0.108%) while Solan (15.707±0.093%) and Mandi (16.6±0.006%) honey exhibits the lowest activity (Table 3.2).

3.19 Determination of ABTS Assay: The antioxidant activity of all honey samples was evaluated using ABTS radical cation activity, and results were shown in table 3.2. The highest ABTS radical cation activity was observed in Kangra (0.847±0.003%) which was statistically significant (0.79±0.006%), whereas Hamirpur (0.187±0.003%) honey exhibits lowest activity, followed by Bilaspur (0.337±0.003%) and Mandi (0.367±0.003%) which was in line with (0.487±0.012%).

3.3: Antioxidant parameters of honey

S.NO	Parameters	Mandi	Bilaspur	Hamirpur	Kangra	Chamba	Solan
1	DPPH (%)	16.6± 0.006	18.817± 0.005	18.757± 0.00	17.583± 0.108	17.717± 0.122	15.707± 0.093
2	FRAP (µ mol/ kg)	2.563± 0.003	2.81± 0.00	3.107± 0.003	2.74± 0.078	3.097± 0.029	3.48± 0.015
3	ABTS (%)	0.367± 0.003	0.337± 0.003	0.187± 0.003	0.847± 0.03	0.79± 0.006	0.487± 0.001

3.20 Determination of antimicrobial activity of honey sample:

Among all studied honey samples Hamirpur honey showed zone of inhibition against for all examined four pathogenic species. Highest zone of inhibition was observed in solan honey which was at the par with Bilaspur honey against *E. coli* whereas Kangra, Mandi and Bilaspur showed no zone of inhibition against *P. aeruginosa* and *S. typhi* as shown in table 3.4. Our current findings of inhibitory bacterial growth were in line with study of Saha *et al*.

Sample No.	**Diameter of zones of inhibition (mm) for honey samples against test isolates**			
	E. coli.	***P. aeruginosa***	***S. aureus***	***S. typhi***
Kangra (1)	7	-UD-	24	-UD-
Mandi (2)	20	-UD-	26	-UD-
Bilaspur (3)	28	-UD-	25	-UD-
Hamirpur (4)	20	20	8	8
Solan (5)	30	16	-UD-	14
Chamba (6)	-UD-	25	-UD-	-UD-
Antibiotic Tetracycline (Positive control)	26	29	28	31

(2018) as antimicrobial inhibition assay showed the honey samples zone of inhibition.

Table 3.4: Antimicrobial assay of 7 honey samples against four different pathogen
Note: UD- Undetected

3.21 D_2O ^1H NMR and ^{13}C NMR Spectrum of Honey

All investigated honey sample by ^1H NMR and ^{13}C NMR revealed three major sugars (α-D-glucose, β-D-glucose and β-D-xylose). Mainly three major anomeric proton signals were observed in all studied samples in the ^1H spectrum. The proton signal in all samples observed at δ_H 5.12-5.15ppm (1H d, *J*- 3.7 Hz) indicated the presence of an α- oriented sugar in all the samples (Duss *et al*., 2000). Similarly, another proton signals at δ_H 4.53-5.57 ppm (2H d, *J*- 7-8 Hz) indicated the presence of two major *β*- oriented sugars in all honey samples, respectively (Duus *et al*., 2000) as shown in table 3.5 and fig (3.5, 3.7, 3.9, 3.11, 3.13 and 3.15).^{13}C NMR experiment is an important experiment which gives the information regarding the total number of carbon atoms and their neighbouring environment. The ^{13}C NMR spectrums of all the honey samples were studied and analysed for the confirmation of present sugar units. The characteristic carbon signals observed at δ_C 63.3 ppm (CH_2-OH), 63.8 ppm (CH_2-OH) confirms the presence of two glucose units and other characteristics signal at δ_c 67.4 ppm (CH_2-O) confirmed the presence of xylose unit in major amount. Similarly, other carbon signals observed in between δ_C 60-80 ppm (CH-OH, CH_2-OH) confirmed that

samples only contain sugar moieties as shown in table 3.5 and fig (3.6, 3.8, 3.10, 3.12, 3.14 and 3.16).

Table 3.5: Characteristics of chemical shifts (^{1}H at 500 MHz, ^{13}C at 125 MHz identified honey samples in D_2O, (δ in ppm and *J* in Hz)

Honey samples	Individual Components	^{1}H	^{13}C
Kangra	α-D-glucose	5.14 (d,3.7)	63.3
	β-D-glucose	4.56 (d, 7.9)	63.8
	β-D-xylose	4.54 (d, 7.9)	67.49
Mandi	α-D-glucose	5.14 (d, 3.7)	63.3
	β-D-glucose	4.56 (d, 7.9)	63.8
	β-D-xylose	4.54 (d, 7.9)	67.50
Bilaspur	α-D-glucose	5.14 (d, 3.8)	63.3
	β-D-glucose	4.54 (d, 8)	63.8
	β-D-xylose	4.56 (d, 8)	67.4
Hamirpur	α-D-glucose	5.15 (d, 3.7)	63.3
	β-D-glucose	4.57 (d, 7.9)	63.8
	β-D-xylose	4.55 (d, 7.9)	67.4
Solan	α-D-glucose	5.12 (d, 3.7)	63.3
	β-D-glucose	4.55 (d, 7.9)	63.8
	β-D-xylose	4.54 (d, 8)	67.5
Chamba	α-D-glucose	5.13 (d, 3.75)	63.3
	β-D-glucose	4.53 (d, 7.7)	63.8
	β-D-xylose	4.54 (d, 7.7)	67.5

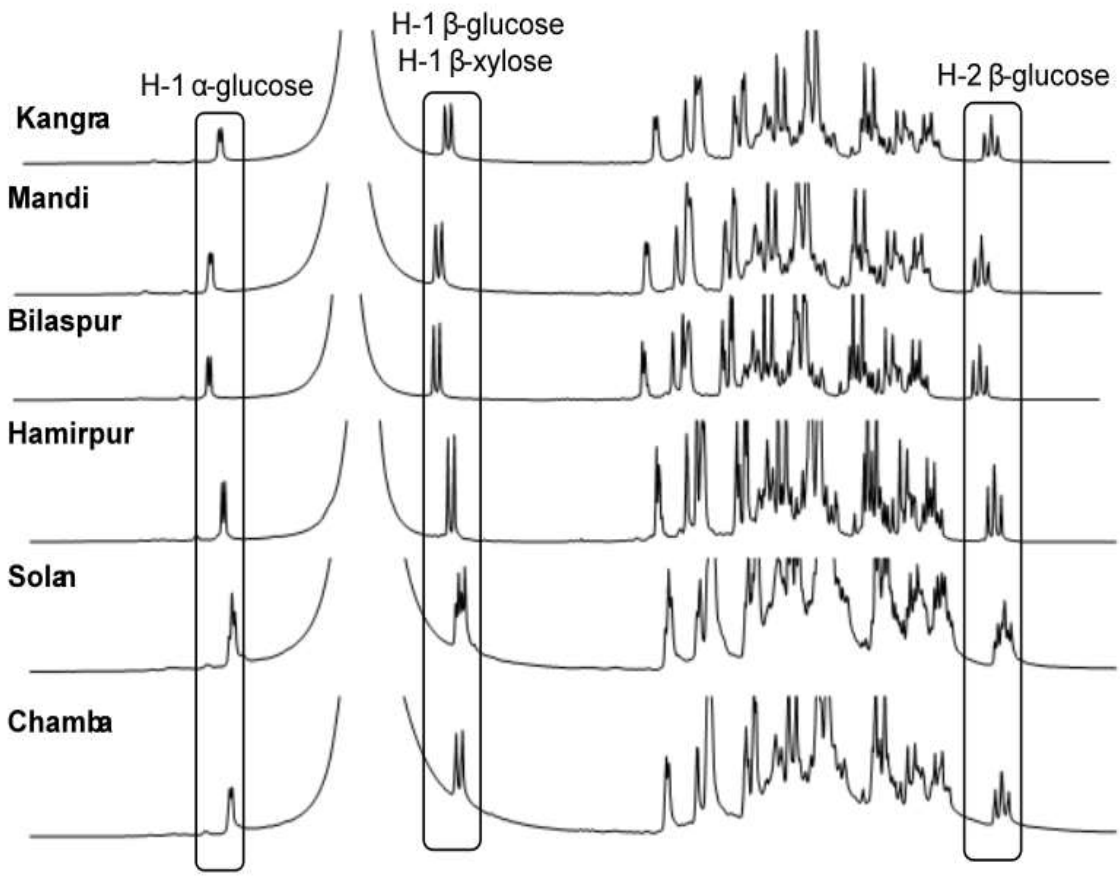

Fig 3.17: ^{1}H NMR spectrum of honey samples collected from different districts of Himachal Pradesh

3.21 Effect of temperature on viscosity of honey:

The viscosities of all studied six honey samples were measured at temperatures ranging from 0°C to 50°C and found to be decreased with increase in the temperature. In current study, Mandi honey sample showed highest viscosity (20.31 Pa.S) at 0°C and the lowest viscosity was observed in Kangra, Hamirpur and Bilaspur honey (0.001Pa.S) at 50 °C as shown in table 3.6.

Table 3.6: Effect of temperature on honey

Samples	**Temperature (°C)**	**Viscosity (Pa s)**		**Temperature (°C)**	**Viscosity (Pa s)**
Kangra	0	16.53	**Hamirpur**	0	18.25
	5	11.43		5	13.15
	10	8.84		10	10.56
	15	4.35		15	6.07
	20	1.71		20	3.43
	25	0.63		25	0.35
	30	0.21		30	0.13
	35	0.12		35	0.08
	40	0.04		40	0.01
	45	0.005		45	0.007
	50	0.001		50	0.001
Mandi	0	20.31	**Solan**	0	15.53
	5	17.62		5	10.53
	10	14.32		10	8.32
	15	10.32		15	5.16
	20	6.52		20	3.73
	25	2.31		25	1.17
	30	0.73		30	0.53
	35	0.21		35	0.15
	40	0.04		40	0.09
	45	0.073		45	0.01
	50	0.012		50	0.007
Bilaspur	0	18.65	**Chamba**	0	17.73
	5	14.73		5	14.26
	10	10.53		10	11.53
	15	6.43		15	7.27
	20	2.18		20	3.42
	25	0.65		25	1.08

	30	0.32		30	0.45
	35	0.15		35	0.08
	40	0.04		40	0.03
	45	0.008		45	0.007
	50	0.001		50	0.002

3.22 Dynamic shear rheological properties

All analysed honey samples in the present study showed Newtonian behaviour. The magnitude of c varied from 0.7284 to 1.9987 with sample Kangra showing a higher value as shown in table 3.7.

Table 3.7: Slopes and intercepts of *loss modulus* (G″) versus angular frequency (*ω*) of different varieties of honey

	T (°C)	n	k	R^2		T	n	k	R^2
Kangra	0	0	0	0	**Mandi**	0	0	0	0
	5	0.231	0.8591	1		5	0.061	0.7412	1
	10	0.474	1.3286	1		10	0.0269	1.0539	1
	15	0.538	1.549	1		15	0.031	1.1978	1
	25	0.585	1.8037	1		25	0.025	1.4155	1
	35	0.572	1.9408	1		35	0.023	1.5598	1
	40	0.572	1.9987	1		40	0.022	1.6172	1
Bilaspur	0	0	0	0	**Hamirpur**	0	0	0	0
	5	0.13	0.7889	1		5	0.042	0.7284	1
	10	0.231	1.1602	1		10	0.118	1.0821	1
	15	0.262	1.3574	1		15	0.142	1.2744	1
	25	0.285	1.5954	1		25	0.16	1.5088	1
	35	0.295	1.7483	1		35	0.168	1.6602	1
	40	0.298	1.8083	1		40	0.21	1.7473	1
Solan	0	0	0	0	**Chamba**	0	0	0	0
	5	0.225	0.8549	1		5	0.065	0.7443	1
	10	0.266	1.1844	1		10	0.296	1.2055	1
	15	0.279	1.3696	1		15	0.358	1.4241	1
	25	0.289	1.5986	1		25	0.403	1.6772	1
	35	0.294	1.7477	1		35	0.421	1.836	1
	40	0.295	1.8067	1		40	0.427	1.8979	1

4. Discussion

4.1 Colour

Honey colour is one of the most important physiochemical criteria that may be used to determine honey quality (Murke, 2018). Natural variation in honey colour ranges from light yellow to amber, dark amber and black. In rare circumstances, red or green hues may also appear (Ibrahim *et al*., 2012). Color of honey also depending upon season; in summer the color of honey is dark while honey is lighter in color in autumn. The color of honey basically depends on the ash content, storage time and temperature (De Silva *et al*., 2016; Szabo *et al*., 2016). The pollen, mineral and phenolic composition of honey affects its hue (Attri, 2011). The colour of obtained data ranged from (white) 25.33±0.00 to (dark amber) 125.29±3.97 mm Pfund. Highly statistically significant difference ($p<0.001$) between the samples was observed. In addition to current study, Koundal and Kumar (2017) also investigated honey of district Kangra to have light amber to extra dark amber color. Our present results were also in accordance to the study of Sharma *et al*. (2021) where color analysis of honey samples ranged in between 67.43±9.28 to 125.29± 6.89 97 mm Pfund. The current study showed positive correlation of colour with pH ($p<0.658$) which was similar to the reports of Eleazu *et al*. (2013). Strong positive correlation was found between colour and FRAP values which were concurrent with the studies of Patrignani *et al*. (2015). In current study, positive correlation was found between colour and ABTS which was found to be similar to the findings of Isla *et al*. (2011). It has been found that polyphenol compounds were significantly correlated with the honey colour, and found that dark colour honey showed highest phenolic compounds and exhibit highest antioxidant property (Bertoncelj *et al., 2007;* Wesołowska and Dżugan, 2017), and also showed positive correlation with pH which was similar to study of Rahaman *et al*. (2013) and Hailu and Belay (2020).

4.2 pH

Since pH has an impact on the stability, texture and shelf life of honey, its analysis is important. None of the investigated honey samples exceed the allowed limit when compared with honey of standards for honey set by FSSAI (2018) i.e. (3.90- 6.10). All studied samples were observed to be highly significant ($p<0.001$). However, current study was similar to Bouhlalia *et al*. (2019) where a linear relationship was observed between pH and acidity and also present obtained results were in line with Attri (2011) and Khalil *et al*. (2012) where pH in the range of 3.62 to 4.5 has been reported. A highly acidic honey sample suggested that sugars may be due to their fermentation into organic acid (Khalil, 2012). The current pH values support the findings of Kamboj *et al*. (2013); Fahim *et al*. (2014) and Umarani *et al*. (2015). Our findings were also found to be in line with studies of Lullah-Deh *et al*. (2018), who studied fresh honey samples from the Mambilla Plateau in Nigeria and reported pH levels to be ranged from 3.22 to 5.00 and of Tigistu *et al*. (2021) where pH levels ranged from 3.42- 4.55. In present study pH showed correlation with acidity which was similiar to the findings of Ratiu *et al*. (2019). Highest correlation was observed with ash content (0.884,

p< 0.001) which was comparable to the finds of Khalafi *et al*. (2016) and Albu *et al*., 2021 also studied similar findings.

4.3 Acidity

One of the main criteria that can influence honey quality is acidity. Honey's acidity ranged from around 3.4 to 6.1 with an average of 3.9, as per National Honey Board (Bogdanov, 2010). In present study, acidity range varied from 0.107±0.00 to 0.170±0.00% which was not more than the FSSAI limit (0.2%) which indicated the absence of undesirable fermentation. All studied samples observed highly statistically significant difference p<0.001between the samples. High acidity increased the antioxidant property of honey which may be due to fermentation of sugars into organic acids which was responsible for two major characteristics of taste and resistance from microbial spoilage. Our findings were similar to Parihar *et al*. (2020) where highest acidity was also found in Solan honey inspite of this acidity showed positive correlation with pH (0.492, p<0.005) which was similar to previous reports of Krishnasree *et al.* (2017); Bouhlali *et al*. (2019) and Ratiu *et al*. (2020).

4.4 Moisture

Honey's moisture content is a significant component in its shelf life, since it helps to keep stability against fermentation and granulation during storage (Cereser *et al*., 2010). This parameter is well established to be linked to the maturity and freshness of samples, regardless of botanic origin or honey type (Manzanares *et al*. 2011). The amount of water in honey impacts its microbiological stability, sensory quality, physical features, and shelf life (Stevenson *et al*., 2015). All studied samples were observed highly to be statistically significant (p<0.001). The moisture content of the honey samples was estimated to be between 17.49±0.006 and 23.05±0.002 %. Moisture content might vary from year to year due to its botanical origin, climatic conditions, processing, storage, hive maturity, and its harvesting season (Sahney and Kumar, 2017). Saxena *et al*. (2010) found that the moisture level of commercial honey brands in India was considerably below the FSSAI's prescribed limit (> 20%), indicating that the honey was mature enough to resist fermentation. In addition, Kamboj *et al*. (2013) found that the moisture content of honey from Himachal Pradesh and its neighboring states, Haryana, Punjab and Rajasthan, varied between 17.08 and 18.89 percent. In contrast, Gairola *et al*. (2013) found high moisture content (>20 percent) for honey from the Uttarkashi district of Uttarakhand, India which was ranged from 19.00 to 25.00. In previous study, Parihar *et al*. (2020) reported highest moisture content in Shimla and Mandi honey whereas lowest moisture content was observed in Hamirpur which was found to be similar to our current findings. Moisture content showed negative correlation with specific gravity (-0.590**; p<0.001) as water content increased the specific gravity decreased close to our investigation comparable results were reported by Attri 2011; *Olugbemi et al*. (2013); Kek and Chin (2018) and Albu *et al*. (2021).

4.5 Total Solid Content

The honey's total solid content shows its purity. The total solids of all six honey samples ranged between 76.93 and 83.48 %, as per results of analyzed samples. In a Solan honey sample, the highest total Solids content was calculated to be (83.48 %). As total solids are inversely proportional to moisture content, the presence of low moisture level might be the reason for high total solid content. Total solid content above or equal to 81% were classified as of higher grade (A and B) (*USDA, 1985, Nyau* et al*., 2013*). In present studied, honey sample from Solan and Hamirpur were found to be of A grade. The highly statistically significant difference $p<0.001$ was recoded. Also, similar findings were reported by Agbawba *et al*. (2011); Iftikhar *et al*. (2011); Khalil *et al*. (2012) were total solid content of honey samples ranged from 75 to 80%. A strong negative correlation was observed with moisture (-0.642**; $p<0.001$) which indicated that as total solid content increased moisture percent decreased which may avoid fermentation of honey. Our current findings were also similar to previous reported studies of Krishnasree *et al*. (2017); Albu *et al*. (2021) and Nemo and Bacha (2021).

4.6 Specific Gravity

Honey's specific gravity is inversely proportional to its moisture content; the denser a honey the less is the humidity it contains (Attri *et al*. 2011). In present study, specific gravity was at 30 $^{\circ}$C ranged from 1.35±0.0 to 1.40±0.0 g/cm^3. All studied samples were observed to be statistically significant ($p<0.005$). The current study results were similar to the findings of Sunkesula *et al*. (2021) for honey samples from Southern India (1.04 to 1.53 g/cm^3). The majority of them met the Codex Alimentarius requirements of 1.39 to 1.52 g/cm3. The present values for specific gravity supported the previous findings of Abdel-Hameed *et al*. 2020 where specific gravity ranged from 1.390±05 to1.42±0.36. Also, these results were in concurrent with Mary *et al*. 2021 where specific gravity ranged between 1.04-1.53 g/cm$^{3.}$

4.7 Refractive index

The refractive method is the most used because it is simple to use and repeat. Refractive index value is correlated with values of water content, solids substances, total soluble solids and specific gravity. Moisture content decreases as refractive index increases, whereas refractive index increases as solid sample increases. In present study, refractive index ranged from 1.459±0.003 to 1.495±0.003. All studied sample observed highly statistically significant difference $p<0.001$.

4.8 Ash content

All studied honey samples have an ash content in accordance with national regulations (Pravilnik *et al*. 2021). The purity of the honey samples was determined by the ash concentration.The present study ranged from 0.2±0 to 0.451%. The investigated samples were found to be within acceptable limits, i.e less than 0.6 %. The ash or mineral content of honey was a factor that affected its colour and flavour. If the ash concentration in honey was

high, the colour would be darker and the flavour would be stronger (Da Silva *et al*. 2016). All studied samples were observed statistically significant p<0.001. Results were comparable with the findings of Umarani *et al*. (2015), Prica *et al*. (2015) and Sharma *et al*. (2021).

4.9 Electrical Conductivity

Honey's electrical conductivity was due to the presence of iron, as well as other mineral elements and organic acid. In this study, the current ranged from 0.137±0.00 to 1.920.01 mS^{-1}. All of the honey samples tested met the codex alimentarius limits (not more than 0.8 mS/cm). All studied samples were observed highly statistically significant p<0.001. our present findings are similar to Sharma *et al*. (2021). The positive correlation was found with colour (0.583*; p<0.005) which is similar to study of Santos *et al*. (2018) and Ratiu *et al*. (2019).

4.10 Total reducing sugar

Honey is mostly composed of major sugars like glucose and fructose, as well as a few other minor ingredients (maltose and sucrose). The varied range of Total reducing sugar is from 67.22±0 to 73.04±0.04 g/ 100g. All investigated samples were within FSSAI limit which is not less than 65%. All studied samples were observed highly statistically significant p<0.001. Our results are found to be in accordance to the studies of Alemu *et al*. (2013) where honey was analyzed in Sekota district of Northern Ethiopia in which reducing sugars were ranged from 63.4 to 89.7 g/ 100g and Sharma *et al*. 2021 ranged from 67 to 68 g/ 100g.

4.11 Carbohydrates

The analysis of honey's carbohydrate content is a quality parameter which might influenced by the storage and heating of honey, thus acts as an indicator of honey freshness. The carbohydrates' presence in the honey totally depends upon the presence of bee flora in that region. In present investigation, the total carbohydrates of the examined honey samples were ranged from 76.24±0.44 to 82.30 g/ 100g. The results of the samples are highly statistically significant p<0.001. Our studies are in accordance with the studies of Kalimi and Sohomic (1964), White (1975) and Khalil, 2012 where total carbohydrates of honey samples were found in the range of 70-80%. Indian honey samples reportedly the range of total carbohydrate is 78.4-82.4% (Saxena et al., 2009) which is found to be similar with our research.

4.12 Sucrose

Sucrose content of procured honey samples ranged from 0.873 to 1.19 g/ 100g which were as per national and international regulations (Codex Alimentations, 2001; EOSC, 2005 and FSSAI 2018). All studied samples observed were highly statistically significant (p<0.001). In previous experimentation by Parihar *et al*. (2020) sucrose content ranged from 2.89 to 3.34 g/ 100g which was more than current investigation. Our current study results were similar to the findings Sharma *et al*. (2021) where *Apis mellifera* sucrose was 1.02 g/ 100g. In current

findings correlation between sucrose and glucose found which was also in agreement to the findings of Ratiu *et al*. (2019).

4.13 Glucose

The sugars present in honey are responsible for certain physicochemical properties such as its viscosity, granulation, characteristics and hygroscopy of honey. Generally, glucose in honey was lower than fructose (Siddiqui and Furgula, 1976). In present investigation glucose varied ranged from 32.79 to 37.67 gg/ 100g. Our results supported the previous investigation of different types of honey by Buba *et al*. (2013); Manzoor *et al*. (2013); EL-Metwally, (2015) and Hegazi *et al*. (2018). Aregay *et al*. (2018) observed similar finding of 36.37±2.14 g/100g glucose content from Godere district. Comparable findings were reported by Tigistu *et al*. 2021.

4.14 Fructose

The fructose content of *A. mellifera* honey sample showed that the highest fructose content varies from 34.43-39.04 g/ 100g. Our investigations are in accordance with the studies of Hegazi *et al*. 2018, the level of fructose was ranged from 23-43 g/ 100g. In all studied samples highly statistically significant difference $p<0.001$ was observed. Current study findings were found similar to Hegazi *et al*. 2018, the level of fructose was ranged from 23-43% which was in accordance to above values. The present result is in line with Aregay *et al*. (2018) reported mean fructose content of 38.64 ± 0.61 g/100g from the Godere district. Current findings result was found accordance with Tigistu *et al*. 2021.

4.15 Fructose: glucose

In present study the fructose glucose ratio of *A. mellifera* honey varied from 0.941±0.0 to 1.15±0.0 g/ 100g results obtain are comparable to study done by Buba *et al*. (2013) who reported the fructose/ glucose ratio in the range of 1.00 -1.45. In previous findings by Parihar *et al*. 2020 studied Himachal honey samples collected from different states showed range from 1.22 to 1.44 g/ 100g which is more than current studies. Honey crystallisation is influenced by fructose glucose ratio. Fructose/Glucose ratio is less than 1.0, crystallization of honey is faster, and when it is greater than 1.0, it is slower (Draiaia *et al*. 2015). Similar findings were reported by Pauliuc *et al*. (2020) where Fructose/Glucose ratio of honey was higher than 1. Another study reported by Kamal *et al*. (2019) Fructose/Glucose ratio was ranged from 1.14-1.34. Our current findings are similar to Afshari *et al*. 2022 were F: G ranged from 0.900- 2.800 with an estimated mean value of 1.32 supported our study. A negative correlation was observed with glucose (-0.668**; $p<0.001$) and positive correlation with fructose (0.681; $p<0.001$) as fructose is more soluble in water then glucose, fructose-glucose ratio indicates ability of honey to crystalize (Amir *et al*., 2010). Our current investigation is similar to Amariei *et al.* (2020).

4.16 FRAP Assay

The FRAP methodology determines honey's reducing power by evaluating the antioxidants' ability to reduce the molecules Fe^{3+}/Fe^{2+} in samples (*Petretto* et al., *2015*). The range of FRAP activity in samples of *Apis mellifera* was 2.563±0.003 to 2.87±3.48±0.015%. According to Gül and Pehlivan (2018) darker honey samples had the highest ferric ion reduction capability, whereas lighter honey had the lowest which supports present investigation. The reduction of ferric ions to ferrous ion was more pronounced when the FRAP was more pronounced at its higher values (Khalil *et al*., 2012).

4.17 DPPH Assay

Honey, like other dietary items, can provide overall hydrogen/electron donating activity through the DPPH radical scavenging function. This was based on an assessment of antioxidants capacity to reduce the DPPH radical. The decrease in absorbance was accompanied by a purple staining of the DPPH (Alves *et al*. 2013). DPPH assay ranged from 15.707±0.093 to 18.817±0 %. The difference in radical scavenging activity might be due to difference in colouration where dark coloured honey samples often exhibit high level of activity in the DPPH (Bertoncelj *et al*. 2007; Blasa *et al*. 2006) which justify our study. The percentage of inhibition exhibited in present study of honey in similiar to Algerian honey, Malaysian (Khalil *et al*. 2011) and Indian honey samples (Saxena *et al*. 2010). Additionally, research by Stagos *et al*. (2018) revealed that tested honey varieties also had potential free radical scavenging activity against DPPH and ABTS radicals which was in in concordant to present investigation. In addition, similar findings were also observed by Larsen and Ahmed (2022).

4.18 ABTS Assay

The ABTS assay is one of the most frequently used analytical method for determining for antioxidant activity. Darker honey often had the highest antioxidant activity and pale honey had the lowest antioxidant activity which was also found in the present study (Beretta *et al*., 2005; Bertoncelj *et al*., 2007). The ability of phenolic compounds to donate hydrogen ions or electrons to free radicals determines their ABTS capabilities (Gasic *et al*., 2014). The DPPH radical scavenging activity of honeys in the ABTS reaction system was substantially lower than the ABTS reaction system. In general, we discovered that honey samples that showed higher effectiveness in DPPH reaction system also showed better inhibition in the ABTS system. Also, according to Isla *et al*. (2011) findings there was a positive correlation with colour (0.507; $p<0.005$) which was found to be comparable to present investigation.

4.19 Antimicrobial assay

According to Tchoumboue *et al*. (2007), microbiological quality in honey suggested hygienic conditions, while bacterial and fungal contamination in honey indicated non-hygienic condition of its handling, processing, and storage settings. The antimicrobial property of honey was determined by using Muller Hinton agar media plates by well diffusion method by

using different pathogenic gram-positive and gram-negative strains of bacteria. Comparatively Chamba honey showed less antibacterial activity. Our current findings of inhibitory bacterial growth were in line with study of Saha *et al*. (2018) as antimicrobial inhibition assay showed the honey samples zone of inhibition. Our present study was in accordance with Chauhan *et al*. (2010), where they reported that raw and processed honey extracts exhibited zone of inhibition ranged from 6.94 to 37.94 mm against different bacterial organisms including, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus spp*., *Salmonella typhi* and *Pseudomonas aeruginosa.*

4.20 NMR

It is a significant analytical method used to identify organic compounds by determining their carbon-hydrogen framework and served as a fingerprinting tool for characterizing different honey samples. In order to characterize organic structures, ^{1}H NMR and ^{13}C NMR was used to determine the type and quality of H and C atoms present in a molecule. ^{1}H NMR experiment was a simple method to maintain the natural ratio of substance to obtain global information regarding the number and nature of protons present in complex samples (Boffo *et al*., 2012). All investigated honey sample by ^{1}H NMR and ^{13}C NMR revealed three major sugars (α-D-glucose, β-D-glucose and β-D-xylose). Boffo *et al*. (2012) also reported α and β-glucose in by ^{1} H-NMR spectra which was similar to our current investigation. Ohmenhaeuser *et al*. (2013) reported D-xylose in the honey. Our results were comparable to previously reported studies of Gerhardt *et al*. (2016) in which ^{1} H-NMR spectra of honey detected sugar region between d 3.0 - 5.5 ppm, typically α and β- glucose and fructose. In addition, xylose was also detected. Gerginova *et al*. (2020) also investigated similar sugar in their studies where 70% of the sugar were found natural sweetness in honey have been found to be contributed by glucose, fructose and sucrose where the composition varied according to the floral sources (Dobre *et al*., 2012). The detection of xylose sugars in the analysed honey samples depicted their significance in promoting health benefits for patients suffering from diabetic ailments, as xylitol was not metabolized in the human body after consumption and was excreted without absorption. This sugar also promoted dental health by preventing the sticking of honey with teeth and also retained high fibre content thus enabling the honey samples as low calories bases for various pharmaceutical and food formulations (Nagel, 2008). The presence of xylose in the given honey samples contributed to their enhanced shelf life due to their antibacterial and antifungal properties.

4.21 Rheology

Honey is a viscoelastic fluid during deformation and flow is dependent on temperature and time. The viscosity decreased as temperature increased because there was less molecular friction and less hydrodynamic force (Mossel *et al*., 2000; Patil and Muskan, 2009; Nayik *et al*., 2016). Changes in viscosity in honey were due to geographical and floral origin also viscosity depends on sugar and protein content. In addition to other characteristics such as honey composition, the water content of honey must be indicated because it has the greatest

influence on honey viscosity. The viscosity is determined by temperature and moisture because average intermolecular forces diminish as temperature rises, kinetic energy rises, and molecules become more mobile, viscosity reduces as temperature and moisture content rise (Patil and Muskan, 2009). The wide range of honey viscosity found around the world can be explained by the fact that chemical composition factors such as moisture content, sugars, and protein levels, which vary with the geographical origins of each honey, have a significant impact on viscosity. These results were similar to the previously reported study by Ahmed *et al*. (2007); Oroian *et al*. (2013) and Boussaid *et al*. (2015) in which the viscosity of Tunisian honey samples decreased slowly with increased temperature. Nayik *et al*. (2016) had similar results to our present findings where similarities were reported to the Indian honey. All studied honey showed Newtonian behaviour and this exceptional shear thickening behaviour could be attributed to the presence of some macromolecular compounds, such as dextran, have a molecular weight in the range of 1,250,000 as validated earlier by Pryce-Jones (1953) and Travnicek and Pridal (2016) for eucalyptus honey.

Conclusion

The analysis of various physiochemical, antioxidant, antibacterial, NMR and rheology parameters in the present study concluded that the honey from various districts of Himachal Pradesh is good quality as most of the analyzed parameters were within in limit range of FSSAI (2018) and International standards. Antioxidant and antibacterial activities provide medicinal and health benefits, all studied honey exhibited high antioxidant properties whereas antibacterial activity showed a zone of inhibition against studied pathogens, Additionally, NMR and rheology studies of honey found that no adulteration or addition of any added sugar/syrups in honey.

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