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Quality Evaluation of *Apis laboriosa* and *Apis mellifera* Honey Collected from Bagmati Province, Nepal

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Abstract

Honey is a natural sweet substance produced by *Apis sp.* from floral nectar or other plant parts which are gathered, modified and stored in the honeycombs by honeybees. The current research was aimed to analyze the quality parameters of locally available honey. Honey samples of *Apis laboriosa* and *Apis mellifera* were collected during spring of 2019 & 2021 and autumn 2021 from the Bagmati province, Nepal. Samples were analyzed their physicochemical and phytochemical properties. The result shows that, the pH was ranged between [4.467±0.0306 - 5.05±0.02], rheological studies showed Newtonian flow and pseudo-plastic type of Non-Newtonian flow, specific optical rotation was ranged between [(+) 5.75±0.4684 - (-) 12.71±0.234], specific gravity was ranged between [1.35±0.00017 - 1.409±0.00022], moisture content was ranged between [19.2% - 25%]. Secondary Metabolite screening showed the honey samples possesses flavonoids, saponins, glycosides, tannins, amino acids, protein and reducing sugar. Total phenolic content was ranged between [1.0427 - 6.86288] gm GAE/Kg honey while total flavonoid content ranged between [0.016755 - 0.353132] gm QE/Kg Honey. IC_{50} obtained from DPPH assay ranged between [649.6465 - 9867.1617] ppm. Properties and qualities of honey are affected by seasonal factors and various floral sources. The samples were in positive correlation between flavanoid content, phenolic content and their respective anti-oxidant potency.

Keywords: Honey, Quality, Physicochemical Properties, Phytochemicals, TPC, TFC, Antioxidant.

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Introduction

Honey is a natural sweet substance produced by honey bee, which can be classified based on type of honey source, floral and extra floral honey[1]. *Apis laboriosa* is world's largest honeybee species with measurement of up to 3.0 cm, which are found at an altitude range from 2,500 m to 4,000 m above the sea level, building their nests commonly in higher altitude, 1,200 m. above the sea level. Honey is harvested twice a year in spring season and in autumn season, with Spring one showing strong medicinal property than autumn one [2].The medicinal property of honey is that, the floral distribution of the region, where *Apis laboriosa* lives; is the plants that belong to *Ericaceae* (Rhododendron) family, which have a psychoactive and hallucinogenic group of phytochemistry known as Grayanotoxins[3]. In Nepal, it is estimated that over 10,000 MT of honey is produced and every year growing the production. Honey is reported to contain at least 181 substances, high nutritional value, high refractive index, high viscosity and Specific gravity [4][5][6]. To the best our knowledge, this is the first comparative study on Nepalese honey to investigate wide range of quality parameters. The present study was aimed to carry out the quality assessment and characterization of honey of *Apis laboriosa* harvested from Bagmati province, Nepal.

Material and methods

Honey Collection

In this study, honey samples of *Apis laboriosa* and *Apis mellifera* were harvested on spring of 2019 & 2021 and autumn 2021 from Bagmati Province, Nepal. The information about samples profile is as given below in the **Figure 1, Figure 2** and **Table 1**.

Physical Properties

In a view to identifying the purity of honey based on physical properties following preliminary tests were performed.

Sand Sinking Test

In this test, 50 g sand was taken from nearby and was sieved through Mesh Size 30. The sand was then allowed to dry completely in oven at a temperature of 95°C for 15 Minutes. The particle distribution profile of the sand was examined using electromagnetic sieve shaker (Model: EMS-8, Instrument Manufacturer: Electropharma). After that, four petri plates were kept in a row.

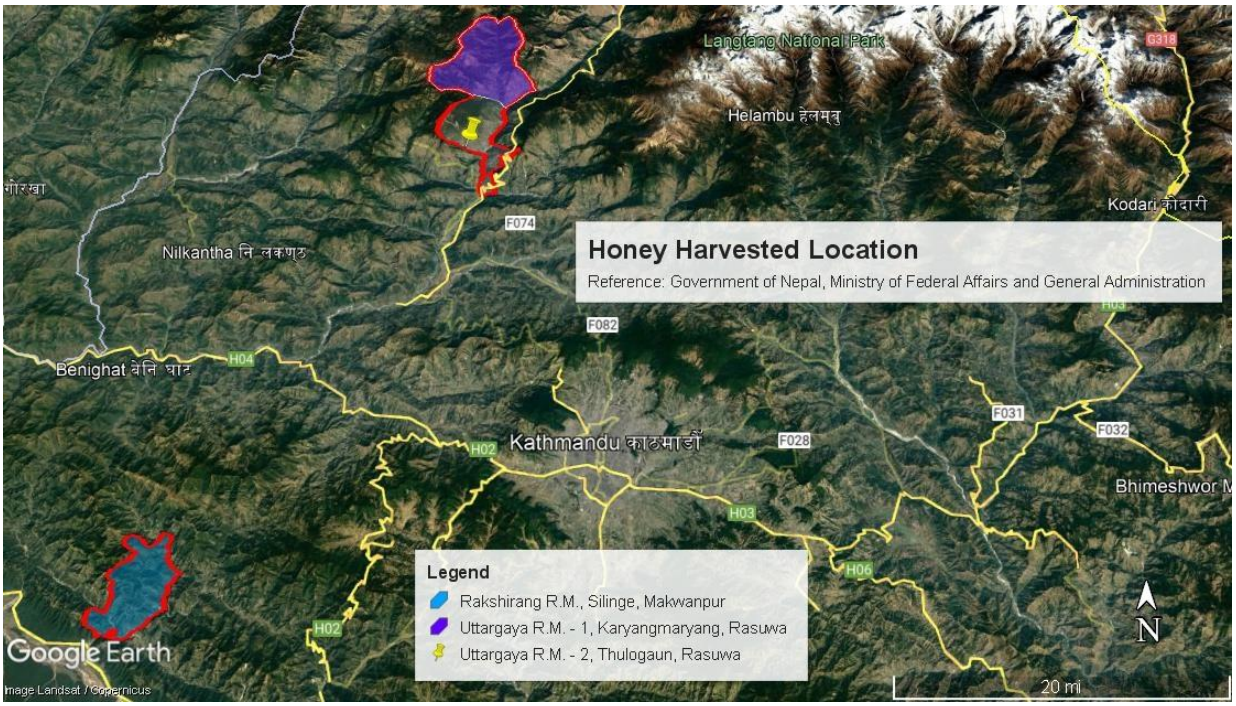


Figure 1. Google Earth Snapshot showing Honey Harvested Location

Table 1. Honey collection site of Bagmati Province, Nepal

Sample No.	Harvested Time		Types of Honey	Honeybee Species	Harvested Location
	Year	Season			
S_1	2019	Spring	Honeydew Honey	*Apis laboriosa*	Uttargaya R.M. – 1, Karyangmaryang, Rasuwa [39.1 Km Perimeter, 71.9 sq. Km Area]
S_2	2021	Spring	General Nectar Honey	*Apis laboriosa*	Uttargaya R.M. – 2, Thulogaun, Rasuwa [28° 1'28.36"N, 85° 9'50.73"E]
S_3	2021	Autumn	General Nectar Honey	*Apis laboriosa*	Uttargaya R.M. – 2, Thulogaun, Rasuwa [28° 1'28.36"N, 85° 9'50.73"E]
S_4	2021	Autumn	*Diploknema butyraceae* Nectar Honey	*Apis mellifera*	Rakshirang R.M., Silinge, Makwanpur [42.7 Km Perimeter, 48.8 sq. Km Area]

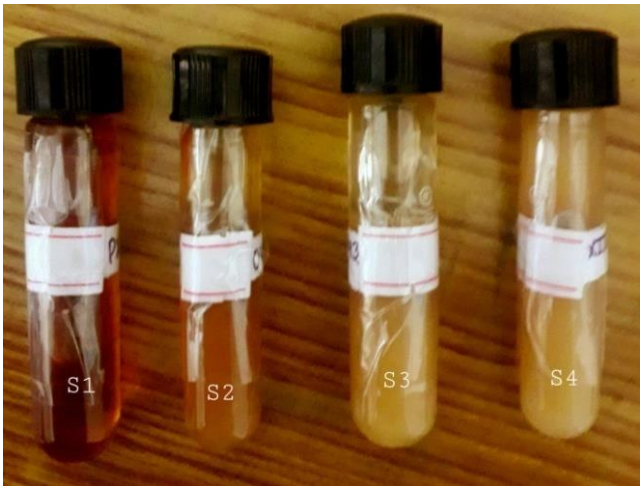


Figure 2.f Different honey samples (From Left to Right; S_1, S_2, S_3, and S_4).

With the help of a tripod stand, the funnel was set and the sand was allowed to flow through the funnel, which created a conical shape of sand over the petriplates. To the sandhill, three drops of each honey sample were placed in each petriplates, and the time required to sink through the sand surface was recorded. Delayed sinking Honey samples are considered to be pure as those honey samples contain less amount of moisture and are not adulterated [7].

Water Sinking Test

In this test, a 5 g of each sample was taken and it was poured into a beaker containing deionized water. After that, the nature by which it interacts with water was observed. Directly sinking honey samples to the bottom of the vessel without mixing with water unless stirred are considered to be pure [7].

Air-Flow Test

In this test, a clean glass stirrer was taken and was dipped into the vessel containing the honey sample. The stirrer was then rotated through the honey sample

and it was raised to a certain height above the honey surface to allow for free-flow of the honey samples. The flowing nature of honey was then observed. Honey samples, which flow in a continuous thread-like pattern, are considered to be pure [7].

Physicochemical Properties

Colour

Colour tone of honey was noted, which also revealed the identity and nature of various floral sources and helped in the differentiation of types of honey.

pH

Firstly, the pH meter (Model: pH 211 microprocessor, instrument manufacturer: Hanna Instruments) was calibrated using Qualigen (Fisher's scientific) buffer tablets of pH 4.0, 7.0, and 9.2. As specified in the label of the buffer tablets, each buffer tablet was dissolved in distilled water to the volume mark of 100 ml volumetric flask. After the device has been calibrated, each honey sample was checked for its pH readings. The pH range of unadulterated honey should lie between the range of 3.5 to 5.5 [8].

Rheological Properties

To make uniform treatment on viscosity, overall samples were maintained at 41±1℃ by using an electric air-heater. Viscosity was analyzed by viscometer (Model: DV-III Ultra Programmable Rheometer, Instrument Manufacturer: Brookfield) attaching spindle size of 63, the rheological properties of Honey were studied [1].

Optical Rotation

For this experiment, 1.0% (w/v) of the Honey sample in distilled water was prepared at first. To the prepared sample, using blank correction as distilled water; the prepared sample was filled in the tube of polarimeter (Model: BK-P2S, Instrument Manufacturer: Biobase Biodustry [Shandong] Co. Ltd.) and its triplicate reading was taken. Honeydew honey should exhibit positive optical rotation while nectar honey exhibit negative optical rotation to the incident plane-polarized light [6]. By using the following equation, specific optical rotation was computed:-

$$[\alpha]_{\lambda}^{T} = \frac{\alpha_{Observed}}{b \times c}$$

Where, $[\alpha]_{\lambda}^{T}$ = Specific Rotation in degree at T℃ and Light Wave-length (λ)

$\alpha_{Observed}$ = Observed Rotation in degree

b = Path-length in the decimeter

c = Concentration in gm/mL

Relative Density

Using the pycnometer (Glassware Manufacturer: Jain Scientific Glass Works [JSGW]), Digital Analytical Balance (Instrument Manufacturer: Bel Engineering), and laboratory thermometer, the density of our four (4) honey samples were determined. The room temperature of the lab during the time of the Experiment was also measured with the help of a Laboratory Thermometer. After that, an empty pycnometer was taken and its mass was noted using digital analytical balance. Now, it was filled with the honey sample and again its mass was weighed. The density of uncontaminated Honey typically ranges between 1.38 and 1.45 gm/ml [9].

$$Density(\rho) = \frac{Wt_{Filled} - Wt_{Empty}}{25}$$

Where, Wt_{Filled} =the mass of Pycnometer after sample filling

Wt_{Empty} =the mass of the empty Pycnometer before filling

Refractive Index

The refractive index of the honey samples was measured by using Abbe Refractometer (Model: SN 4040, Instrument Manufacturer: Guru Nanak Instruments, New Delhi). Sample holding prism of the instrument was cleaned well by rinsing it, with the help of ethanol and soft tissue paper. After that, a drop of honey sample was then loaded into the lower Sample prism. Now, the upper sample prism was interlocked with the lower sample prism which facilitated contact between the two Sample prisms and ultimately formed a film layer of honey sample. By viewing through the eye-piece, coarse scale adjustment and fine scale adjustment knobs were rotated and readings were noted. Refractive index of unspoiled honey typically ranges from 1.474 to 1.504 indicating the presence of water content in honey from 25% to 13% respectively [10].

Pollen Contents

Honey sample was prepared in a conical flask and it was left to shake in incubator shaker (Manufacturer: Biobase Biodustry [Shandong] Co. Ltd.) at 100 Revolution per Minute (RPM) for 15 Minutes at 45℃. The stock solution was then poured into the centrifugation tube. After that, it was allowed to centrifuge at 5000 RPM for 10 minutes in Centrifugation Apparatus (Model: NF 200, Manufacturer: Nüve Laboratories). The settled precipitate was scraped out and finally, it was observed in the microscope for determination of its shape and size.

34

Qualitative Screening Test of Phyto-chemical Classes

Plant metabolites were screened according to previously established methods [11-13]

Total Phenolic Content

Total phenolic content was determined by Folin-Ciocalteu (Thermo Fisher's Scientific India Pvt. Ltd.) method, according to previously published method [12].The honey stock solution was prepared at 10% in water. A portion of 1 ml of the honey and 0.8 ml of 10 % aqueous reagent and followed by 2 ml of 15% sodium carbonate was added. Final volume was made by adding water. Mixture was incubated for 2 hrs, and absorbance was measured at 765 nm against the blank. A standard curve of gallic acid was prepare for quantification, using a concentration range of 250, 500, 750, 1000 and 1250 ppm and result were expressed as mg Gallic acid/Kg honey. Calculation of TPC was done using the following formula expressed in mg Gallic Acid Equivalent (GAE) per Kg of Honey sample,

$$TPC = \frac{GAE(mg/l) \times V(ml) \times 10^{-3}\ (l/ml) \times Df}{Wt_{Sample}(gm) \times 10^{-3}(kg/gm)}$$

where TPC =Total Phenolic Content (in mg GAE/Kg Honey)
GAE = Gallic Acid Equivalent (in mg/l)
V = Total Volume of Methanol Extract (in ml)
Df = Dilution Factor

Total Flavonoid Content

Total Flavonoids content in honey was determined by a calorimetric method according to previous method. Briefly, 1.0 gm of honey sample was taken, which was dissolved in 10 ml 80% Ethanol to make sample stock solution. The sample stock solution was then allowed to incubate in incubator shaker (Biobase Biodustry [Shandong] Co. Ltd.) at 100 RPM for 15 Minutes at 45℃. Total 1.0 ml of supernatant sample stock solution was pipette out to which 0.2 ml of 10% (w/v) aqueous Aluminum Chloride was then added and subsequently, 0.2 ml of 1 M. Aqueous Potassium Acetate and 3 ml of 80% Ethanol was added. Finally, volume make-up was done to the mark adding sufficient Distilled Water and absorbance reading was measured at the wavelength of 415 nm against Blank Solution from the standard stock solution.[12].TFC was done using the following formula expressed in mg Quercetin Equivalent per Kg of honey;

$$TFC = \frac{QE(mg/l) \times V(ml) \times 10^{-3}\ (l/ml) \times Df}{Wt_{Sample}(gm) \times 10^{-3}(kg/gm)}$$

where, TFC is Total Phenolic Content (in mg Quercetin/Kg Honey)
QE is Quercetin Equivalent (in mg/l)
V is Total Volume of Ethanol Extract (in ml)
Df is Dilution Factor

Anti-Oxidant DPPH Assay

DPPH assay was estimated using the 2,2-diphenyl-1-picrylhydrazyl hydrate radical (DPPH) (Glentham Life Science Ltd, UK)) according to previous method of Kačániová[14 - 15]. The honey samples were diluted in Methanol at concentrations of 400, 800, 1200, 1600 and 2000 ppm solutions and from each dilution 0.3 ml was mixed with DPPH. The mixtures were vortexed, left in dark room temperature for 60 min and the absorbance was measured at 517 nm under UV-Visible spectrophotometer (UV 1800, Manufacturer: Shimadzu Scientific Instruments) correcting baseline Blank correction by Methanol. The Inhibition % is given by the relation

$$Inhibition\ \% = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100\%$$

Where $A_{Control}$ = mean Absorbance reading of 0 ppm solution against Methanol as Blank
A_{Sample} =e mean Absorbance reading of 400, 800, 1200, 1600, and 2000 ppm solutions

Results and Discussions

Preliminary Purity Test

Sand Sinking Test

S_1 took shortest period of time among all the samples while S_4 took longer period of time to sink through Sand-hills. Various time (in Seconds) taken by samples to sink through the Sand surface is as shown in **Table 2**.

Table 2. Time of Sand Sinking Test

S.N.	Sample	Time (in Seconds)
01.	S_1	62.33±2.08
02.	S_2	197.33±2.52
03.	S_3	262.33±2.517
04.	S_4	900±0

Water Sinking Test

All of our honey samples went down to the bottom of the cup without mixing up with the water except when stirred, which showed that all our honey samples complied the Water Sinking Test as mentioned in literatures mentioned above.

Air Flow Test

All the honey samples went down like a thread without breaking ranging 0.5 to 3 seconds as a continuous thread, which also showed that all our honey samples

complied for the Air Flow Test as mentioned in literatures mentioned above.

Confirmatory Purity Test

Colour

From colour shade analysis of Honey Samples with that of reference shade of Yellow colour, our different honey samples demonstrated varieties of colour as listed in **Table 3**.

Table 3. Different Shades of honey as per base Yellow colour

S.N.	Sample	Shade of Yellow Colour
1.	S_1	Amber
2.	S_2	Light Amber
3.	S_3	White
4.	S_4	Extra-Light White

pH

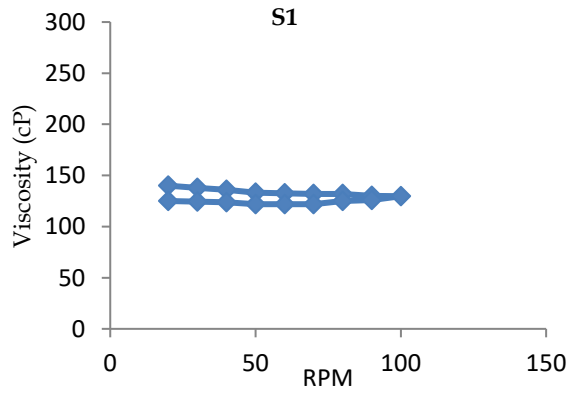
Presence of carbohydrate in dominant amount is the reason to which Honey shows slightly acidic nature. Due to the reason, Honey has a good Anti-Microbial potency. Among four samples, S_3 showed highly acidic with pH of (4.467±0.0306) where S_1 showed lowest pH with the value of (5.05±0.02). Various values of pH shown by different honey samples are tabulated down in **Table 4**.

Table 4. Various pH readings of different Honey Samples

S.N.	Sample	pH
1.	S_1	5.05±0.02
2.	S_2	4.833±0.0379
3.	S_3	4.467±0.0306
4.	S_4	4.767±0.0416

Rheological Properties

Among the samples, only S_1 showed Newtonian type of flow property, while rest of the samples showed Pseudo-plastic flow property upon checking Rheological properties of Honey Samples using Brookfield Viscometer. Plot of Rotation per Minute (RPM) Versus Torque% and RPM Versus Viscosity (in centi-Poise) of different Honey Samples as shown in **figure 3-6**.



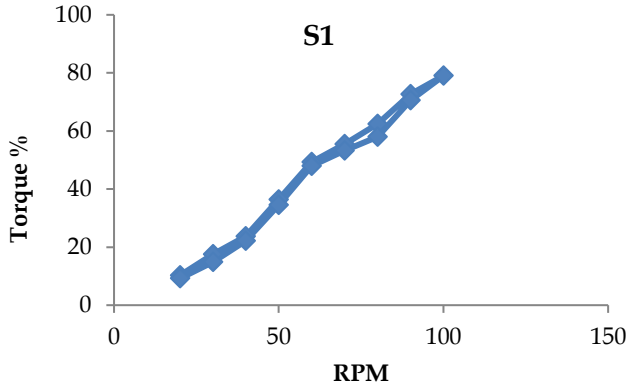
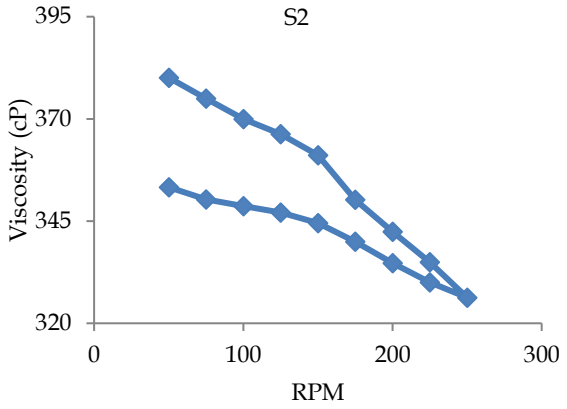


Figure 3. RPM Versus Torque% and RPM Versus Viscosity (cP) (i.e. Rheological Properties of S1)



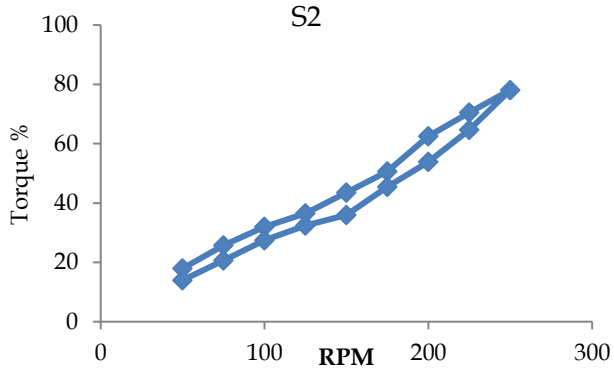
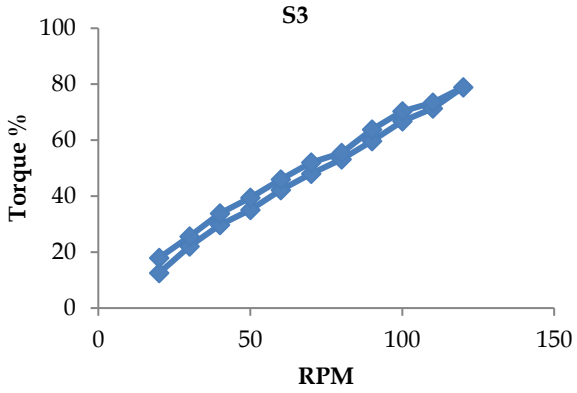


Figure 4. RPM Versus Torque% and RPM Versus Viscosity (cP) (i.e. Rheological Properties of S_2)



36

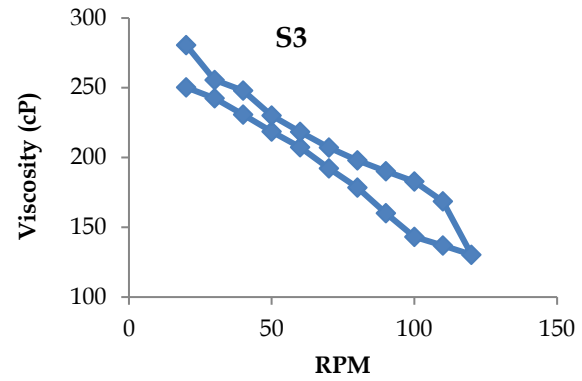


Figure 5. RPM Versus Torque% and RPM Versus Viscosity (cP) (i.e. Rheological Properties of S_3)

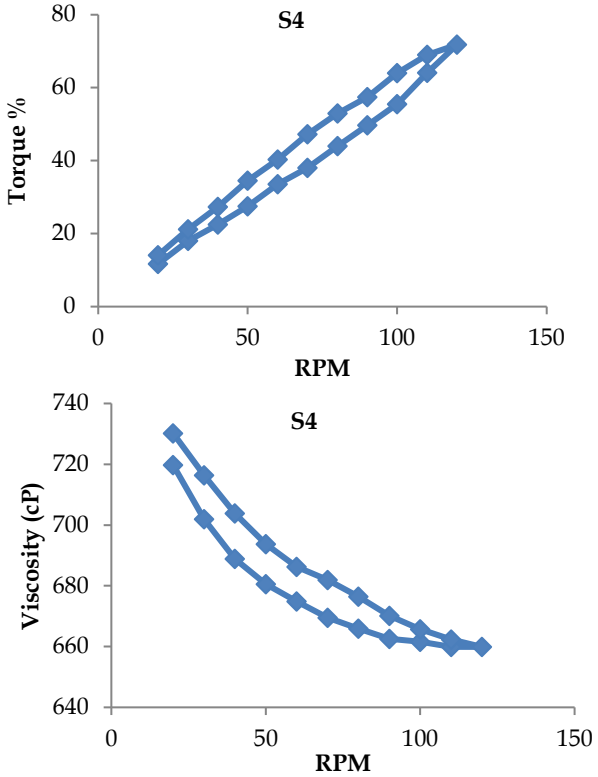


Figure 6. RPM Versus Torque% and RPM Versus Viscosity (cP) (i.e. Rheological Properties of S_4)

Specific Optical Rotation

From the literatures, it is established fact that Honeydew Honey and Adulterated Honey only shows positive Specific Optical Rotation, while Nectar Honey shows negative Specific Optical Rotation to the plane polarized light. S2 showed highest angle of rotation among the four samples while S1 showed positive angle of rotation. Various values of degree of Rotation are shown in **Table 5**.

Specific Gravity

Honey is denser than water with 1.3 to 1.4 folds. S_3 and S_1 are the respectively highest and lowest dense Honey Samples among the samples involved in this study. **Table 6**. shows different values of Specific Gravity which was obtained during analysis.

Table 5. Specific Optical Rotations of different Samples at Concentration of 1.0% (w/v) in Water.

S.N.	Sample	Specific Optical Rotation
1.	S_1	(+)5.75±0.4684
2.	S_2	(-)12.71±0.234
3.	S_3	(-)8.79±0.3098
4.	S_4	(-)2.299±0.3098

Table 6. Specific Gravity of samples observed at Room Temperature of 14°C.

S.N.	Sample	Specific Gravity
1.	S_1	1.35±0.00017
2.	S_2	1.404±0.0016
3.	S_3	1.409±0.00022
4.	S_4	1.376±0.00497

Refractive Index and Moisture Content

Moisture Content in Honey Samples is indicated by the Refractive Index values of Honey Samples. S_1 has highest Refractive Index and thus Moisture Content and S_4 has lowest Refractive Index and Moisture Content upon analysis of four different samples. **Table 7** is listed with different observations of Refractive Index and Moisture Content of all four honey samples involved in the study.

Table 7. Refractive Index and Moisture Content (in %) of Samples

S.N.	Sample	Refractive Index	Moisture Content (in %)
01.	S_1	1.473	25
02.	S_2	1.476	24.2
03.	S_3	1.476	24.2
04.	S_4	1.489	19

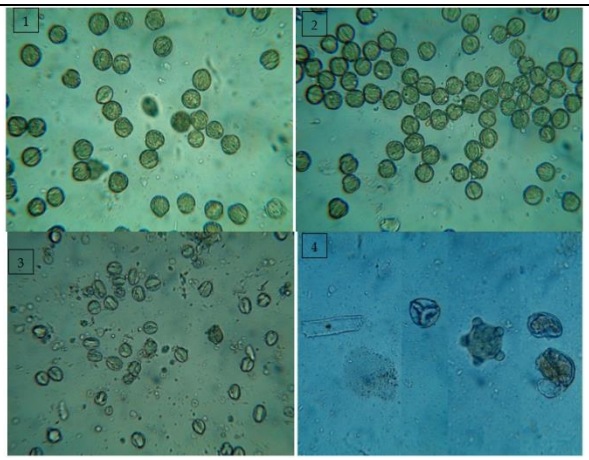


Figure 7. Microscopic View of Pollen Content at 400x Magnification of S_1(1), S_2(2), S_3(3) andS_4(4).

Microscopic Studies of Pollen Contents

Upon viewing microscopic slides of different honey samples, we found out the spring season harvested honey containing the same kind of pollen and it was different from autumn harvested sample. For the case of S_4, as it is nectar honey of *Diploknema butyraceae*; the honey sample exhibit different kind of pollen contents in it, which is totally different from all of the honey samples. **Figure 7** contains the pictures of Microscopic Slides of different Honey Samples.

Secondary Metabolites

Upon performing qualitative screening of secondary metabolites in honey samples; there was positive test for flavanoids, glycosides, saponins, tannins, reducing sugar, terpenoids, amino acids and proteins. There was negative test for alkaloids and phlobatanins. **Table 8** shows the summary of results for all four honey samples as obtained from qualitative screening of secondary metabolites.

Table 8. Qualitative Screening of Secondary Metabolites in different Honey Samples

S.N.	Name of Test	S_1	S_2	S_3	S_4
01.	Mayer's Test for Alkaloid	-	-	-	-
02.	Flavanoid Test	+++	+++	+++	+++
03.	Browntoger's Test for Glycosides	+	+	+	+
04.	Foam Test of Saponins	++	++	++	++
05.	Ferric Chloride Test for Tannins	+	+	+	+
06.	Benedict's Test for Reducing Sugar	++	+++	++	+++
07.	Xanthoproteic Test for Amino Acids and Protein	+++	+++	+++	+++
08.	Terpenoids Test	+++	+++	+++	+++
09.	Phlobatannins Test	-	-	-	-

- Negative, + Weak Positive, ++ Moderate Positive, +++ Strong Positive

Quantitative Determination of Secondary Metabolites

TPC and TFC

Upon plotting of standard calibration plot of Gallic acid for TPC and Quercetin for TFC, we obtained following graphs as shown in figures with R^2 of 0.9658 and 0.981 respectively for the linear regression keeping Concentration (in ppm) along X-axis and mean absorbance along Y-axis. Mean absorbance is the average of absorbance reading taken from triplicate data.

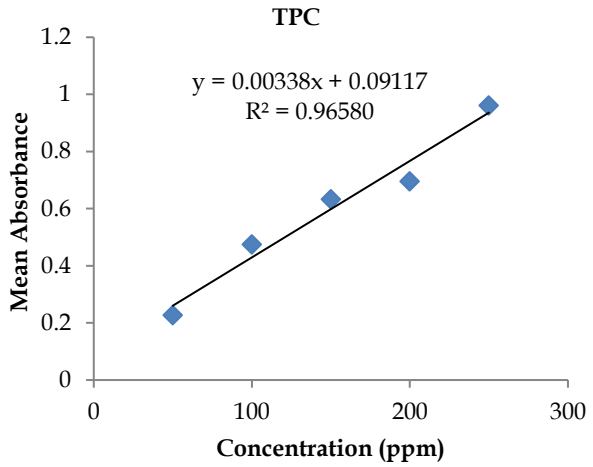


Figure 8. Standard Calibration Curve of Gallic Acid for TPC

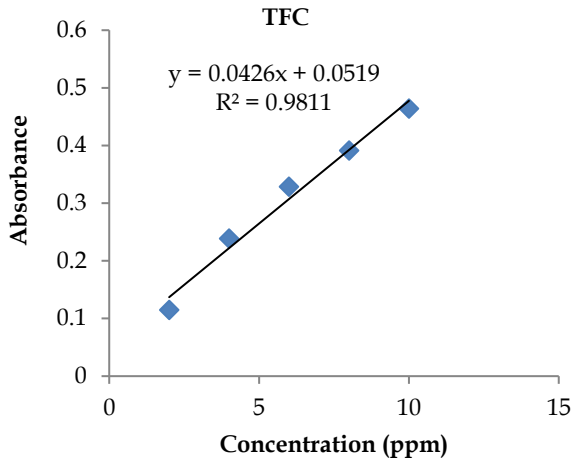


Figure 9. Standard Calibration Curve of Quercetin for TFC

Using above Standard Calibration Curve and linear Regression equation of line, TPC and TFC was found to be highest in S_1 and lowest in S_3. **Table 9** shows the results of estimated TPC and TFC of different Honey Samples.

Table 9. Quantitative Determination of Secondary Metabolites

S.N.	Sample	TPC (gm GAE/ Kg Honey)	TFC (gm QE/ Kg Honey)
01.	S_1	6.862884943	0.3531322456
02.	S_2	1.626269056	0.1607248345
03.	S_3	1.042708154	0.01675598334
04.	S_4	2.615476423	0.1883074849

TPC = Total Phenolic Content and TFC = Total Flavanoid Content

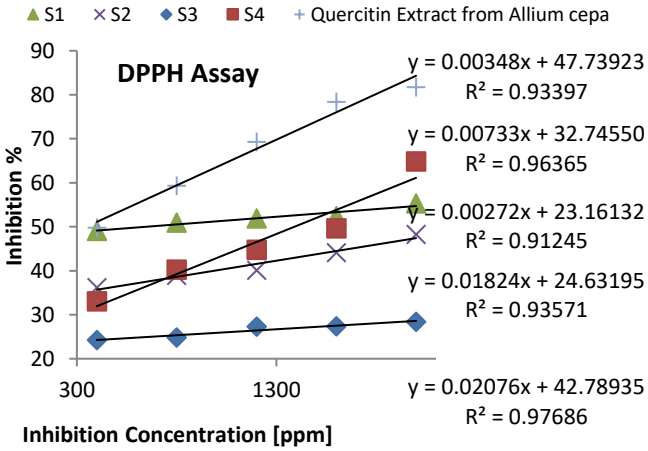


Figure 10. Concentration Versus Inhibition% Graph from DPPH Assay (Anti-Oxidant of different samples at varying Concentration)

Table 10. Results of Anti-Oxidant Assays (IC_{50} Values and Times Potency with the respect to Standard)

S.N.	Samples	IC_{50} (in ppm)	Times Potency
01.	Quercetin	346.9112586	1.000
02.	S_1	649.6465517	0.534
03.	S_2	2353.956344	0.1475
04.	S_3	9867.161765	0.0353
05.	S_4	1390.792215	0.2497

38

Anti-Oxidant DPPH Assay

Upon plotting, Concentration (in ppm) Versus % Inhibition of different Honey Samples and similarly, Standard of Quercetin Extract extracted from *Allium cepa*, we obtained various IC_{50}value from the plot, through which we became able to compare between the times potency value of Anti-Oxidant ability of different samples to that with Standard Quercetin Extract. From the observation, we found out that S_1 had maximum potency with 0.534 times and S_3 has minimum potency with 0.0353 times of standard Quercetin Extract from Allium Cepa. **Figure 10** is the plot of inhibition concentration (in ppm) versus % inhibition of different Samples and Standards involved in the study. **Table 10** is the result of IC_{50} value and times potency of different honey samples obtained from the calculation.

Conclusions

Different tests performed during this research complied with the various standard research articles published in different journals. Potency of honey is affected by the types of honey and seasons at which it has been harvested. Honey with lower anti-oxidant potency can be used in formulation of topical cosmetological products or as daily dietary supplement. Honey with higher values of anti-oxidant potency can be used in combination with other different active ingredients for the formulation of different therapeutic products.

Conflict of Interest

The author declares no conflict of interest.

Authors Contribution

All authors have equal contribution

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