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# Antioxidant and Antimicrobial Activities of Four Characteristic Honey Samples Produced in West Bengal (India)

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**ABSTRACT**

*Four different honey samples, viz. Sundarban honey, Litchi honey, Cumin honey and Eucalyptus honey, produced in the Southern part of West Bengal (India), were tested for their antioxidant and antimicrobial properties. Among the six standard antioxidant assay protocols (viz. ABTS radical decolorization assay, DPPH radical decolorization assay, assay for total phenolis content, FRAP assay, hydroxyl radical scavenging assay and assay for inhibition of lipid peroxidation) applied in the present study, Sundarban honey gave the best result, in terms of gallic acid equivalents. Greater antioxidant potential of the sample was reflected in its' antibacterial property, as it produced highest zone of inhibition against all the five bacteria (viz. Escherichia coli, Klebsiella aerogens, Staphylococcus aureus, Salmonella typhi and Bacillus cereus) used in the study. Eucalyptus honey was the second best in the study protocols. The honey samples possessed very low hydroxyl radical scavenging or anti-lipid peroxidation activities, indicating that the samples might produce peroxide themselves for their antibacterial activities.*

**Keywords**: Honey; Antioxidant; Antimicrobial; Lipid peroxidation

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## INTRODUCTION

Honey is a low-cost natural product that can be used for different purposes. Now, commercially honey is used in various industries for product formation and this trend is increasing day by day as industrialists are finding honey to be cheap source of sweetening agent without any side-effects as in case of synthetic sweeteners. Honey showed physiological effects on blood health indicators as well as effects on hepatitis A, cardiovascular health and radiation mucositis patients [1]. Medicinal importance of honey has been documented in the world's oldest medical literatures, and in holy book like The Quran, the colourful colloid was described to be a remedy with tremendous benefits that was used in folk medicine [2]. Honey is produced in most of the countries of the world. Ayurvedic as well as Yunani medicine have been using honey as a vital medicine for centuries. Scientists of today also accept honey as a very effective medicine without any side effects for all kinds of diseases [3]. A few phenolic acids were detected from honey samples, in addition to flavone and flavanones as well as organic acids, using modern methods like UPLC-MS/MS. Particularly honey content of flavonoids and polyphenols (including phenolic acids) might be related to the antioxidant activity of the substance [4]. Besides vitamins, these compounds were most probably the key constituents contributing to the antioxidant capacity of honey samples. Since the ancient times, it has been known to possess antimicrobial property as well as wound-healing activity. The healing property of honey is believed to be due to the fact that it maintains a moist wound condition and its high viscosity helps to provide a protective barrier to prevent infection. Honey has anti inflammatory, antioxidant and immune boosting property attributed to the high sugar concentration and the resulting osmotic effect, low pH and acidity, and hydrogen peroxide [5]. Its immunomodulatory property is relevant to wound repair too. The antimicrobial activity in most honeys is also due to the enzymatic production of hydrogen peroxide. However, another kind of honey, called non-peroxide honey,

displays significant antibacterial effects even when the hydrogen peroxide activity is blocked. Its mechanism may be related to the low pH level of honey and its high sugar content (high osmolarity) that is enough to hinder the growth of microbes [6]. The moisture absorbing quality of honey helps breads, cakes, cookies and candies stay fresh longer. In nut-shell, honey is a primeval remedy for the management of infected wounds, which has recently been rediscovered by the medical science [7].

There is a large variation in the antimicrobial activity of some natural honeys, which is due to spatial and temporal variation in sources of nectar [8]. Thus, for honey to be used as an antimicrobial alternative it has to be first tested in laboratory to determine its antimicrobial spectrum. Secondary metabolites of plant origin present in honey are one factor contributing to its antioxidant activity [9]. Many publications attest to honey's antimicrobial properties but the mechanisms by which it acts are incompletely studied. To date, few clinical reports have defined the specific type of honey applied to infected wounds, burns or ulcers. Similarly, *in vitro* investigations of antimicrobial activity have used uncharacterized honey samples. Although it appears that the honey from certain plants has better antibacterial activity than from others, there is not enough evidence for such definite conclusion to be justified because the data are from small numbers of samples. Thus it is important that when honey is to be used as an antimicrobial agent, it is selected from honeys that have been assayed in the laboratory for antimicrobial activity. The current study was planned to analyze the four different honey samples produced in the southern parts of the state of West Bengal (e.g. Sundarban honey, Litchi honey, Cumin honey and Eucalyptus honey) for any differences in their antioxidant and antimicrobial activities. The antimicrobial activity was tested against the five different pathogenic bacteria (i.e., *Escherichia coli, Klebsiella aerogens, Staphylococcus aureus, Salmonella typhi* and *Bacillus cereus*).

## EXPERIMENTAL SECTION

### Materials and Methods

2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), ABTS, were obtained from Sigma, USA. 2,2′-Diphenyl-1-picryl hydrazyl (DPPH) and Muller-Hinton agar were obtained from Himedia, India. Analytical grades of thiobarbituric acid (TBA), ascorbic acid, gallic acid, Folin-Ciocalteau's solution, sodium hydroxide and sodium carbonate were obtained from Merck, India. All other reagents and chemicals used were of analytical grade procured from local sources. Deionized distilled water was used in the entire study.

### Collection and Processing of Samples

The honeys were collected from a Government honey processing organisation of West Bengal. The honeys were stored in normal room tempareture at 25°C and experimented within 7 days of procurement. The four different honey samples used in the study were Sundarban honey, litchi honey, cumin honey and eucalyptus honey. The samples were marked as SH, LH, CH and EH, respectively, and were taken in different sterile micro-tubes. Experiments were performed with aqueous solution of the samples. For this, 100 µl of honey was taken in a micro-tubes and 900 µl of distilled water was added. Then they were mixed well by shaking with hands for about 5 minutes.

### ABTS Radical Decolorization Assay

The ABTS assay was performed using a previously described procedure [10]. The oxidant was generated by persulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid. The oxidant solution was mixed with the sample/standard solutions in such a way that total volume of the solution reached 1 ml. The absorbance at 734 nm in a Systronics spectrophotometer (model – 2202) was read at room temperature, 4 minutes after mixing. The results were expressed as Gallic acid equivalents.

### DPPH Radical Decolorization Assay

The DPPH assay was performed using a previously described procedure [10]. DPPH solution (0.1 mM) was mixed with sample/standard solution and the decrease in absorbance of the mixture after 20 minutes of incubation in the dark was monitored at 517 nm in a Systronics spectrophotometer (model – 2202). The results were expressed as Gallic acid equivalents.

### Estimation of Total Phenolics Content

Total phenolics compound contents were determined by the Folin-Ciocalteau method [11]. The samples/standards were mixed with Folin-Ciocalteau reagent (1:10 diluted with distilled water) for 5 min and aqueous sodium carbonate (1 M) was then added. The absorbance of the reaction mixture was then measured at 765 nm in a UV-Vis

spectrophotometer (model – Systronics 2202). Gallic acid was used as standard. The results were expressed in terms of gallic acid equivalent/ml honey.

**Ferric Reducing Antioxidant Power (FRAP)**

Ferric reducing antioxidant power of the samples was estimated with a previously described procedure [12]. Briefly, a maximum of 100 µl of extract solution or standard was mixed with 1.9 ml of FRAP reagent and incubated at 37°C for 30 mins. FRAP reagent was prepared by mixing 0.1 M aqueous acetate buffer (pH 3.6), TPTZ solution and ferric chloride solution. After the stipulated time period, absorbance was measured at 593 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Gallic acid is used as standard. Results were expressed as Gallic acid equivalents (GAE) in terms of gallic acid equivalent/ml honey.

**Hydroxyl Radical Scavenging Assay**

Hydroxyl radical scavenging potentials of the samples were estimated with a previously described procedure [13]. Briefly, 10 mM each of $FeSO_4.7H_2O$, EDTA, 2-deoxy-D-ribose and $H_2O_2$ solutions were prepared in water. Each solution of above four with sample/standard solution was mixed in a test tube to get a final volume of 1 ml and incubated at 37°C for 90 mins. $H_2O_2$ solution was added last. After the incubation, 2.8% (w/v) aqueous TCA solution and 1% (w/v) aqueous TBA solution were added to the reaction mixture and kept at boiling water bath for 20 mins. Development of the pink chromophore was measured at 532 nm in a UV-Vis spectrophotometer (model – Systronics 2202). Results were expressed as Gallic acid equivalents (GAE) in terms of gallic acid equivalent/ml honey.

**Inhibition of Lipid Peroxidation *in vitro***

A 10% (w/v) of fresh chicken liver homogenate was prepared using ice-cold KCl (0.15 M) in a Teflon tissue homogenizer and the test system containing homogenate of protein content was adjusted to 500 µg/ml. In the control system to 1ml of tissue homogenate, the lipid peroxidation was initiated by the addition of $FeSO_4$ (25 µM), ascorbic acid (100 µM) and $KH_2PO_4$ (10 mM). The volume was made up to 3 ml with distilled water and incubated at 37°C for one hour. Then 1 ml of 5% TCA and 1 ml of 0.67% TBA in 50% acetic acid was added to this reaction mixture and the tubes were boiled for 20 mins in a boiling water bath. The extent of inhibition of lipid peroxidation was evaluated by the estimation of Thiobarbituric acid reactive substances (TBARS) level by measuring the absorbance at 532 nm. The results were expressed as gallic acid equivalents (GAE) in terms of gallic acid equivalent/ml honey [14].

**Antimicrobial Activity**

**Determination of zone of inhibition:**

Antimicrobial activity was measured by agar well diffusion method. The different pathogenic bacteria used in the study were *Escherichia coli, Bacillus cereus, Staphylococcus aureus, Salmonella typhi* and *Klebsiella aerogens*. Each bacterial isolates was previously grown on sterile Muller Hinton Agar plate at 35 ± 2°C for 24 hours. After that, each of the isolates was inoculated with 100µl of standardized inoculums of each bacterium (in triplicates) and spread with sterile cotton swabs. Wells are 6 mm sizes were made with sterile borer into agar plates containing the bacterial inoculums. Each plate was punched to make 4 wells of 6 mm diameter with the help of a sterile cork borer at different sites of the plates. Different working dilutions of four different honey extracts were prepared in sterile water. For different honey extracts, 500 mg/ml, 400 mg/ml, 300 mg/ml, 200 mg/ml, 100 mg/ml and 50 mg/ml dilutions were prepared. From these different dilutions as well as raw honey, 50 µl solution was poured into the wells of the respective culture plates. The plates thus prepared were left at room temperature for ten minutes allowing the diffusion of the extract into the agar. After incubation for 24 hrs at 35°C, the plates were observed. If antibacterial activity were present on the plates, it would be indicated by an inhibition zone surrounding the well containing the different dilutions of different extracts. The zone of inhibition was measured and expressed in millimeters. Antibacterial activity was recorded if the zone of inhibition was greater than 6 mm. The antibacterial activity results were expressed in term of the diameter of zone of inhibition and <9 mm zone was considered as inactive; 9-12 mm as partially active; while13-18 mm as active and >18 mm as very active [15].

**Determination of minimum inhibitory concentration (MIC):**

The minimum inhibitory concentration (MIC) of active extract was evaluated by tube dilution method. The MIC of all the extract was determined by dilution of the extract to various concentrations (5.000 to 0.150 mg/mL) as NCCLS. Decreasing concentrations of aqueous extract were prepared in serial two-fold dilutions using Soybean casein digest broth (SCDB). 100 µl standard inoculum of the microorganism was added to an equal volume (5 ml) of

each concentration and to a tube of the growth medium without extract that served as growth control. An uninoculated tube of SCDB was incubated to serve as a negative growth control. In one tube, water was added to SCDB which served as solvent control. After overnight incubation at 37º C, the tubes were examined for turbidity indicating growth of the microorganisms. The lowest solution of the extract that inhibited growth of the microorganism as detected by the lack of visual turbidity (matching the negative growth control) was designated the minimum inhibitory concentration [16].

**Statistical Analysis**

Values were expressed as mean ± standard deviation of four replicates for each experiment. The analyses were done using the software – SPSS Statistics 17.0 (IBM Corporation).

## RESULTS AND DISCUSSION

It is noteworthy that the botanical origin of honey has the greatest influence on its antioxidant activity, while processing, handling and storage affects its antioxidant capacity only to a minor degree [17-19]. The qualitative and quantitative composition of honey (including the antioxidants constituent and the other phytochemical substances) is a reflection of the floral source as well as the variety of the particular honey [8]. The antioxidant activity has been shown to strongly correlate with the content of total phenolics. Earlier researches have reported that dark honey has a higher total phenolic content and consequently a higher antioxidant capacity [20]. Fractionation guided studies have shown that the antioxidant activity is located in both the ether and the aqueous fractions, indicating that the flavonoids or their glycosides of honey might be available in the human system, where they might exert different physiological effects [21]. The following is the result obtained from ABTS radical scavenging assay.

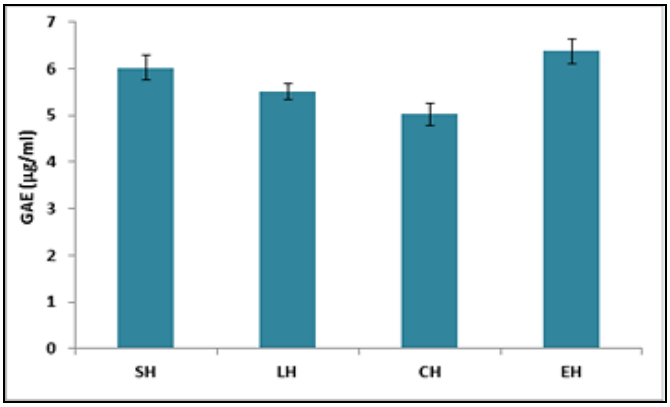


**Figure 1: ABTS radical scavenging activities of four different honey samples. Results are expressed as µg gallic acid equivalent (GAE)/ml honey**

The result of the assay indicated that all the honey samples showed significant radical scavenging activities (Figure 1). Amongst all, eucalyptus honey showed highest activity, followed closely by SH. CH showed the lowest activity.

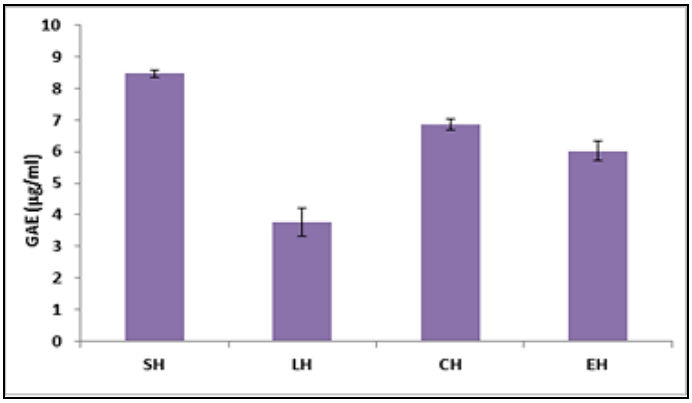


**Figure 2: DPPH radical scavenging activities of four different honey samples. Results are expressed as µg gallic acid equivalent (GAE)/ml honey**

The results of DPPH assay (Figure 2) indicated that all the honey samples showed significant radical scavenging ability. It was also found that SH sample given the highest scavenging. The excellent radical scavenging abilities of the honey samples, particularly by SH, substantiated the earlier finding by Blasa et al. [21]. Results from ABTS and DPPH assays established the fact that both the polar and non-polar fraction of honey contributed to the overall antioxidant capacities of the samples as ABTS assay indicates activities of the polar components, whereas DPPH assay indicates activities of non-polar components [10]. It can also be interpreted from the two scavenging assays that SH contained fair amounts of antioxidants of both higher and lesser polarities. However, non-polar antioxidative bioactives were lesser in LH and EH samples.

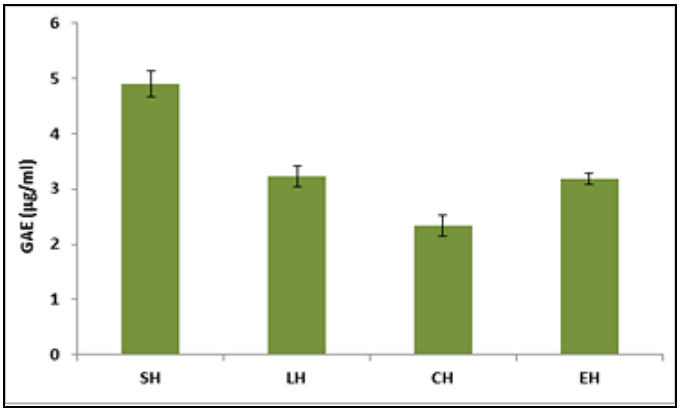


**Figure 3: Total phenolics contents of four different honey samples. Results are expressed as µg gallic acid equivalent (GAE)/ml honey**

The result of this assay indicated that SH honey sample had higher polyphenolics content in comparison to other honey samples (Figure 3).

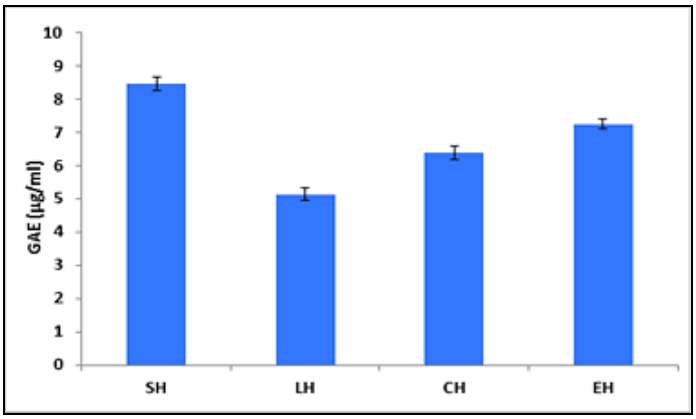


**Figure 4: FRAP of four different honey samples. Results are expressed as µg gallic acid equivalent (GAE)/ml honey**

The result of FRAP assay indicated that SH honey sample had higher ferric reducing potential in comparison to other honey samples (Figure 4). Results of FRAP assay and TPC assay indicated that the mechanism involved in SH samples for radical neutralization were mainly electron-transfer.

The result of hydroxyl radical scavenging assay indicated that SH honey sample had higher radical scavenging potential in comparison to other honey samples (Figure 5).

The result of this assay indicated that SH honey sample had the higher lipid peroxidation protecting potential in comparison to other honey samples (Figure 6). The result is also at par with the hydroxyl radical scavenging assay (Figure 7), which is directly related with the inhibition of lipid peroxidation. Interesting results were obtained from the antimicrobial assays with the four honey samples. In the present study, the four honey samples inhibited the growth and multiplication of the all tested bacterial strains, e.g. *Escherichia coli*, *Bacillus cereus*, *Salmonella typhi*, *Staphylococcus aureus* and *Klebsiella aerogenes.* Both the raw and water extract of the samples showed promising results. Sundarban honey showed most potent activity against *Escherichia coli* amongst all the tested strains. Between the honey samples, SH was better against all the tested strains. Litchi honey and cumin honey were found to be better than Eucalyptus honey. However, the samples were not very effective against the tested strain of *Staphylococcus aureus*.

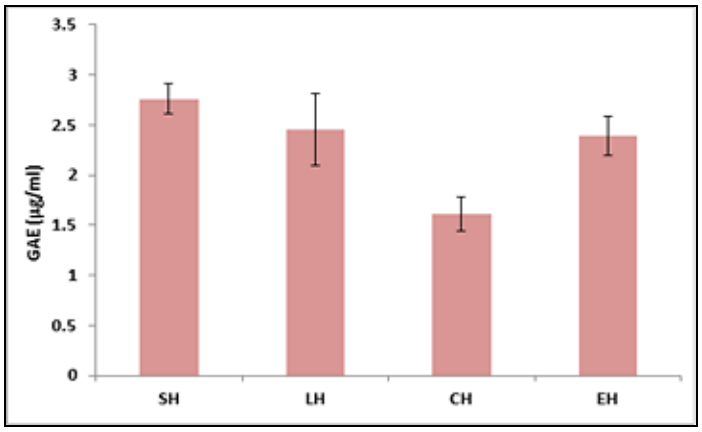


**Figure 5: Hydroxyl radical scavenging activities of four different honey samples. Results are expressed as µg gallic acid equivalent (GAE)/ml honey**

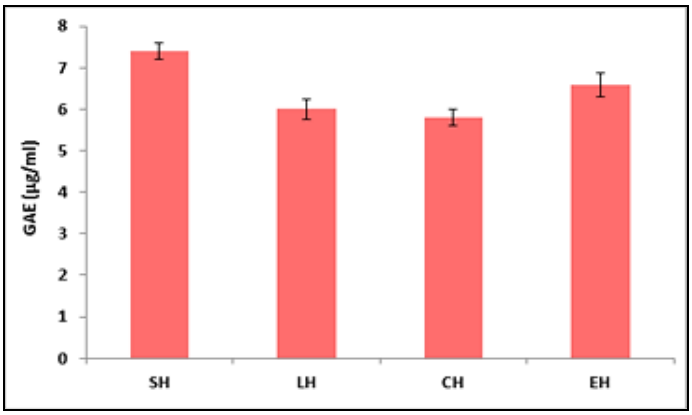


**Figure 6: Inhibition of lipid peroxidation by four different honey samples. Results are expressed as µg gallic acid equivalent (GAE)/ml honey**

The MICs of various types of honeys for various pathogenic bacterial strains have been determined by many authors. In the present study, MIC for the honey samples was determined against some bacterial strains that usually produce food-borne or GI-diseases.

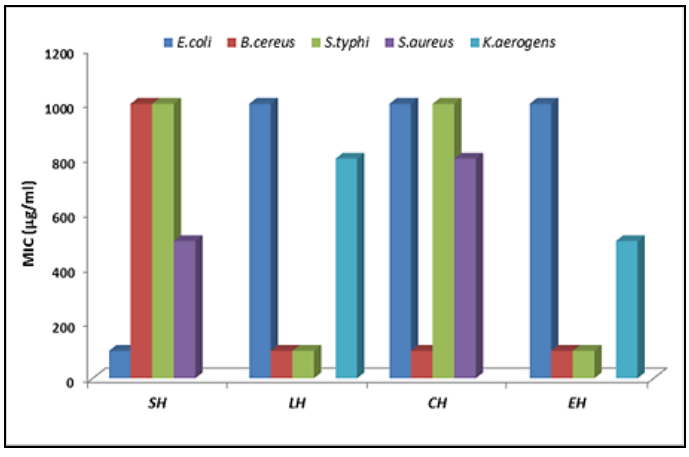


**Figure 7: Minimum inhibitory concentrations (MIC, µg/ml) of four different honey samples against five tested microorganisms**

A large number of *in vitro* and clinical studies have confirmed the broad-spectrum antimicrobial activity of honey, which may be due to low pH, osmotic effect (due to high sugar concentration), presence of bacteriostatic and bactericidal factors like hydrogen peroxide, lysozyme, polyphenols, phenolic acids and flavonoids, methylglyoxal and small peptides like abaecin, defensin-1, apidaecin, and hymenoptaecin as well as to immune modulating and

anti-inflammatory properties [22]. A study with thirteen different honey samples showed excellent antibacterial activities against *E. coli* and *P. aeruginosa* when tested *in vitro* [23]. The authors indicated non-peroxide antibacterial activities for the honey samples. In another interesting study, methylglyoxal supplemented honey samples showed potent antibacterial activity against biofilm production by *S. aureus*, indicating probable role of the compound against pathogenicity of the prokaryotes [24]. In the present study, we have observed that the honey samples possessed very low anti-lipid peroxidation activities (due to their low gallic acid equivalent values). This indicated that the samples might produce peroxide themselves for their antibacterial activities.

## CONCLUSION

The present study analyzed four different honey samples, produced in the southern parts of the state of West Bengal (e.g. Sundarban honey, Litchi honey, Cumin honey and Eucalyptus honey), for their antioxidant and antimicrobial activities. The antimicrobial activity was tested against the five different pathogenic bacterial strains (i.e., *Escherichia coli, Klebsiella aerogens, Staphylococcus aureus, Salmonella typhi* and *Bacillus cereus*). Among all the honey samples, Sundarban sample possessed the highest antioxidant potential, albeit the gallic acid equivalent was a bit low. The same sample showed best antibacterial activity in the antibiogram patterns (Table 1). Eucalyptus honey was second in the list. The honey samples possessed very low anti-lipid peroxidation activities, indicating that the samples might produce peroxide themselves for their antibacterial activities.

**Table 1: Antibiogram patterns of four honey samples against five different microorganisms. Results are expressed as average of three readings**

| Sample | Concentration (mg/ml) | Zone of inhibition (mm) | | | | |
|---|---|---|---|---|---|---|
| | | ***Escherichia coli*** | ***Bacillus cereus*** | ***Salmonella typhi*** | ***Staphylococcus aureus*** | ***Klebsiella aerogens*** |
| SH | Raw | 34 | 23.6 | 29.3 | 17.6 | 26 |
| | 0.5 | 32.3 | 20.3 | 28 | 14.6 | 22.6 |
| | 0.4 | 31 | 18 | 24.6 | 13 | 21 |
| | 0.3 | 29.6 | 14.3 | 22.3 | 11.6 | 18.3 |
| | 0.2 | 25.3 | 11 | 19 | 9 | 15.6 |
| | 0.1 | 23 | 9.6 | 18.3 | - | 11.6 |
| | 0.05 | 20.3 | 8 | 15.3 | - | 8.6 |
| LH | Raw | 30.3 | 28.3 | 25 | 18 | 22 |
| | 0.5 | 28.3 | 24 | 22.6 | 14.6 | 20.3 |
| | 0.4 | 26.3 | 22 | 20.3 | 13 | 19 |
| | 0.3 | 25 | 20.6 | 18 | 10.6 | 17.6 |
| | 0.2 | 21.6 | 17.3 | 14 | 9 | 15 |
| | 0.1 | 20 | 14 | 11.6 | - | 12 |
| | 0.05 | 18.3 | 10 | 9 | - | 10.3 |
| CH | Raw | 31.6 | 26 | 27.3 | 15.6 | 24 |
| | 0.5 | 29.3 | 24 | 26 | 13.3 | 21.6 |
| | 0.4 | 28 | 21.3 | 23.6 | 11.6 | 18.3 |
| | 0.3 | 25 | 20 | 21 | 9 | 16 |
| | 0.2 | 22.3 | 18.3 | 18 | - | 13.3 |
| | 0.1 | 20.6 | 16 | 15.3 | - | 10 |
| | 0.05 | 17 | 12.6 | 12 | - | - |
| EH | Raw | 30 | 28 | 31.6 | 14 | 26 |
| | 0.5 | 27.6 | 23.6 | 28 | 10.6 | 23.6 |
| | 0.4 | 25 | 21.3 | 23 | 10 | 11.3 |
| | 0.3 | 22 | 20 | 21 | 8 | 18 |
| | 0.2 | 21.3 | 18.6 | 19.3 | - | 15.6 |
| | 0.1 | 18.6 | 15.6 | 16 | - | 13.3 |
| | 0.05 | 16 | 11.3 | 14.3 | - | 10 |

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