BIBECHANA
Vol. 22, No. 1, April 2025, 52-62

ISSN 2091-0762 (Print), 2382-5340 (Online)
Journal homepage: http://nepjol.info/index.php/BIBECHANA
Publisher:Dept. of Phys., Mahendra Morang A. M. Campus (Tribhuvan University)Biratnagar

Physicochemical, antioxidant, and antimicrobial study in Nepalese honey

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Abstract

Five honey samples, mostly from western Nepal, were analyzed for physicochemical properties, TPC, TFC, antioxidant, and antimicrobial assays. The results were found to be within the acceptable range, while some samples had higher moisture, HMF, and total ash levels. The TPC was in the range of 8.420 to 9.920 mg GAE/g and the TFC was between 0.039 to 0.103 mg QE/g. The IC_{50} values were calculated of which Forest honey was found to have the lowest IC_{50} value i.e. 7.735 ± 0.008 mg/mL. Additionally, samples also showed a large zone of inhibition against four pathogens. Besides, the correlation between physicochemical parameters, TFC, TPC, and antioxidant activity was established by using Pearson's correlation and PCA. It displayed that the variables IC_{50}, reducing sugar, HMF, and TFC in samples were positively correlated with one another and inversely with total ash (%), acidity as formic acid (%), and moisture (%).

Keywords
Physicochemical parameters, Antioxidant, Antibacterial.

Article information
Manuscript received: June 4, 2024; Accepted: January 16, 2025
DOI https://doi.org/10.3126/bibechana.v22i1.74118

1 Introduction

Bees naturally generate honey as a food item through the collection of either flower nectar from the family: Apidae [1] or the waste of insects that consume living plant parts and insect secretion. The honey sourced from living plant parts and insect secretion is commonly categorized as honeydew honey [2]. Since ancient times, honey has been utilized to cure illnesses such as eye diseases, infections, asthma, and fatigue [3]. It is composed of minor amounts of organic acids, 15-17 % water, and approximately 80-85 % sugars, vitamins, proteins, phenols, and flavonols [4]. Mainly, fructose, glucose, and sucrose are three kinds of carbohydrates found in honey. In addition, honey contains nearly all minerals in minute amounts including potassium, sodium, phosphorus, sulfur, calcium, iron, manganese, magnesium, silicon, and copper. The most prevalent mineral element is potassium, which accounts for almost one-third of the overall amount [5]. The presence of potassium, cadmium, and nickel indicates honeydew honey [6]. It is suggested that these minor elements of honey are associated with various biological activities to exemplify antioxidant, antibacterial, antiviral, anti-inflammatory, antiulcer, antihypertensive, vasodilator, antihypercholesterolemic, and antitumor activity [7]. It contains various enzymes which include diastase, invertase, catalase, glucosylcerami-

dase, glucose oxidase, α-amylase, β-glucosidase, α-glucosidase, and proteases. Diastase breaks down starch into dextrin and maltose contributing to the honey quality, while invertase transforms sucrose into glucose and fructose [8]. Glucose oxidase is involved in the conversion of glucose to δ-gluconolactone, followed by hydrolysis of gluconic acid and hydrogen peroxide, providing honey with antimicrobial properties [9].

Honey could be the best choice for use in medicinal value, as in recent times, the multi-drug resistance problem has arisen and this problem can be solved by the natural products singly or in combination with drugs. Besides therapeutic benefits, some honey e.g. Mad honey can also pose toxicity leading to a serious food safety concern. Such toxicity and hallucinating effects are due to the honey bees collecting poisonous plants nectar like *Rhododendron* species, *Coriaria arborea*, and *Tripterygium wilfordii* Hook F.. Mad honey contains natural plant poisons, such as pyrrolizidine, tutin, triptolide and grayanotoxins [10]. The chemical makeup, taste, color, and biological activities are primarily determined by its floral origin, species of bees, climate, and geographic area, however can additionally be altered by the weather circumstances, as well as their handling, packing, and storage [11]. In Nepal, five honeybee species are found. *Apis cerana* and *Apis mellifera are suitable* for hive management and honey production whereas *Apis laboriosa*, *Apis dorsata*, and *Apis florea* are wild species [12]. Among them, only *Apis mellifera* is an exotic species, while the remaining four species are indigenous to Nepal [13]. The majority of the honey produced in Nepal is the multi-floral origin, but some are also unifloral origins like chiuri, mustard, buckwheat, rudilo, sunflower, and litchi honey, as well as honeydew honey from pine, spruce (Salle Maha), and oak (Dalle Maha) trees mostly in mountain region [12].

For the import and export of honey, the global standard is contained in the Codex Alimentarius. The Codex Alimentarius standard, European Honey Directive, and National Standard are the standards that set rules for food safety, regulation, and honey authenticity [14]. It has been reported that the various physicochemical parameters including moisture, sucrose, reducing sugar, total ash, acidity as formic acid, hydroxymethylfurfural (HMF), fructose/glucose ratio, etc are widely used to analyze botanical authentication of honey. In honey, glucose and fructose are present in higher amounts whereas twenty-two different compounds such as isomaltose, maltose, sucrose, etc are present in small amounts. Further, a nondigestible molecule (fructooligosaccharides) supports the growth of intestinal bacteria and helps to enhance the human digestive system. Thus, it is also named as prebiotics [15, 16]. Honey also contains low-fat, water-soluble vitamins and vitamin C in higher amounts, whereas the fat-soluble vitamins and vitamin B are present in small amounts [17].

Nepal has a vast array of honey types and huge potential for honey production, yet it has not fully reached its capabilities in honey harvesting due to limited research and investment in Nepal. Thus, a key goal of this study is to explore the physicochemical parameters, antioxidant properties, and antimicrobial test, and conduct principal component analysis on varieties collected which include local, market-sourced, and forest honey.

2 Materials and Methods

2.1 Chemicals

We acquired quercetin (CAS No: 117-39-5) from Sigma-Aldrich in Germany. Gallic acid (CAS No: 5995-86-8) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (CAS No: 1898-66-4) were purchased from Molychem in India and HiMedia (India), respectively. Growth medium (MHA and MHB) were obtained from Sigma-Aldrich. Other chemicals were bought from Fisher Scientific (India).

2.2 Collection of honey samples

Samples of honey were collected from multiple places, including Pokhara, Rukum, Lamjung, and Kathmandu (**Table 1**). The honey types studied were Forest honey, Mad honey, Mustard honey, Chestnut honey, and Indian brand-marketed honey.

2.3 Physicochemical parameters

The physicochemical parameters included by the International Honey Commission for the quality control of honey such as moisture, sucrose, reducing sugar, total ash, acidity as formic acid, Hydroxymethyl Furfural (HMF), and fructose/glucose ratio were analyzed [18].

Moisture

In honey, the moisture content was analyzed by operating the refractometric method with the help of an Abbe refractometer [19].

Total ash content

Five grams of honey were subjected to a muffle furnace at 55 °C for five hours. Afterward, the ash was obtained by cooling it to room temperature and then weighed [20].

Acidity as formic acid

Acidity as formic acid is calculated by using the titrimetric method [20].

Hydroxymethylfurfural

The UV-spectrophotometric method was employed to analyze and determine the levels of HMF [18]. Firstly, 25 mL water and 5 g honey were added to a 50 mL volumetric flask. Further, solution I and II of 0.5 mL carrez solution were added on, mixed, and diluted with water. After filtration, 5 ml of filtrate was taken in two test tubes. In one test tube, distilled water of 5 ml was added, while the other test tube received 5 ml of a 0.2% sodium bisulfite solution as the reference. The contents within each tube were effectively blended in a vortex mixer. Subsequently, the sample's absorbance was determined at 284 nm, and the absorbance of the reference was assessed at 336 nm. Finally, the HMF (mg/100 g) content was computed based on the measurements.

Table 1: List of honey samples collected for the study from different regions of Nepal.

S. N.	Name of Sample	Floral Source	Botanical Classification	Floral Location	Local Name
1	Mustard honey	Mustard	*Brassica napus*	Pokhara	Tori
2	Forest honey	Forest	Unspecified	Rukum	Vire Rukum
3	Mad honey	Multi-floral	*Rhododendron arboreum* and forest	Lamjung	Vire Pokhara
4	Indian brand marketed honey	Market	Unspecified	Kathmandu	Market honey
5	Chestnut	Chestnut	*Castanea sativa*	Pokhara	Katus

pH

The pH of honey was measured by a pH meter. 3 g of sample of honey was dissolved in 30 mL of water and mixed by agitating for 30 minutes. At ambient temperature, the pH of the samples was determined [21].

Reducing sugar

The AOAC (2005) method was used to analyze reducing sugar content in honey [20]. 1 gm of honey was added in 10 mL lead acetate solution (20%) taken in a volumetric flask of 250 mL which was diluted up to the mark and filtered. The filtrate (25 ml) was combined with 100 mL of distilled water in a 500 ml volumetric flask. A few drops of 10 % potassium oxalate solution were added until no more precipitate formed. In a 250 ml conical flask, 5 ml each of Fehling A and B were combined with 10 ml of distilled water, boiled, and using the sample solution to titrate and find the amount of reducing sugar content.

Sucrose

The Fehling solution method was employed to determine the sucrose content in various types of honey [22]. The quantity of sucrose present was determined using the provided formula below:

$$Sucrose\ \% = (Total\ reducing\ sugars\ \% - Reducing\ sugars\ \) \times 0.95$$

2.3.1 Glucose ratio

The glucose percentage was determined using an iodometric method, and this amount was deducted from the percentage of reducing sugars to calculate the fructose percentage and the fructose-to-glucose ratio. The calculations were performed using the formulas provided below [23].

$$Glucose\ \% = \frac{N\ of\ thiosulphate\ \times (blank - titre)\ \times a \times 100\ 0.1\ N\ \times b}{2 \times 0.1N \times b}$$

where a=0.009005, b= weight pf sample

$$Fructose\ \% = Reducing\ sugar\ \% - Glucose\ \%$$

$$Fructose\ :\ \ Glucose\ ratio\ =\ \frac{Fructose\ \%}{Glucose\ \%}$$

Impurities

50 mL of water was used to dilute each honey sample of 10 g and blended in a shaker for about 30 minutes. Filtration was done and the sample obtained was kept in the oven at 103 ^{o}C for 10 minutes and the dried filter paper was weighed [21]. By using the formula the percentage of impurity was measured.

$$I = \frac{m_2}{m_1} \times 100\%$$

'I' represents the quantity of impurities (%), 'm_1' represents the mass of the sample taken for analysis (g), and 'm_2' represents the mass of residue left on the filter paper after drying (g).

2.4 Total Phenolic Content

As per the study, the total phenolic content was determined using the Folin-Ciocalteu method with fewer adjustments [24]. Gallic acid was taken as a standard (1 mg gallic acid dissolved in 1 mL ethanol to create a gallic acid solution). It was serially diluted ranging from 10 to 100 µg/mL to prepare solutions of different concentrations. For each honey sample, 5 mg/mL of honey solution was prepared by dissolving in 70 % ethanol, 20 µL (at a concentration of 5 mg/mL) was mixed with 100 µL of Folin-Ciocalteu's reagent (diluted 1:10 with water), and 80 µL of 1 M aqueous sodium carbonate solution (Na_2CO_3). After about 30 minutes at ambient temperature, the mixture's absorbance at 765nm was analyzed using a microplate reader. The extracts' total polyphenolic content was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of the extract. This analysis was done in triplicate.

2.5 Total Flavonoid Content

As outlined by Pham et al., the aluminum chloride colorimetric method was utilized to determine the total flavonoid content (TFC) with minor alternations [25]. In brief, a concentration of 5 mg/mL of honey was made ready adding 70 % ethanol. Subsequently, 20 µL of each extract was combined with 100 µL of distilled water and 60 µL of ethanol. Subsequently, 10 µL of 10% $AlCl_3$ and 10 µL of 1M CH_3COOK were added to each well. Quercetin was used as the standard reference. After that, the mixture was left at room temperature for 30 minutes in the shade, after which the absorbance was measured at 415 nm using a microplate reader. The overall flavonoid concentration within the samples was expressed as milligrams of quercetin equivalent per gram of dry weight (mg QE/g) of the extract. This analytical process was conducted in triplicate.

2.6 Antioxidant Activity

The assessment of antioxidant activity was conducted by employing the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) [25]. 100 µL of DPPH solution in methanol (0.1 mM) along with 100 µL of honey solution made in methanol at different concentrations (1000, 500, 250, 125, and 62.5 µg/mL) were loaded into a 96-well plate. Then, it was kept in the shade for 30 minutes and the absorbance was recorded at 517 nm. Further, Quercetin was employed as a standard. Here, a reduction from DPPH radical (purple) to diphenyl picryl hydrazine (yellow) was observed at varying concentrations. The antioxidant used for the determination of the ability to inhibit DPPH radical was obtained by the below equation:

$$\% \quad scavenging \quad = \quad \frac{A_0 - A_t}{A_0} \quad \times \ 100\%$$

Here, "A_o" represents the absorbance of control, while "A_t" corresponds to the absorbance of DPPH when exposed to a test or reference sample.

2.7 Antibacterial Activity

The antibacterial activity of honey samples at various concentrations (10% W/V, 20% W/V, 50% W/V, and 75% W/V) against four distinct bacterial strains (namely: *E. coli, S. aureus, S. shigella, K. pneumoniae)* was examined through the agar well diffusion method on MHA plates [26]. A 0.5 McFarland equivalent of microorganism inoculum in Mueller Hinton broth (MHB) was spread on the MHA plate's surface using an autoclaved cotton swab. Wells were bored by the clean and well-sterilized cork-borer in cultured MHA media. Then, a concentration of 100mg/mL of honey extracts was filled into the wells. For the positive and negative control, neomycin and distilled water were used respectively and incubated at 37^{o}C for 18 to 24 hours. Finally, the zone of inhibition (ZOI) was observed and measured by a ruler in mm.

2.8 Statistical Analysis

The MS-Excel was used to do a Karl Pearson correlation between the variables such as physicochemical parameters, TPC, TFC, and antioxidant activity. Additionally, to correlate several physicochemical data (such as moisture content, sucrose, reducing sugar, total ash, acidity as formic acid, HMF, fructose/glucose ratio, and pH), TPC, TFC, and antioxidants. Further, using R studio Principal Component Analysis (PCA) was performed. PCA is a dimensionality reduction technique that transforms a set of numerous variables into a smaller set that retains most of the information from the original large data [27]. Pearson correlation helps to determine the degree and direction of correlation. **Table 2** shows the degree of correlation as follows [28].

Table 2: The degree of correlation is represented by the numerical values.

Amount of r	Strength of the Correlation
$0.0 < 0.1$	No correlation
$0.1 < 0.3$	Low correlation
$0.3 < 0.5$	Medium correlation
$0.5 < 0.7$	High correlation
$0.7 < 1$	Very high correlation

Note: 'r' indicates Karl Pearson's correlation coefficient.

3 Results and Discussion

3.1 Physicochemical-parameters-1

The following Physicochemical parameters of all samples were determined and the results were mentioned below (**Table 3**).

3.1.1 Moisture

The standard set by the Codex Alimentarius must be below 20%. The honey sample above the set standard will undergo deterioration by fermentation [29]. The water content of mustard, market, and chestnut honey fell within the accepted range, while forest honey (24.5%) and mad honey (25.7%) exceeded the standard range. The level of moisture is determined by factors like the relative humidity of the plant source, processing techniques, and storage conditions [30]. Moisture plays a crucial role in regulating microbial growth and the potential spoilage of honey through fermentation [31].

3.1.2 Total ash content

The total ash content in samples is useful for the identification of honey. Here, the total ash content was from 0.1 to 0.7 in which all samples were within the standard limit except Forest honey with a 0.7 value and the lowest shown was market honey [32]. Out of five samples, one sample (forest honey) was out of range.

3.1.3 Acidity as formic acid

Inorganic ions, organic acids (sulfate, chloride, and phosphate), esters, and lactones are present in honey as free acidity[30]. Vitamin C, protein, and phenolic acids are the proton donors and help in identifying the quality of honey. As the honey worsens its free acidity increases and fermentation is seen by the conversion of sugar to organic acids. Acidity as % formic acid consists of 0.001 to 0.01%. The lowest value of acidity as % formic acid is of Indian brand market honey and the highest is of Mustard and Forest honey which are present in an acceptable range.

3.1.4 Hydroxymethylfurfural (HMF)

The HMF of various samples was from 2 to 87 mg/kg of which the lowest was that of Forest honey and the highest was of Indian brand market honey which exceeded the given standard value. HMF levels serve as a common measure for assessing the freshness of honey, as it is typically undetectable or found in minute amounts in newly harvested kinds of honey. However, its content tends to increase due to processing and aging [33]. Indian brand market honey is processed honey and is made available in the market after a prolonged period, this could potentially account for the elevated levels of HMF observed in market honey.

3.1.5 Reducing sugar

The reducing sugar of different samples of honey was from 63.8 to 74.3 %, in which the highest amount of reducing sugar was present in market honey and the least in Mad honey. The reduced sugar content in all the analyzed samples is up to the standard range. The amount of sugar present in honey is influenced by factors such as the source of nectar from different types of flowers, the prevailing climate, additionally the methods used in handling and keeping [34].

3.1.6 Sucrose

Among the five honey samples, the sucrose content ranged from 0.1% to 7.2%. The lowest sucrose content (0.1%) was found in Mad honey, while the highest content (7.2%) was observed in chestnut honey. The sucrose content of different honey from different places was within the standard range (10).

3.1.7 Fructose/Glucose ratio

The chestnut honey exhibited a fructose/glucose ratio of 1.34, while the other honey samples showed a ratio of 1.2. The carbohydrate composition of honey is responsible for its capacity to retain moisture, prolong shelf life, react in microwaves, and enhance the development of colors and flavors. The fructose-to-glucose ratio determines the ability to crystallize honey. Honey crystallizes slowly if the fructose/glucose ratio exceeds 1.3 whereas it occurs quicker when the proportion is less than 1.0 [35].

3.1.8 pH

The honey samples' pH values varied from 3.26 to 5.80 depending on organic acids and these organic acids are responsible for the aroma and antimicrobial activity of honey. All the honey samples were within the standard pH range except the chestnut honey. The pH difference is based on the maturity period, the concentration of minerals, and chemical composition [36]. The pH of the floral and wild honey in this research work shows a resemblance with the range of pH in a study by Alves et al. [37].

3.1.9 Impurities

The impurities percentage in the samples was in the range of 1.11%-6.77%. The highest impurities were found in market honey. This might be due to the adulteration, additives, and inclusion of other substances during processing.

Table 3: Physicochemical parameters and impurities in five honey samples.

S.N.	Name of Sample	Moisture (%)	Total Ash (%)	Acidity as Formic Acid	pH	Impurity (%)	HMF	Reducing Sugar (%)	Sucrose (%)	Fructose/Glucose Ratio
1	Mustard honey	21.3	0.2	0.01	3.26	2.50%	40	68.5	3.4	1.2
2	Forest honey	24.5	0.7	0.01	4.22	4.10%	2	63.9	0.2	1.2
3	Mad honey	25.7	0.4	0.004	4.42	4.66%	12	63.8	0.1	1.2
4	Market honey	19.2	0.1	0.001	4.78	6.77%	87	74.3	1.0	1.2
5	Chestnut honey	20.2	0.4	0.004	5.80	1.11%	-	68.1	7.2	1.34
6	Standard Range	≤ 23	≤ 0.5	-	3.5-5.5	-	≤ 40	≥ 60	≤ 10	≥ 0.95

3.2 TPC, TFC, and Antioxidant Activity

The TPC, TFC, and free radical scavenging activity were assessed in various honey extracts, revealing TPC value in a range of 8.420 ± 0.703 to 9.920 ± 0.649 mg GAE/g, while TFC value was less than 1 mg QE/g in all the analyzed honey samples. Among the collected samples, the antioxidant activity (based on IC_{50} value) of honey samples followed the order: Forest honey > Mad honey > Chestnut honey > Mustard honey > Indian brand Market honey. In comparison to the standard quercetin (IC_{50} value $= 0.34 \pm 4.11$), all analyzed honey samples displayed lower antioxidant activity. These values are compared with data from similar studies on honey's antioxidant properties and found similar results [38–41]. The TPC, TFC, and antioxidant activity (IC_{50} values mg/mL) are displayed in **Fig 1, Fig 2, Fig 3,** and **Table 4**.

Table 4: TPC, TFC, and IC_{50} values of different honey samples.

Honey Samples	**TPC (mg GAE/g)**	**TFC (mg QE/g)**	**IC_{50} (mg/mL)**
Mustard honey	8.976 ± 1.395	0.053 ± 0.004	11.628 ± 0.004
Forest honey	9.809 ± 1.345	0.039 ± 0.011	7.735 ± 0.008
Mad honey	9.106 ± 1.473	0.044 ± 0.008	10.2 ± 0.002
Indian brand market honey	9.920 ± 0.649	0.103 ± 0.066	12.51 ± 0.003
Chestnut honey	8.420 ± 0.703	0.055 ± 0.012	10.92 ± 0.005
Quercetin	-	-	0.34 ± 4.11

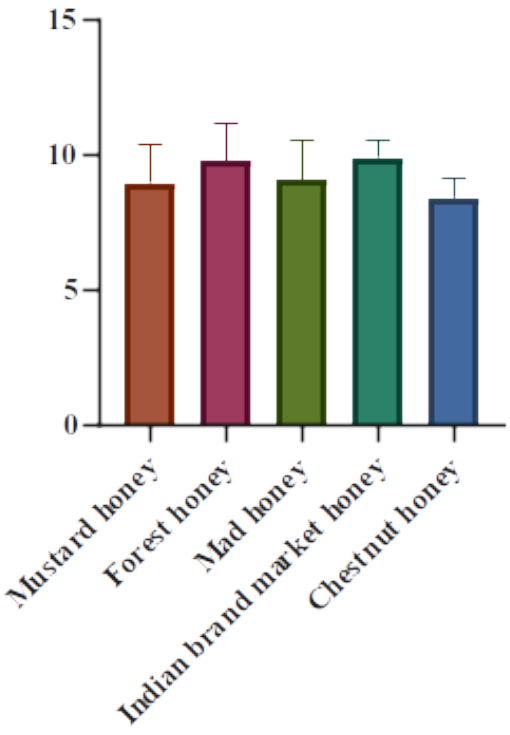

Figure 1: Bar diagram of TPC of honey samples.

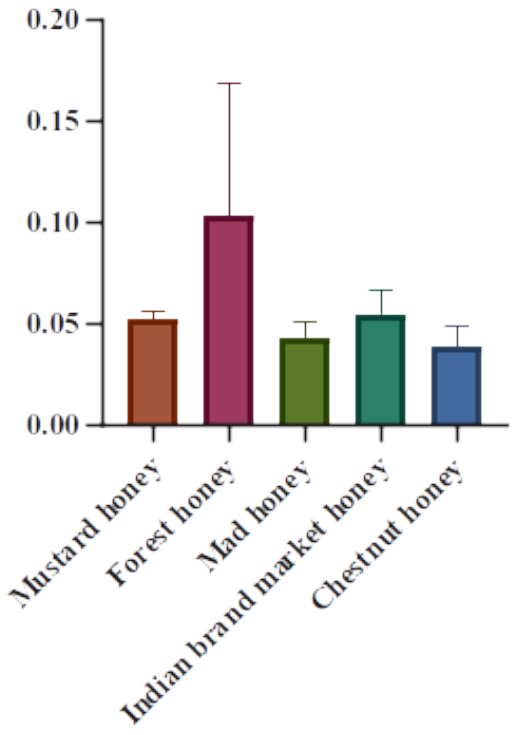

Figure 2: Bar diagram of TFC of honey samples.

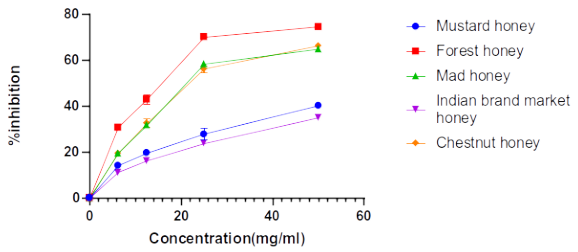

Figure 3: The plot of % inhibition of DPPH Vs concentration of different honey samples.

3.3 Correlation of bioactive compound and antioxidant activity

We observed the normal distribution of the response variable (antioxidant activity) ($p>0.05$) using the Shapiro-Wilk normality test [42] The linear correlation between the two variables was assessed by using the Karl Pearson correlation coefficient. The results of the correlation between the variables are provided in **Table 5**.

The moisture (%) exhibited a moderately negative relation with sucrose and HMF, while it displayed a strongly negative relationship with reducing sugar content (%), TFC, and IC_{50} value. The sucrose (%) had a minimal connection with acidity in the form of formic acid (%) and TFC. Sucrose (%) demonstrated a highly negative correlation with TPC (-0.816), but a notably positive correlation with the ratio of Fructose/Glucose ratio (0.897). The percentage of reduced sugar exhibited a positive correlation with TFC, HMF, and IC_{50} values. The proportion of total ash (%) showed no significant correlation with the fructose/glucose ratio, TPC, and pH. However, it revealed a strong negative correlation between TFC and IC_{50} value. HMF displayed a markedly positive correlation with TFC and IC_{50} values. HMF has an extremely strong positive relationship with the TFC (0.909).

The relationship between TPC and TFC is positive, suggesting that most of the phenolic compounds in these honey types are flavonoids. In the provided **Table 5**, TPC and TFC exhibit a moderately negative correlation with the IC_{50} value for DPPH free radical scavenging. This implies that higher TPC and TFC levels correspond to lower IC_{50} values, indicating that as the phenolic and flavonoid content increases, the honey's ability to neutralize free radicals improves. This connection between phenolic/flavonoid content and antioxidant activity aligns with the findings of numerous studies.

Conversely, the correlation between TFC and IC_{50} value (a measure of the level of antioxidants) is positive according to the table. This suggests that antioxidant activity isn't solely attributed to flavonoids but might be influenced by other factors such as HMF, enzymes, proteins, and minor constituents. In certain instances, flavonoids might even interact with other compounds that possess antioxidant properties, potentially diminishing overall antioxidant effectiveness. Similar outcomes have been observed in prior honey-related research [43–45].

Table 5: Karl Pearson's correlation between physicochemical parameters, TPC, TFC, and IC_{50}.

Correlation	Moisture (%)	Sucrose (%)	Reducing Sugar (%)	Total Ash (%)	Acidity as formic acid (%)	HMF (mg/kg)	Fructose/Glucose ratio	TPC (mgGAE/g)	TFC (mgQE/g)	pH	IC_{50}
Moisture (%)	1										
Sucrose (%)	-0.577	1									
Reducing Sugar (%)	-0.907	0.209	1								
Total Ash (%)	0.685	-0.160	-0.827	1							
Acidity as formic acid (%)	0.390	-0.058	-0.548	0.502	1						
HMF (mg/kg)	-0.605	-0.253	0.873	-0.828	-0.447	1					
Fructose/Glucose ratio	-0.395	0.897	0.049	0.097	-0.250	-0.431	1				
TPC (mgGAE/g)	0.097	-0.816	0.216	0.036	-0.027	0.520	-0.743	1			
TFC (mgQE/g)	-0.759	-0.027	0.952	-0.767	-0.696	0.909	-0.083	0.400	1		
pH	-0.315	0.489	0.174	0.093	-0.670	-0.195	0.792	-0.304	0.227	1	
IC_{50}	-0.752	0.326	0.833	-0.980	-0.573	0.742	0.099	-0.180	0.754	0.076	1

3.4 Principal Component Analysis of Different Variables

In this analysis using Principal Component Analysis on the dataset, 50.4% of the variation is explained by the first principal component (PC1), and 31.4% is explained by the second principal component (PC2). The scree plot, shown in **Fig 4**, visually displays the percentage of variation represented by each principal component. It reveals that there are two prominent principal components that, when combined, account for 81.8% of the total variance present in the five distinct samples of honey. The two-dimensional graph of two prominent principal components that includes 81.8% of the total variation of the data is shown in **Fig 5**.

Fig 5 is a variable correlation plot that shows the relationship between the variables of honey samples. HMF, TFC, reducing sugar, and fructose/glucose showed a better representation of variables. Reducing sugar, TFC, and HMF again fructose/glucose ratio, pH, and sucrose are positively correlated. Kowalski et al. also showed a positive correlation with fructose/glucose ratio, pH, and sucrose[46]. IC_{50} is highly positively correlated with each other and negatively correlated with Total Ash (%), acidity as formic acid, and Moisture (%). Total ash and f/g ratio, acidity as formic acid, and TPC are approximately right-angled indicating no correlation between them.

A PCA biplot is shown in **Fig 6**, Honey samples Mustard, Chestnut, Forest, Mad, and market honey were given by points 1, 2, 3, 4, and 5 respectively and the variables were represented by the vectors. Based on this biplot, Points: 2, 4, and 5 have a major impact on the variability of the data and in principal components, and observations 2 and 3 share similarities in terms of their variability in their respective datasets. The components of biplot Dim 1 and 2 gave 81.8% of the variance in the descriptive profile of five different samples. Kowalski et al. showed the first two components of 68.67% of the variance [45].

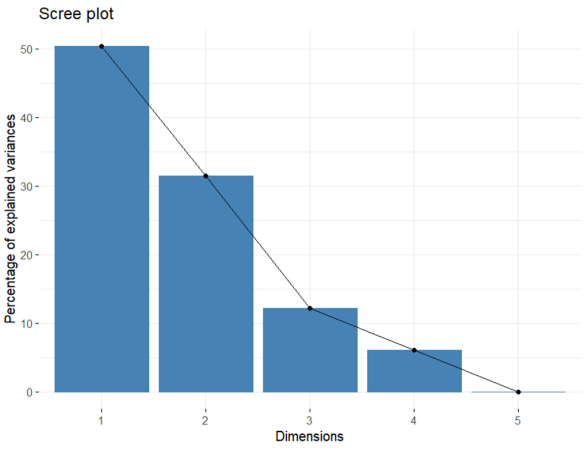

Figure 4: Scree plot of the principal components.

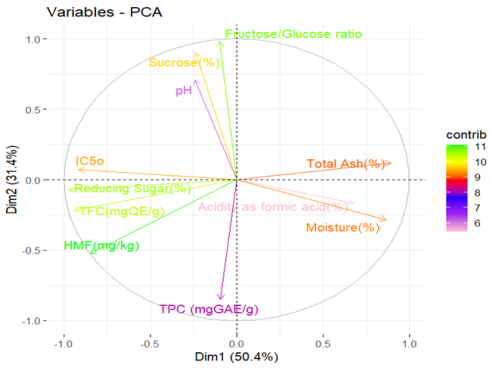

Figure 5: The principal component plot of variables.

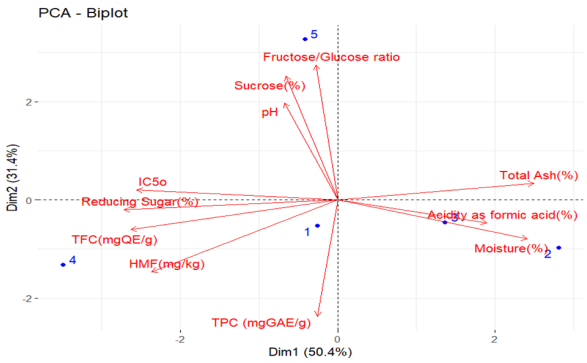

Figure 6: Principal component biplot of observations and variables.

3.5 Antibacterial Activity

The agar well diffusion method was employed to assess the susceptibility of four bacterial strains (*E. coli, S. aureus, S. shigella,* and *K. pneumoniae*) to various concentrations of samples, as outlined in **Table 6**. The largest zone of inhibition (ZOI), measuring 36 mm, was observed at a 50% concentration of two honey samples (Chestnut honey and Forest honey) against E. coli. Mustard honey and Market honey exhibited no significant impact on *E. coli.* However, market honey demonstrated the highest inhibitory effect against the other three bacterial species: *S. aureus* (34 mm), *S. shigella* (35 mm), and *K. pneumoniae* (30 mm). Mustard honey was also ineffective against *S. aureus* but showed a moderate effect against *S. shigella* and *K. pneumoniae.* This study found antimicrobial activity at 50% v/v of sample concentration of almost all samples to the four microorganisms (*E. coli, S. aureus, S. shigella, K. pneumoniae*). Fj et al. have also reported similar antimicrobial activity in all the analyzed honey samples at a concentration greater than 60% v/v against the four different microorganisms (*E. coli, S. aureus, P. aeruginosa, and B. subtilis*) [46, 47].

In general, the zone of inhibition tended to be slightly larger for gram-negative bacteria in contrast to gram-positive bacteria. This divergence in results can be attributed to the structural characteristics of the bacteria and their distinct mechanisms of toxicity. The major antibacterial element in honey is primarily hydrogen peroxide which disturbs the structure and function of bacteria resulting in their diminished viability due to the oxidation of their constituent molecules [48]. The relative amounts of glucose oxidase, which bees create, and catalase from flower pollen affect its levels [9].

Table 6: Zone of inhibition of honey samples at different concentrations.

Samples	E. coli (mm)				S. aureus (mm)				S. shigella (mm)				K. pneumoniae (mm)			
	10%	20%	50%	P.C.	10%	20%	50%	P.C.	10%	20%	50%	P.C.	10%	20%	50%	P.C.
Chestnut honey	12	22	36	14	8	8	9	12	12	24	26	13	0	9	10	16
Forest honey	18	20	36	18	0	0	0	10	0	28	29	12	25	28	28	15
Mad honey	10	22	32	19	12	22	25	0	0	22	28	11	0	0	0	0
Mustard honey	0	0	0	0	0	0	0	23	22	28	30	22	25	25	30	20
Market honey	0	0	0	0	18	22	34	23	24	28	35	20	28	30	30	20

4 Conclusion

A comprehensive examination of five honey samples gathered from diverse regions of Nepal was conducted and considerable variability was found in the physicochemical parameters, TFC, TPC, antioxidant activity, and antibacterial activity. It might be due to unique geographical regions and botanical origins. The pH and impurities of all the samples examined were within the range of the quality standard except for moisture (due to premature harvest), ash content (indicates honeydew honey), and HMF (due to processing and storage conditions). Indian brand market honey displayed comparatively lower antioxidant properties among the samples, possibly due to ingredient loss during processing and packaging, and exhibited lower antioxidant activity. The samples were very efficient in opposition to the tested gram-negative bacteria displaying a high zone of inhibition, 36 mm at a 50% concentration of two types of honey (chestnut honey and Forest honey(R) against *E. coli*. Market honey exhibited noteworthy inhibitory effects against *S. aureus* (34 mm), *S. shigella* (35 mm), and *K. Pneumoniae* (30 mm). The findings of this study demonstrated the important physiochemical parameters, TPC, TFC, and potential of honey ranging from its antioxidant properties to its antimicrobial activity, suggesting it is a natural therapeutic agent. Further study is necessary to identify the bioactive substances, their particular targets, and their mechanisms of operation as antioxidant and antibacterial agents.

Abbreviations

MHA Muller Hinton Agar
TPC Total Polyphenol Content
TFC Total Flavonoids Content
GAE Gallic Acid Equivalent
QE Quercetin Equivalent
DMSO Dimethyl Sulphoxide
DPPH 2, 2 Diphenyl-1-picrylhydrazyl
IC_{50} Concentration provides 50% Inhibition
MHB Muller Hinton Broth
ZoI Zone of Inhibition
mg Milligram
µL Microliters
DFTQC Department of Food Technology and Quality Control
IHC International Honey Commission
MGO Methylglyoxal
HMF 5-hydroxymethylfurfural
PCA Principal Component Analysis
F/G(f/g) Fructose/Glucose ratio
CFU/mL Colony-forming unit per milliliter
MDR Multidrug-resistant
ROS Reactive oxygen species

Conflicts of Interest

The authors declare no conflicts of interest.

Authors contributions

Conceptualization, N.P.; methodology, A.A.; writing-original draft preparation, N.K.T, A.A.; supervision, N.P, Review, A.G.; All authors have read and agreed to the published version of the manuscript.

Acknowledgments

The authors would like to express their gratitude towards the DFTQC, Babarmahal, Kathmandu, for laboratory support.

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