A study on antibacterial and antioxidant activity of different honey samples in relation with their bioactive components

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ABSTRACT:

Honey, a sweet natural product known for its nutritive value and beneficial health effects, is produced by honeybees (*Apis mellifera* L.). Honey exhibits potent antimicrobial activity and thus its use in modern medicine represents a good alternative treatment to fight multidrug resistant pathogens. The current analysis assessed the antibacterial and antioxidant properties of different honeys in relation with their bioactive components. Antimicrobial activity was examined against six bacteria using agar well diffusion assay. Its related attributes like sugar concentration and hydrogen peroxide were also analyzed. Antioxidant activity was examined using reducing power assay and polyphenols were analyzed using Folin ciocalteu and aluminium chloride method. Results revealed that compared to Patanjali and Raw honey, Dabur honey showed most effective antibacterial activity. *Bacillus cereus* showed the lowest susceptibility to all types honey samples. *Proteus vulgaris* showed high susceptibility to all types of honey. Raw honey has more antioxidants compared to processed Dabur and Patanjali honey samples and was darkest in color. Phenolic concentration of honey samples were ranged from 300 – 425mg/kg of honey. Dabur honey consists the lowest while the raw honey consists the highest amount of polyphenols. In conclusion, all the three tested honey samples exhibited antimicrobial and antioxidant activity with varying potency. Results suggested that presence of hydrogen peroxide and high sugar content in honey accounted, at least in part, for the antibacterial activity. In addition, polyphenolic contents were also found significantly related to its antioxidant activity.

Keywords: Honey, antibacterial activity, antioxidant activity, polyphenols

INTRODUCTION:

Honey is a natural product with many attributes that are useful for humans. It has widely accepted as food and medicine by all generations, traditions and civilizations, both ancient and modern (Fikselova *et. al*., 2014). The composition of honey can vary significantly depending upon its floral source, bee species, mode of harvesting and post harvested conditions (Singhal *et. al*., 1997). Honey contains about 70 – 85% of sugars, where fructose

and glucose are the major sugars. It also consists of other minor components such as proteins, vitamins, organic acids, enzymes, minerals and phenolics (Roshan *et. al*., 2017).

Honey exhibits potent antimicrobial activity, thus its use in modern medicine represents a good alternative treatment to fight multidrug resistant pathogens (Kus *et. al*., 2016). The antimicrobial activity of honey may be attributed to its low pH, high sugar concentration, presence of bactericidal factors like hydrogen peroxide, lysozyme, polyphenols, methylglyoxal and bee defense (Israili, 2013). Antimicrobial potency of honey is variable depending on its source, storage conditions and processing.

Antioxidants are the substances that protect the cells of the body from damage caused by unstable molecules known as free radicals. The antioxidant property of honey is because of enzymatic substances like glucose oxidase, catalase and non-enzymatic substances like ascorbic acid, carotenoids and more than 150 polyphenols including phenolics and flavonoids (Khalil *et. al*., 2012). Most studies on the effects of honey are concentrated on the activities of bioactive compounds, especially phenolic compounds, in the human organism. Polyphenols has also been reported to affect color of the honey (Alvarez Suarez. *et. al*., 2010). Therefore, the current analysis assessed the antibacterial and antioxidant properties of different honeys in relation with their bioactive components.

METHODOLOGY:

The laboratory work was carried out from 27th July 2019 to 15th February 2020 at the Microbiology Department of Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management & Commerce, Gujarat, India.

Sample collection:

Present study was carried out using three types of honey. Two commercial (Processed) honey – Dabur and Patanjali honey was purchased from the retail store in Vapi, India. Raw (Unprocessed) honey was collected from the Himalayan region, north India. All the three samples were kept under refrigeration at 5°C.

pH of honey:

10gm of honey was taken in 100ml beaker and mixed with 75ml D/W. It was properly mixed and reading was taken in pH meter (Sereia *et. al*., 2017).

Color intensity:

For identifying the color of all the honey samples, a method according to Al-Farsi *et. al*., (2018) was used wherein honey samples were diluted to 50% using distilled water, mixed properly and centrifuged at 3200 rpm for five minutes. Then the absorbance was measured at 635 nm and the color intensity was determined using the Pfund scale. Pfund value (mm) = −38.70 + 371.39 × Abs and expressed in millimeters on a Pfund scale (Fell, 1978).

Polyphenol compounds:

In our study, all the three honey samples were studied for their polyphenol compounds consists of phenolics and flavonoids compounds.

Total content of phenolic compounds (TCPC):

The total phenolic compounds in the honey sample were measured using the Folin-Ciocalteu method (Socha *et. al*., 2009). The honey stock solution was prepared with concentration 0.04gm/ml in distilled water. 0.5 ml of stock solution was combined with 0.3 ml of Folin-Ciocalteu reagent and then added with 2 ml of 15% sodium carbonate. The final volume was made up to 5 ml by adding distilled water to it. The mixture was properly mixed, incubated for 2 hours and absorbance was measured at 789nm. Experiment was performed in triplicates; values of mean and standard deviation were calculated. Standard graph of catechol was prepared for the quantification.

Total content of flavonoids (TCF):

Total flavonoids content in the honey samples were determined using a colorometric method (Al-Frasi *et. al*, 2018). Honey stock solution was prepared with concentration of 0.4gm/ml in distilled water. 0.1 ml of stock solution was combined with 0.5 ml of distilled water followed by 0.1 ml of 5% sodium nitrate and the mixture was incubated for 6 minutes. The mixture was added with 0.15 ml 10% aluminum chloride and incubated for 5 minutes. Lastly, 0.2 ml of 1M Sodium hydroxide was added to the mixture and properly mixed. The absorbance was measured at 510 nm. Experiment was performed in triplicates; values of mean and standard deviation were calculated. Standard graph of quercetin was prepared for the quantification.

Sugar estimation

Phenol sulphuric acid method was used for sugar estimation (BeMiller, 2003). 100mg of honey sample was weighed into boiling tube, 5ml 2.5N HCl was added to it and was hydrolyzed for 3 hours by keeping it in boiling water bath. After 3 hours content was allowed to cool at room temperature and neutralized with solid sodium carbonate until effervescence

ceases. 0.1 and 0.2 ml of sample solution was then taken into test tubes and volume was made up to 1ml by adding distilled water. 1ml 5% phenol solution followed by 5ml of 96% sulfuric acid was added and mixed properly. After 10 minutes, again the content was shaken well and placed in water bath for 20 minutes at 25-30°C. Absorbance was measured at 490nm. Glucose was used as the standard for the calculation.

Hydrogen peroxide content:

Hydrogen peroxide content was measured by using a titrimetric method (https://www.nrc.gov/docs/ML0329/ML032960470.pdf). 1ml of 50% diluted honey was taken in flask and added with 100 ml distilled water. 10ml (1:4) sulfuric acid and small pinch of manganese sulfate ($MnSO_4$) as a catalyst was added to the flask. The solution was titrated using 0.1N potassium permanganate ($KMnO_4$) till the pink color persists in the solution.

Formula: $\% H_2O_2 = \frac{\text{ml of } KMnO_4 \times N \times 1.701}{\text{ml of sample aliqout}}$ (where N= normality of $KMnO_4$)

Antibacterial activity:

Antibacterial activity was performed using Fikselova *et. al*. (2014). The potential antimicrobial activities of 3 samples against six bacteria (*Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa, Serratia marcescens, Staphylococcus aureus* and *Bacillus cereus*) were studied using the agar well diffusion assay. The concentration of microbial inoculum was set in the range of 10^6 CFU/ml. Petri plates containing 15ml of Muller Hinton agar were swabbed with the inoculum all over the surface. Subsequently, three equidistant wells having 6 mm diameter were punched into the inoculated medium using a sterile cup borer. Wells were filled with the 0.1ml of honey samples and were incubated at 37° C for 24 hours. After incubation, the zone of inhibition was measured.

Antioxidant activity:

Antioxidant activity was performed using Alzahrani *et. al*. (2012). Reducing power assay is a convenient rapid screening method for measuring the antioxidant potential. 2.5ml of honey was mixed with 2.5 ml of 0.2M phosphate buffer and 2.5 ml 1% potassium ferricyanide and was incubated at 50°C for 20 min. After incubation, the mixture was added with 2.5 ml of 10% trichloroacetic acid and centrifuged for 10min at 3000 rpm. The upper layer (1 ml) was combined with 1 ml of distilled water and 0.5 ml of 0.1% ferric chloride. The absorbance was measured at 700nm. Standard graph of ascorbic acid was used for the quantification.

RESULTS AND DISCUSSION:

pH profile:

In the present study, among all the three honey samples, Dabur honey was more acidic in nature followed by Raw and Patanjali honey (Table 1). In general, honey is acidic in nature irrespective of its variable geographical origin.

Table 1: pH of honey samples

Honey samples	pH of honey
Dabur	3.3
Patanjali	4.0
Raw	3.5

The pH values were in agreement with the results of Algerian, Brazilian, Spanish and Turkish honeys (Ouchemoukh *et. al*., 2007 ;Ozcan and Olmez, 2014).The pH values of the tested honey samples as shown in table 1, were found to be similar recorded by Nayik *et. al*., (2019) for the four different honey samples that ranged from pH 3.0 to 4.3, collected from Kashmir Valley.

Color assessment of honey:

In our study, raw honey was appeared to be darkest honey while Patanjali honey was lightest in color (Figure 1).

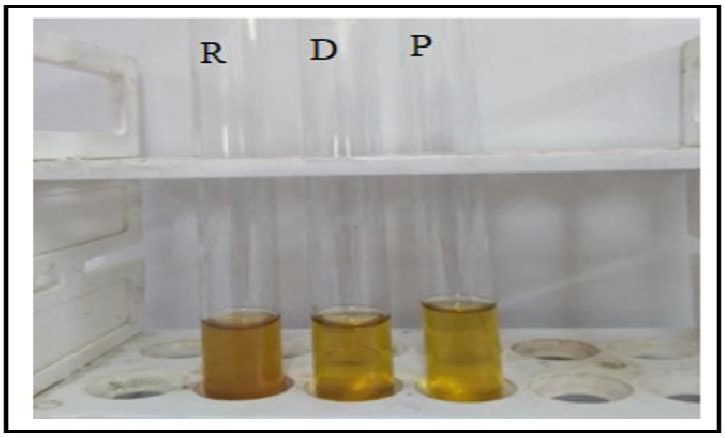

Figure 1: Color of honey samples

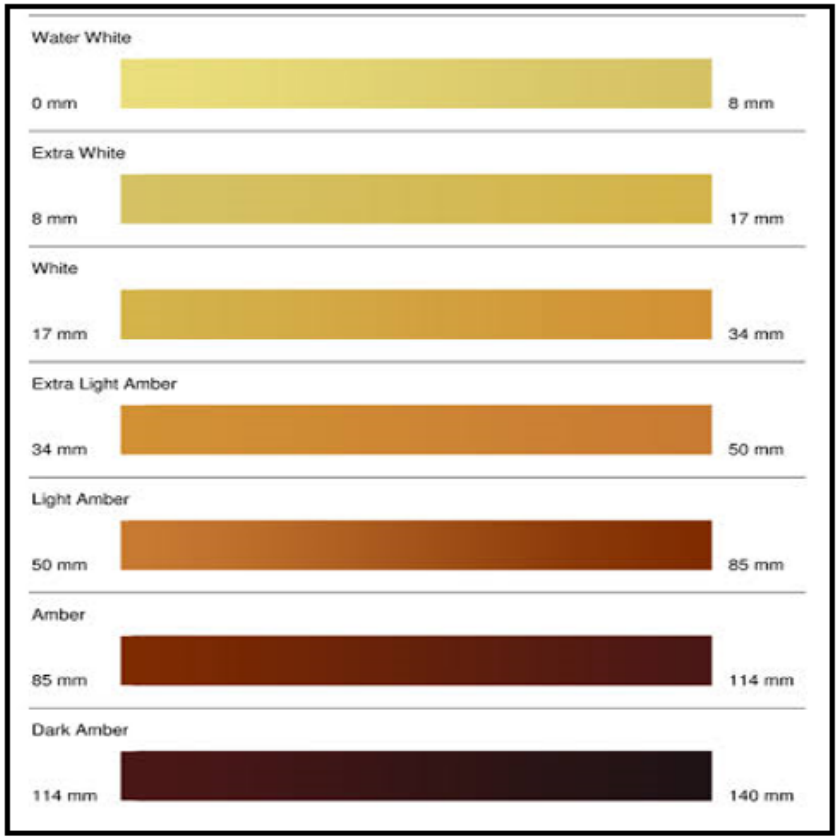

Figure 2: Pfundscale (Dominguez & Centurión, 2015)

Table 2: Color assessment of honeys according to Pfund scale

Honey samples	**Color**
Dabur	36.5mm (Extra light amber)
Patanjali	26.49mm (White)
Raw	83.17mm (Light amber)

Color values are presented in Pfund values (mm) and Pfund scale (Dominguez & Centurión, 2015) (water white, extra white, white, extra light amber, light amber, amber and dark amber) is used in order to classify honey colors. Dabur honey has Pfund value 36.5mm which is in the range of extra light amber, Patanjali honey has Pfund value 26.49mm which is in the range of white. Raw honey has Pfund value 83.17mm which is in the range of light amber which is the darkest of all (Table 2). The color of each honey is due to pigments such as carotenoids and flavonoids which is dependent on the botanical and geographical origin of the product (Terrab *et. al*., 2003). Several studies revealed that dark honeys have higher values of phenolic content and antioxidants than lighter honey (Al-Farsi *et. al*, 2018).

Polyphenols in honey:

Polyphenols are one of those compounds which are responsible for the antioxidant activity of honey. Results revealed that the Dabur honey consists the lowest amount of polyphenols while the raw honey consists the highest amount of polyphenols (Table 3).

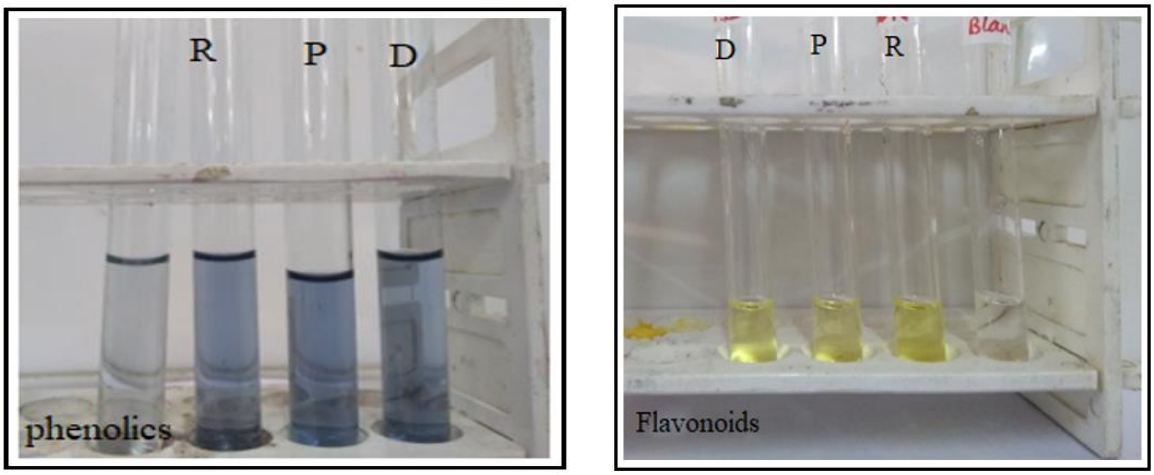

Figure 3: Color intensity of phenolics and flavonoids in honey samples

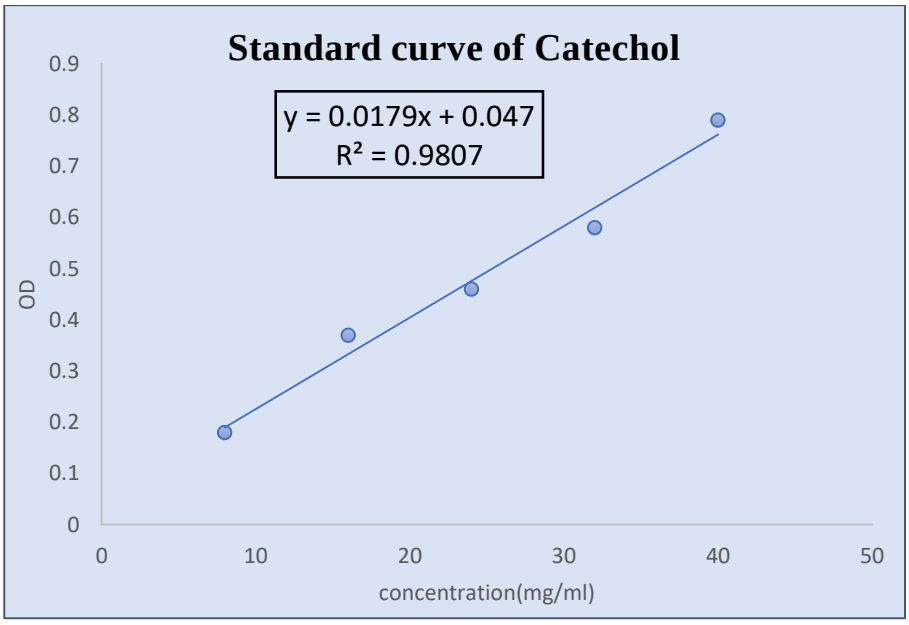

Figure 4: Standard curve of Catechol

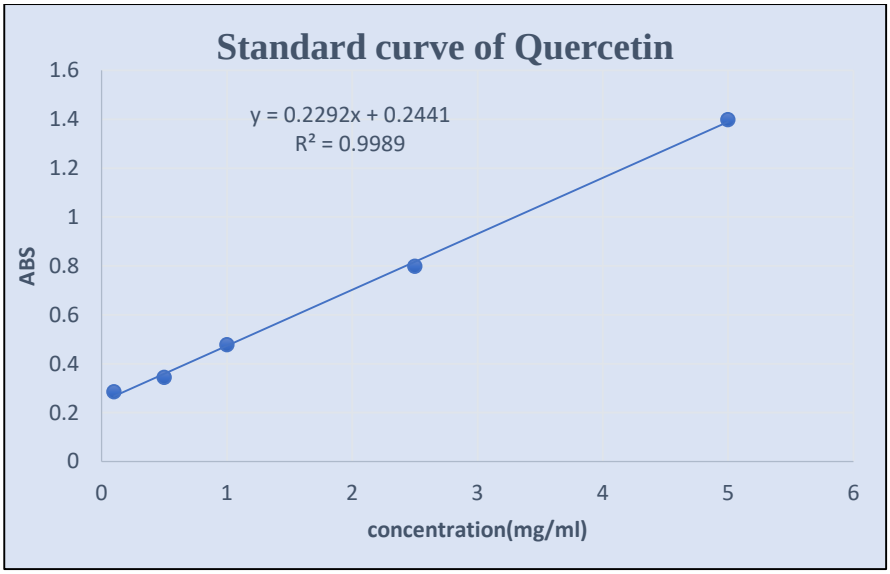

Figure 5: Standard curve of Quercetin

Table 3: Phenolics and Flavonoids content of honey samples

Honey samples	**Total phenolics (mg/kg honey)**	**Total flavonoids (mg/kg honey)**
Dabur	305 ± 20.3	333 ± 52
Patanjali	405.8 ± 17	272.2 ± 90
Raw	376.4 ± 20	515 ± 52.4

Phenolics and Flavonoids are the types of polyphenols present in honey. In the current study, phenolic concentration of honey samples ranged from 300 – 425mg/kg of honey (Table 3). These findings were similar to the study carried out by Pontis *et. al*. in 2014. The Flavonoids results of honey, in this study ranged from 270 – 520 mg/kg, which was higher than the results obtained from the northeast of Brazil, which ranged from 2.5 to 83mg/kg of honey (Liberato *et. al*., 2011).

Sugar estimation:

Results showed that highest amount of sugars were present in Dabur honey (805 gm/kg) and lowest were present in Raw honey (755 gm/kg) (Table 4). Among the different attributes in honey responsible for the antimicrobial activity, concentration of sugars is one of the important attribute.

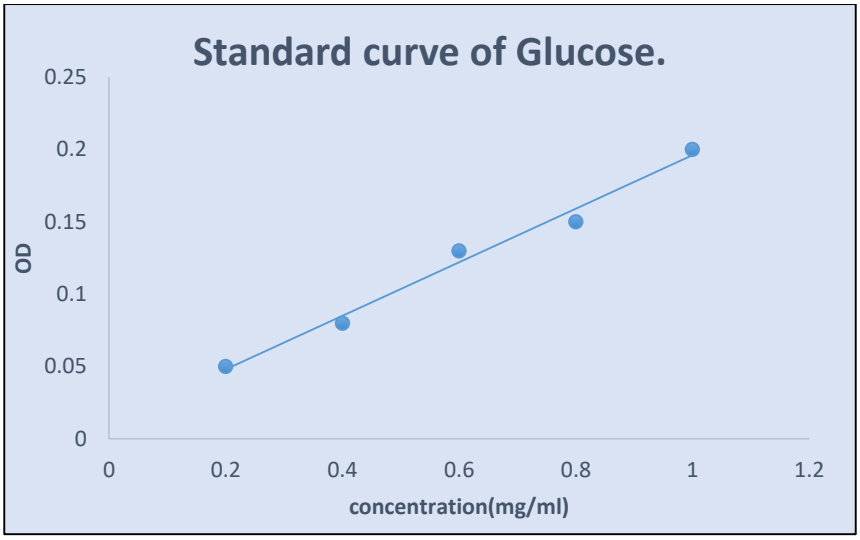

Figure 6: Standard curve of Glucose

Table 4: Results of sugar estimation

Honey samples	**Abs (490nm)**	**Sugar concentration (gm/kg honey)**
Dabur	1.45	805
Patanjali	1.40	777
Raw	1.36	755

Sugars are the main components of honey which depends mostly on floral and geographical origins and less on seasonal, processing and storage conditions (Dobre *et. al*., 2012). Glucose and fructose are the majorly the sugars present in honey. In 2019, Nayik *et. al*., in their studies, have characterized four different honey from Kashmir Valley on the basis of their physiochemical property and carbohydrate profile and, in their studies, they have found the sugar concentration ranged from 73% to 78%, the results were comparable to the results observed in our study.

Hydrogen peroxide content:

In our study, raw honey was found to release more hydrogen peroxide (0.20%) on dilution, followed by Patanjali which was followed by Dabur honey (Table 5).

Table 5: Percentage of hydrogen peroxide in honey samples

Honey samples	**Hydrogen peroxide percent**
Dabur	0.10%
Patanjali	0.17%
Raw	0.20%

The literature suggested that the rate of hydrogen peroxide production by glucose oxidase in honey depends largely on the degree of honey dilution and that little or no production of hydrogen peroxide occurs in full strength honey (White *et. al*., 1963). The levels of H_2O_2 in honey may differ between different types of honey, regardless of its botanical and geographical origin (Brudzynski *et. al*., 2011).

Antibacterial activity:

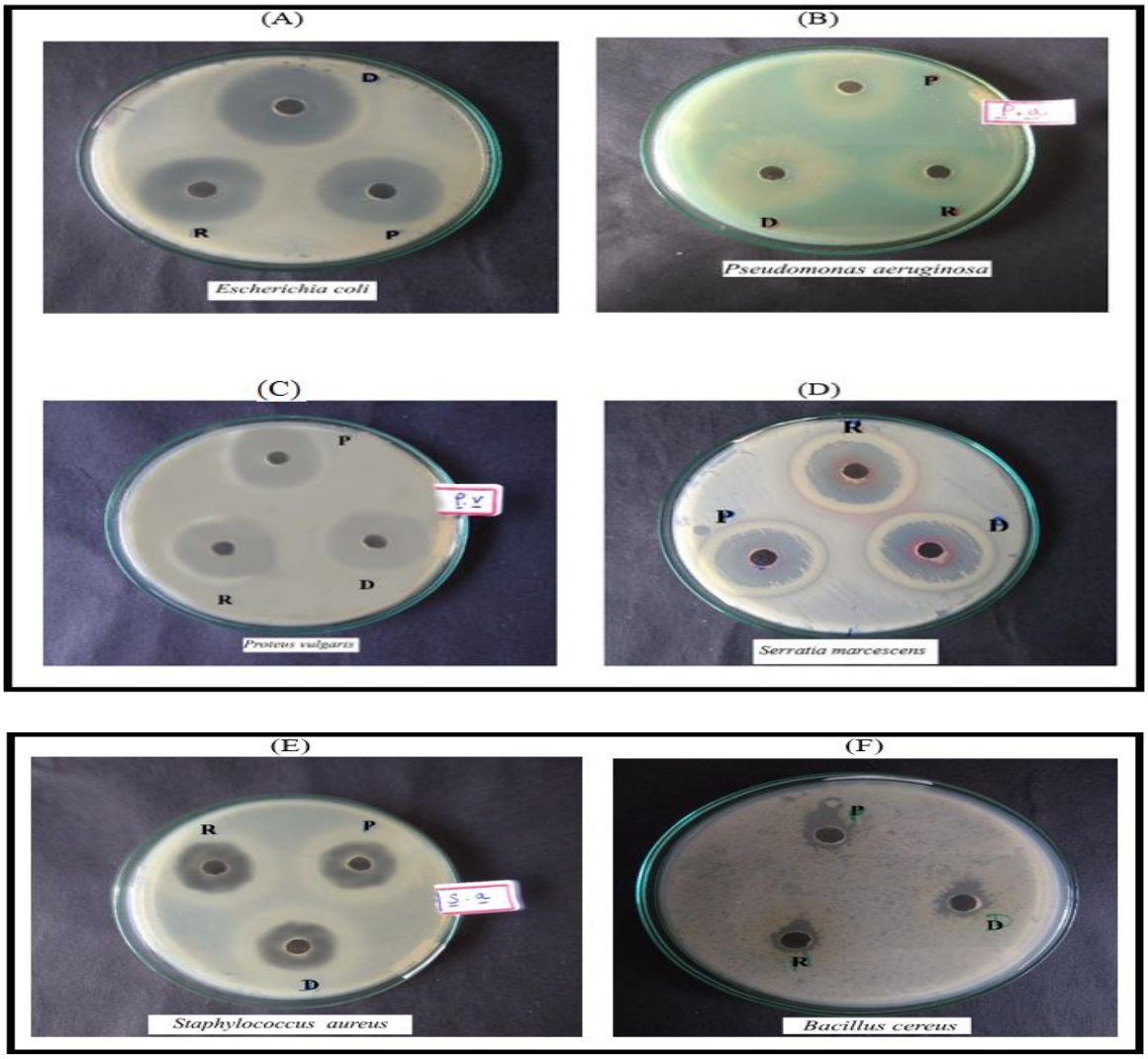

Figure 7: Zone of inhibition showing antibacterial activities of honey samples

All the three types of honey were found to inhibit the growth of all the tested microorganisms. Dabur honey was found to have highest antibacterial activity compared to other two honey samples (Figure 7).

Compared to Patanjali and Raw honey, Dabur honey showed most effective antibacterial activity. In our study, *Staphylococcus aureus* was found less susceptible to Dabur honey compared to other two honeys. *Bacillus cereus* showed the lowest susceptibility to all types honey samples. *Proteus vulgaris* showed high susceptibility to all types of honey. Patanjali and raw honey showed quite similar antibacterial activity (Figure 7).

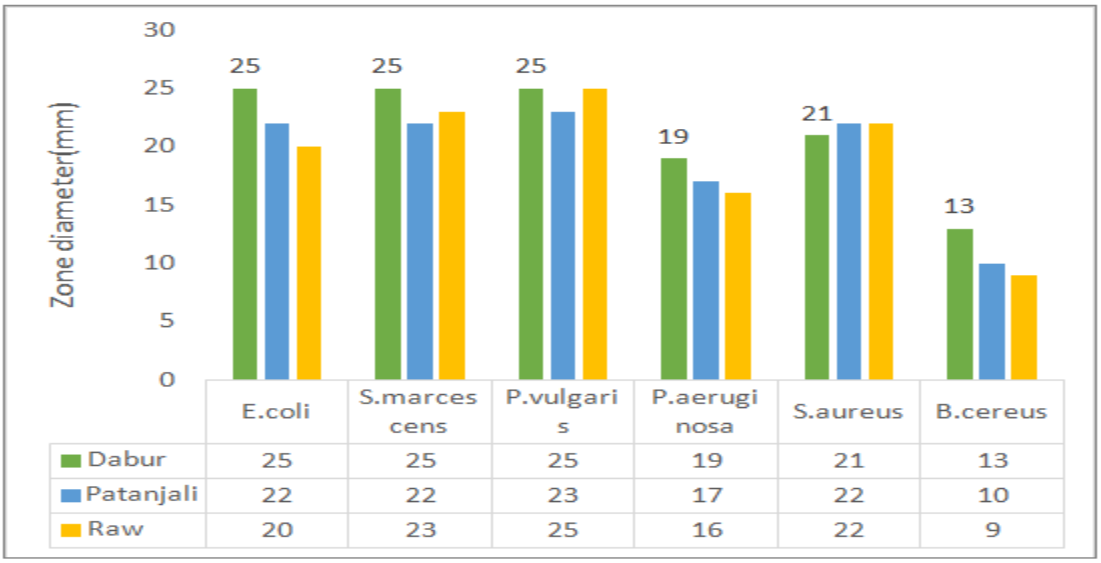

Figure 8: Antibacterial activities of all three tested honey samples

In the present study, Dabur honey gave highest inhibition zone (25mm) against *Serratia marcescens*, *Proteus vulgaris* and *Escherichia coli*. Lowest inhibition zone (13mm) was found against *Bacillus cereus*. Compared to Raw and Patanjali honey, Dabur honey gave bigger inhibition zones against all the tested bacteria except *Staphylococcus aureus*. Patanjali honey gave highest inhibition zone (23mm) against *Proteus vulgaris* and lowest inhibition zone (10mm) against *Bacillus cereus*. Raw honey showed highest inhibition zone (25mm) against *Proteus vulgaris* and lowest inhibition zone (9mm) against *Bacillus cereus* (Figure 7 and 8).

In 2003, Miorin *et. al*. have also suggested that effectiveness of honey depends on difference in chemical composition, bee species and geographic region. In 2014, Elbanna *et. al*. have studied and stated that types of honey exhibited various degrees of antibacterial activity

against different bacteria. Different species of bacteria differed in their sensitivity to honey, wherein *Salmonella enteritidis* was found to be the most sensitive one followed by *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli*, respectively. In the contrary, in our study, the most sensitive bacterium amongst all the selected microorganisms was *Proteus vulgaris*, which was followed by *Escherichia coli* and *Serratia marcescens* respectively.

In the present study, variation in potency of antibacterial activity found indicates the different botanical and geographical origin and also processing methods. The similar findings were also reported by Olga *et. al*. in 2012 who stated that the chemical composition and bioactivity of honey are greatly affected by the botanical and entomological source, as well as by the climatic and geographical location.

In present study, highest sugar concentration was present in Dabur honey and it also exhibited most effective antibacterial activity, so it can be said that high sugar concentration is one of the reasons for the antimicrobial activity. Acidic pH and hydrogen peroxide are also responsible for antibacterial activity.

Antioxidant activity

Reducing power assay is the only one that directly estimates antioxidants in a sample, and is based on the ability of the analyte to reduce the Fe^{3+}/Fe^{2+} couple. All the honey samples tested exhibited the reducing power. In the present study, the highest antioxidant activity was exhibited by Raw honey (3.6gm/kg) compared to both the commercial honey tested. Lowest antioxidant activity was detected in Dabur honey (2.3gm/kg) (Table 6).

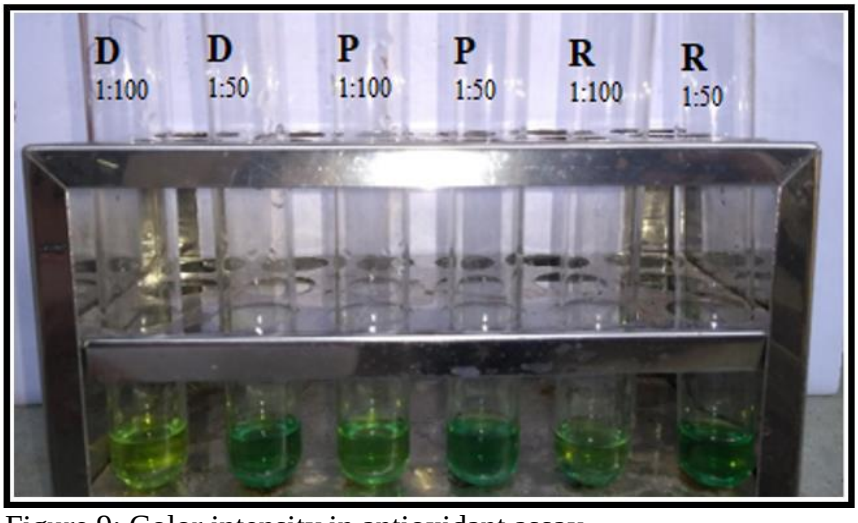

Figure 9: Color intensity in antioxidant assay

Table 6: Antioxidant activities of tested honey samples

Honey samples	**Abs (1:50) 700nm**	**Abs (1:100) 700nm**
Dabur	0.33	0.10
Patanjali	0.43	0.17
Raw	0.44	0.19

In the current study, results showed that Raw honey has more antioxidants compared to processed Dabur and Patanjali honey samples (Table 6) and was found in accordance with the study carried out by Wang *et. al*., (2004) where they have reported that processed buckwheat honey has 33.4% lesser antioxidant activity than raw buckwheat honey. In 2012, Alzahrani *et. al*. have evaluated the antioxidant activity of honey from geographical origin using reducing power assay and found highest antioxidant activity in Manuka honey.

Conclusion:

In conclusion, all the tested honey samples exhibited potent antibacterial and antioxidant properties. Raw honey has lowest sugar concentration (755gm/kg), highest antioxidant activity and polyphenols providing it with the darkest color compared to commercial honeys. It also exhibited good antibacterial activity. Dabur honey exhibited highest antibacterial activity; it also had highest sugar concentration (805gm/kg) and lowest pH. It consists of lowest polyphenols and antioxidant activity. Patanjali honey exhibited antibacterial activity similar to Raw honey and, also had highest pH. It consists of second highest polyphenols after Raw honey and also has second highest antioxidant activity. In spite of having second highest polyphenols color of the Patanjali honey was the lightest among the three. Significant relation was observed between antioxidant activity and polyphenolics content in all the three honey samples. Due to the processing techniques carried out in processed honey, not much affect was seen on their biological properties. From the results obtained, it can be said that Raw and commercial honeys have good biological properties in different proportions. Moreover, antimicrobial activity of all the three tested honey samples suggests that honeys under analysis may have a relevant role as antibacterial natural products to reduce the effects of bacterial infections and contribute for better food.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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