



Research Article

PHYTOCHEMICAL SCREENING, PHYSICOCHEMICAL PROPERTIES AND TOTAL PHENOLIC CONTENT OF BITTER HONEY SAMPLES

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ABSTRACT

Honey is a nutritional food used in medicine since antiquity because of its broad spectrum of therapeutic activities. The aim of this present study was to screen the phytochemical compounds present in bitter honey samples of Nilgiris and to determine the physicochemical properties and total phenolic content. Ethno medical survey conducted in Nilgiris revealed the medicinal importance of bitter honey among Alu Kurumba tribes. The Phytochemical screening showed positive results for carbohydrates, tannins, amino acids, saponins and flavonoids. The results of physicochemical parameters are within the limits described by FSSAI except for reducing and total reducing sugar in the second sample. The total phenolic content present in bitter honey samples were found to be 1136.64 ± 36.497 mg/Kg equivalent of gallic acid and 1626.46 ± 75.003 mg/Kg equivalent of gallic acid which indicates that both the bitter honey samples can be a good source of antioxidants when compared to sweet honey.

Key words: Bitter honey, Physicochemical, Total Phenolic Content

INTRODUCTION

Honey is a natural sweet substance produced by honey bees from the nectar of blossoms or from secretions of living plants, which is collected and transformed by the honey bees after combining with specific substance of their own, deposited, dehydrated and finally stored in the honey combs.¹ Honey is consumed by the humans since ancient times due to its high medicinal and nutritional value.²

The major components of honey comprises of complex mixture of carbohydrate; whereas other constituents like proteins, minerals, vitamins, phenolic acids, flavonoids, enzymes, organic acids and other volatile compounds constitutes the minor components of honey.³

The chemical composition and biological activities of honey varies depending upon its nectar source and geographical origin.⁴ it is essential to have knowledge on the physicochemical properties of honey for assessing the quality of honey.⁵ Physicochemical properties of honey like colour, moisture content, sugars, protein etc depends on the nectar and pollen origin. It is also helpful in distinguishing natural honey from artificial honey and also provides a parameter for characterization of honey.⁶ Bitter honey is harvested in Nilgiris especially in Kotagiri. The local Alu Kurumba tribes use this honey traditionally as nutraceuticals and also to cure stomach aches. Since characterisation of bitter honey samples are not available, the present research work is proposed to study the phytochemical screening, physicochemical parameters and total phenolic content of bitter honey samples from Nilgiris.

MATERIALS AND METHODS

Bitter honey sample harvested during the month of May were collected from the Alu Kurumba tribes of Nilgiris, Ooty while one

sample were purchased from the local market. Both the honey samples were stored in an airtight container to avoid moisture absorption. All the chemicals used for this study are of analytical grade.

Qualitative phytochemical analysis of bitter honey samples

The preliminary phytochemical analysis of bitter honey samples were carried out using the following standard method.⁷⁻⁸

Test for carbohydrate: Mix 2ml of Benedict's reagent to 2ml of test solution and boil in a water bath. The formation of red precipitate indicated the presence of carbohydrate.

Test for tannins: To 2ml of test solution add few drops of 5% ferric chloride solution. The formation of blue –green colour indicated the presence of tannins.

Test for amino acids: Mix 2ml of test solution with 1ml of 5% Ninhydrin solution. The formation of purple colour indicated the presence of amino acid.

Test for Saponins: 10ml of distilled water was added to the sample and mixed vigorously. The appearance of frothing which lasts more than 5 minutes indicated the presence of saponins.

Test for flavonoids: 2ml of 2% Sodium hydroxide was added to the test solution; a concentrated yellow colour was produced which decolourises after addition of 2 drops of acid, indicated the presence of flavonoids.

Test for Steroids: To 5ml of the solution 2ml of chloroform and concentrated H₂SO₄ was added. No red colour in the chloroform layer indicates the absence of steroids.

Test for alkaloids: To the sample solution 1ml of Dragendorff's reagent was added. Absence of an orange red precipitate indicated the absence of alkaloids.

Physicochemical Analysis

All the physicochemical parameters were carried out according to the standard analytical methods prescribed by the A.O.A.C (1990).⁹

Colour Intensity: The colour intensity of bitter honey samples was determined using UV-Visible Spectrophotometer (Thermofischer).¹⁰ The absorbance of 50% w/v samples were measured at 450 and 720nm and the intensity was calculated using the formula,

$$ABS_{450} = (ABS_{450} - ABS_{720}) \times 1,000 \text{ mAU}$$

Moisture content: Moisture content of honey samples were determined by refractometric method using ATAGO refractometer at 20°C, according to the relationship between honey water content and refractive index.¹¹

Specific gravity: The specific gravity of honey samples were determined using specific gravity bottle. It is the ratio of the weight of sample to that of equal volume of water.¹²

pH: The P^H of the bitter honey samples were determined using digital P^H meter (Deep vision). One gram of honey sample was diluted with distilled water and the pH were measured after calibrating the pH meter with standard solution of buffers.¹³

Determination of sugars: The reducing sugar, total reducing sugar in honey samples was carried out using Lane Eynon titrimetry.¹⁴

Determination of reducing sugars: Accurately weighed amount of honey (about 25g) was transferred into 250ml volumetric flask and 10ml of neutral lead acetate was added and made up to the volume with distilled water and filtered. From the above solution 25ml was pipetted out and 100ml of water was added and finally potassium oxalate until there is no further precipitation. The final volume was adjusted to 500ml and filtered. This solution was transferred into a burette and was titrated against Fehling's reagent (A and B) using methylene blue indicator until the blue colour changes to brick red.

Determination of total reducing sugars: An aliquot of 50ml of clarified de-leaded filtrate was transferred to 100ml volumetric flask. To this 5ml of Concentrated hydrochloric acid was added and allowed to stand at room temperature for 24 hours. The solution was further neutralised with Sodium hydroxide solution and made up to the volume. Finally this solution was transferred to burette and was titrated against Fehling's solution similar to the procedure described in the determination of reducing sugars.

Ash content: Ash content of the sample were determined by using muffle furnace by placing 5g of honey sample in a crucible in a muffle furnace (Technico) at 550°C for 4 hours and then measuring the ash in an electronic weighing balance. Preheating of honey up to darkness is necessary to avoid foaming. The ash content of honey depends on the materials gathered by the bees during foraging and it is quality criterion of botanical origin of honey.¹⁵

Electrical conductivity: A standard solution of potassium chloride was prepared and the conductivity meter (Roy instruments) was calibrated using the solution. A 20%w/v

solution of honey samples was prepared and the conductance was measured at 20°C.¹⁶

Conductivity (mS/cm) = Cell constant x Conductance measured

Acidity (formic acid): Total acidity of the honey samples were determined using volumetric method. About 10gms of the honey sample was taken in a titration flask and dissolved it in 75ml of water and mixed thoroughly. It was then titrated against standard 0.05N Sodium hydroxide solution using phenolphthalein as indicator. The percentage of acidity as formic acid was calculated using the equation,

$$\text{Acidity (\%)} = (0.23 \times V) / M;$$

Where V=volume of 0.05N Sodium hydroxide consumed and
M= weight of the honey sample taken ¹⁴

Fiehe's test: To the ethereal extract of honey sample, 2ml of freshly prepared solution of resorcinol in Hydrochloric acid was added and the colour change was noted. The formation of cherry red colour within one minute indicates the presence of invert sugar.¹⁷

HMF Content: The hydroxyl methyl furfural content of honey samples were determined by using spectroscopic method (white 1979). It is an indicative of poor storage and overheating of honey. The absorbance of samples after adding Carrez 1 and Carrez 2 solutions and Sodium bisulphite were measured at 284nm and 336nm using UV-Visible Spectrophotometer (Thermofischer).¹⁸

Total Protein: The total protein content in the bitter honey samples were measured using Kjeldhal method (Kelplus- Supra LX). To about 0.4 g of the sample 5ml of Concentrated Sulphuric acid was added and subjected to digestion; sodium sulphite and copper sulphate was used as a catalyst in this process. The solution was further distilled after adding 40% Sodium hydroxide. The distillate was collected in a flask containing 4% of boric acid. It was finally titrated with 0.1N Hydrochloric acid. The percentage of nitrogen quantified was converted into nitrogen by multiplying with 6.25 as conversion factor.⁹

Total phenolic content: The total phenolic content of bitter honey samples were measured using Folin-Ciocalteu reagent method. Briefly 1ml solution (0.1g/ml) of bitter honey sample was mixed with 5ml of Folin - Ciocalteu reagent and 4ml of 10% Na₂CO₃, kept in dark for about 90 minutes. After incubation the absorbance of the reaction was measured at 765nm against distilled water as a blank using UV-Visible Spectrophotometer (Thermofischer). Standard calibration curve of gallic acid was determined at a concentration from 5 -150 µg/ml the total phenolic content was expressed in mg/kg equivalent of gallic acid.¹⁹

STATISTICAL ANALYSIS

The statistical analysis was carried out using statistical package for social science (SPSS) Software and the result represents the mean ± standard deviation. Comparisons were carried out by one way analysis of variance (ANOVA) and Duncan's Multiple Range Test with P<0.05 level of significance.

RESULTS

Phytochemical screening: The obtained results on phytochemical screening of bitter honey samples are displayed in table 1.

Table 1: Qualitative Phytochemical Screening of Bitter Honey Samples

Sl.No	Phytochemical	Sample 1	Sample 2
1	Carbohydrate	+++	+++
2	Tannins	++	++
3	Amino acids	++	++
4	Saponin	+++	+++
5	Flavonoid	+	+
6	Steroid	-	-
7	Alkaloid	-	-

Key: ++ = Relatively presence, + = Presence, - = Absence

Physicochemical parameters: The results of physicochemical parameters were represented in table 2.

Table 2: Physicochemical Parameters of Bitter Honey Samples

Parameters	Sample 1	Sample 2
Colour intensity (mAU)	1611.33±28.308 ^a	2804.00±50.715 ^a
Moisture Content (%)	21.30 ±0.100 ^a	18.13±0.153 ^a
Specific Gravity (mg/ml)	1.37±0.006 ^a	1.39±0.006 ^a
pH	4.83±0.006 ^a	4.85±0.010 ^b
Reducing Sugar (%)	64.72±0.237 ^a	43.02±0.493 ^a
Total Reducing Sugar (%)	68.18 ±0.235 ^a	46.86±1.009 ^a
Ash Content (%)	0.50±0.011 ^a	0.39±0.039 ^a
Electrical Conductivity(mS/cm)	0.31±0.011 ^a	0.33±0.011 ^b
Acidity (%)	0.08±0.002 ^a	0.13±0.007 ^a
Fiehe's Test	Negative	Negative
HMF(mg/kg)	BDL	11.6±0.32
Total Protein(mg/g)	2.888±0.085 ^a	4.756±0.065 ^a

BDL: Below detectable limit

Values are the Mean ± Standard deviation of triplicate determinations.

Values with different superscript alphabets in the same row are significantly different at P<0.05 level of significance.

Total Phenolic Content

The results of total phenolic content of bitter honey samples were displayed in table.3. The calibration curve of gallic acid is represented in figure1.

Table 3: Total Phenolic Content of Bitter Honey Samples

Sample	Total Phenolic Content (mg/Kg Of Gallic Acid equivalent)
Sample 1	1136.64±36.497
Sample 2	1626.46±75.003

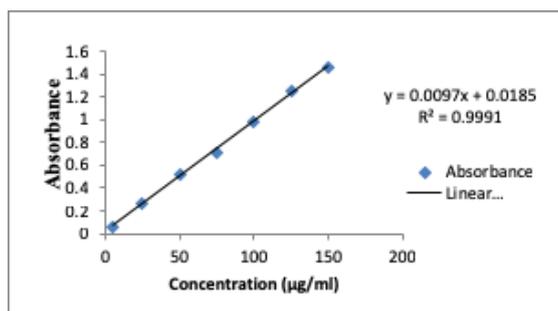


Figure 1: Calibration Curve of Gallic Acid

DISCUSSION

The preliminary phytochemical screening of bitter honey samples revealed the presence of carbohydrates, tannins, amino acids, saponins and flavonoids whereas alkaloids and steroids were not detected in both the honey samples.

The colour intensity of analysed bitter honey samples were found to be 1611.33±28.308mAU and 2804.00±50.715mAU .The colour of honey determines the presence of flavonoids, phenolic compounds, and pigments present in it. The moisture content of the bitter honey samples investigated was found to be 21.30

±0.100 and 18.13±0.153. The low moisture content is one of the most important parameter which plays a critical role in its quality. The Moisture Content values were found to be within the FSSAI limits (Not more than 20 percent).

The specific gravity of bitter honey samples were found to be 1.37±0.006mg/ml and 1.39±0.006 mg/ml within the limits as described by FSSAI. (Not less than 1.35). The bitter honey samples were found to be acid with a P^H of 4.83±0.006 and 4.85±0.010, which is found to be within codex limit (3.40-6.10). The P^H of this honey samples was found to be low enough to inhibit the growth of microorganism.

The reducing sugar and total reducing sugars of first sample of bitter honey was found to be $64.72 \pm 0.237\%$ and $68.18 \pm 0.235\%$ respectively. Both the values are within the FSSAI limits. But the reducing sugar and total reducing sugars of other sample of bitter honey was found to be $43.02 \pm 0.493\%$ and $46.86 \pm 1.009\%$ which is lower than the acceptable limit (not less than 65 percent). The ash content in honey is influenced by the chemical composition of nectar that varies according to the different botanical sources of honey. The total ash content of bitter honey samples were found to be $0.50 \pm 0.011\%$ and $0.33 \pm 0.011\%$ and were within the FSSAI limit (not more than 0.5%).

The electrical conductivity of two samples of bitter honey was found to be 0.31 ± 0.011 mS/cm and 0.33 ± 0.011 mS/cm. The conductivity of both the samples of bitter honey was found to be within the codex alimentarius limits (not more than 0.8 mS/cm). The acidity of bitter honey samples was found to $0.08 \pm 0.002\%$ and $0.13 \pm 0.007\%$ which is not more than the FSSAI limit (0.2%) which indicates the absence of undesirable fermentation. The presence of organic acids, like gluconic acids which is produced from the nectar during ripening and inorganic ions such as phosphate and chloride contributes the acidity of honey. It indicates the absence of undesirable fermentation.

Fiehe's test determines the presence of commercial invert sugar in honey. Both the bitter honey samples tested showed negative results for Fiehe's test for invert sugar.

The hydroxyl methyl furfural is an indicator of the freshness of honey and both the samples were found to be within the FSSAI limits (not more than 80mg/kg); below detectable limit in first sample and 11.6 ± 0.32 mg/kg in the second sample. These values were very low which indicates that the storage conditions and harvesting process of both the honey samples are of good quality. The total protein content of both the samples of bitter honey was found to 2.888 ± 0.085 and 4.756 ± 0.065 mg /g which indicate the nutritional importance of bitter honey.

Total phenolic content present in bitter honey samples was found to be 1136 ± 36.50 mg/kg and 1626 ± 74.93 mg/kg, which indicates that it is rich in antioxidants.

CONCLUSION

Since characterisation of bitter honey from Nilgiris is not yet available, this study for the first time provides a preliminary evaluation of its phytochemical and physicochemical properties. It was found to be that bitter honey samples are rich sources of important phytochemicals of pharmacological significance. The free reducing sugar and total reducing sugar of the second sample of bitter honey was found to be lower than the limits described by FSSAI. The HMF content in both the samples of bitter honey showed that the values are in acceptable range as described by FSSAI and Codex standards. Honey contains trace amount of proteins which is usually originated from the pollen. There were significant differences between total protein content of two bitter honey samples. The total phenolic content of two samples of bitter honey were found to be higher in both the samples which indicates that bitter honey can be considered as valuable natural source of antioxidants.

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