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Resolving a mechanism of honey antibacterial action: Polyphenol/H_2O_2-induced oxidative effect on bacterial cell growth and on DNA degradation

Madhavi Rane*[1], Kalyani Deshmukh[2], Paurnima Mulik[3], Nihal Mulani[4], and Rajeshree Patwardhan[5]

[1,2,3,4]Department of Microbiology, Abeda Inamdar Senior College, Azam Campus, Pune 411001 (India)
[5]Department of Microbiology, H.V.Desai College, Pune 411011 (India)
*Address for correspondence
E-mail: drmadavidrane@gmail.com

Abstract

Honey has an established function as an antibacterial agent that has a broad spectrum of activity against gram-positive and gram negative bacteria such as *Salmonella spp.*, *Staphylococcus spp.*, *Escherichia coli*, *Bacillus spp*. Large number of honeys from different geographical locations and different botanical origins show growth inhibitory action. Several bioactive compounds have been identified in honey which contributed to its antibacterial action. The commonly accepted list of contributors includes hydrogen peroxide, catalase to hydrogen-peroxide ratio, polyphenols, antioxidants, DNA degradation and protection assay, Minimum Inhibitory Concentration and Minimum Bactericidal Concentration. Removal of H_2O_2 by catalase eliminated bacteriostatic activities caused by both phenolics and H_2O_2 suggesting that the growth inhibition resulted from the coupling chemistry between these compounds. Both phenolics and H_2O_2 were involved in DNA degradation by honey. In conclusion the aim of study was to find out the antibacterial activity of honey by DNA degradation assay,Hydrogen peroxide assay and phenolic content.

Traditional uses of natural honey for humans are traced to some 8000 years ago as depicted by Stone Age paintings. The ancient ayurvedic civilization considered honey one of nature's most remarkable gifts to humans. Traditionally, honey is a boon to those with weak digestion, also it has been emphasized that the use of honey is highly beneficial in the treatment of irritating cough also as valuable in keeping the teeth and gums healthy ,effective against wounds, bacterial infections ,gut abnormalities it is an everyday household product that can provide various healing benefits. Honey has an established

function as an antibacterial agent against broad spectrum of gram-positive and gram negative bacteria such as *Salmonella spp., Staphylococcus spp., Escherichia coli, Bacillus spp.* Many bioactive compounds have been identified in honey which contributed to its antibacterial action. This includes hydrogen peroxide, polyphenols, and antioxidant. Structural and functional diversity of these bioactive components implies that they can exert their degrading action to the bacterial cell through different mechanisms.Although, a generally accepted view is that degradative action of hydrogen peroxide produced in honey are the main mechanisms responsible for the antibacterial activity of honeys. The bacteriostatic efficacy of honey H_2O_2 was strongly influenced by two factors.

Susceptibility of bacteria to oxidative stress mediated by

1. H_2O_2
2. By honey phytochemical components.

We also made an important finding that honey has the ability to degrade DNA of both bacterial and plasmid origin. This DNA degrading activity was due to the presence of honey H_2O_2 but also required the use of other honey components because H_2O_2 alone did not cause DNA degradation[2]. Other components such flvonoids, polyphenols ,antioxidants and also the honey enzymes which are invertase, diastase, glucoseoxidase, catalase, and acid phosphatase. The activity of glucose-oxidase is usually related to the honey antimicrobial properties. The enzyme catalyses the conversion of glucose to gluconolactone, which is further converted to gluconic acid and hydrogen peroxide (H_2O_2). The resulting products are responsible for antimicrobial activity. Catalase enzyme has the role of converting peroxide into water and oxygen. Due to this the transportation of nutrients to organism and its presence will reduce the antibacterial activity of the honey, which means honey with a high catalase activity has a low antibacterial peroxide activity[1]. The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration of an antimicrobial component that is bacteriostatic which prevents the visible growth of bacteria. MICs are used to find the antimicrobial efficiency of various compounds by calculating the effect of decreasing concentrations of antibiotic/antiseptic over a defined period in terms of inhibition of microbial population growth. Thus the aim of study was to establish a correlation between the color, flavonoids, and phenolic contents, and antioxidant capacity of the honey samples. Darker honey samples had higher amounts of phenolic compounds, flavonoids and increased antioxidant activity. We tried to resolve the coupling chemistry between polyphenols and H_2O_2 which resulted in phenolic auto-oxidation and a generation of radical species which was the mechanism responsible for DNA degradation by honey[2].

Collection of honey sample :

Samples were collected from different regions of Western Maharashtra, India. (Fig.1 and Table-1).

2. Catalase assay
Materials

Reagents used for CAT assay was:-

1. (0.065 M) Hydrogen Peroxide (H_2O_2)
2. 60 mmol/l Sodium-Potassium Phosphate Buffer, pH 7.4
3. 32.4 mmol/l Ammonium Molybdate ($(NH_4)_6\ Mo_7O_{24}\ .4\ H_2O$)

All reagents and honey were brought to 37°C. Sample, blank 1, blank 2, and blank 3 test tubes were prepared by adding Honey, Ammonium Molybdate,Phosphate buffer and Substrate as H_2O_2 and then pipetted into test tubes.

Reagent	Samples(µl)	Blank 1(µl)	Blank 2(µl)	Blank 3(µl)
Honey	50	-	-	-
Substrate	1000	1000	1000	-
Phosphate Buffer	-	-	50	1050
Ammonium Molybdate	-	1000	1000	1000
Honey	-	100	-	-

Tubes were incubated for 60 second at 37°C.

Ammonium Molybdate	1000	-	-	-

Absorption was read at 405 nm against blank 3

Calculation of CAT Activity :

$$\text{Honey CAT activity (kU/L)} = \frac{A_{(\text{Blank 1})} - A_{(\text{Sample})}}{A_{(\text{Blank 2})} - A_{(\text{Blank 3})}} \times 271$$

Where:

- Blank 1=Control, Blank 2=Standard, Blank 3=Reagent Blank, and 271=Constant.[1]

3. a) *Minimum Inhibitory Concentration (MIC):*

The MIC of the honey was determined using broth dilution technique. :

Two-fold serial dilutions 4 of the honey were prepared by adding 2ml of the honey into a test tube containing 2ml of Nutrient broth, thus producing solution containing 50v/v of the extract. The process continues serially up to test tube No. 5, hence producing the following concentrations; 3.125,6.25,12.5,25,50v/v. Test tube No. 6 do not contain extracts and served as negative control. Exactly 0.5ml of 0.5 McFarland equivalent standards of test organisms (*Staphylococcous aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Salmonella abony* NCTC 6017, *Bacillus spp.*) were introduced into the test tubes and incubated at 37 °C for 24 hours. After incubation the test tubes were observed for growth by checking for turbidity.[3]

b) *Antibactrial activity of honey:*

The agar well diffusion technique were used to screen for antibacterial activity of honey .The standard inoculums (0.5 MacFarland Standard) were introduced into the surface of the Mueller Hinton agar plate

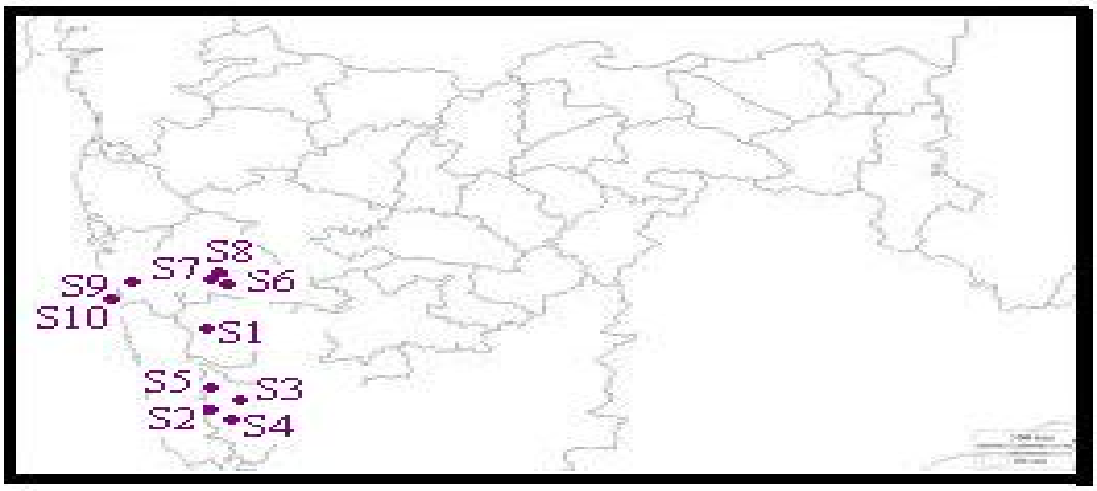

Fig. 1. Map of Western Maharashtra.

Samples	Region
S1	Mahabaleshwar
S2	Gaganbavda
S3	Radhanagari
S4	Kolhapur

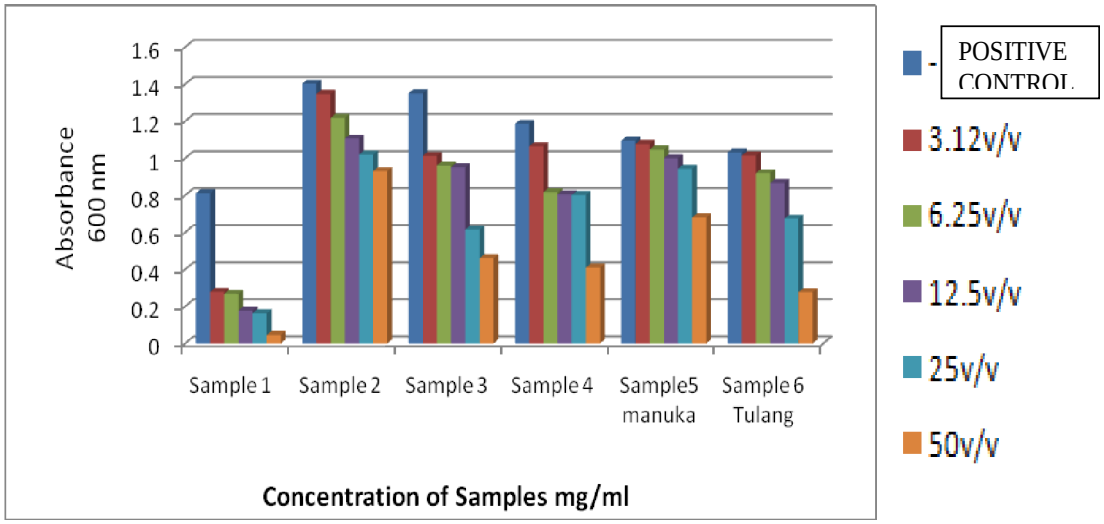

Fig. 2. Distribution of bacteriostatic activity (MIC) among 6 honey samples against *Bacillus* spp.

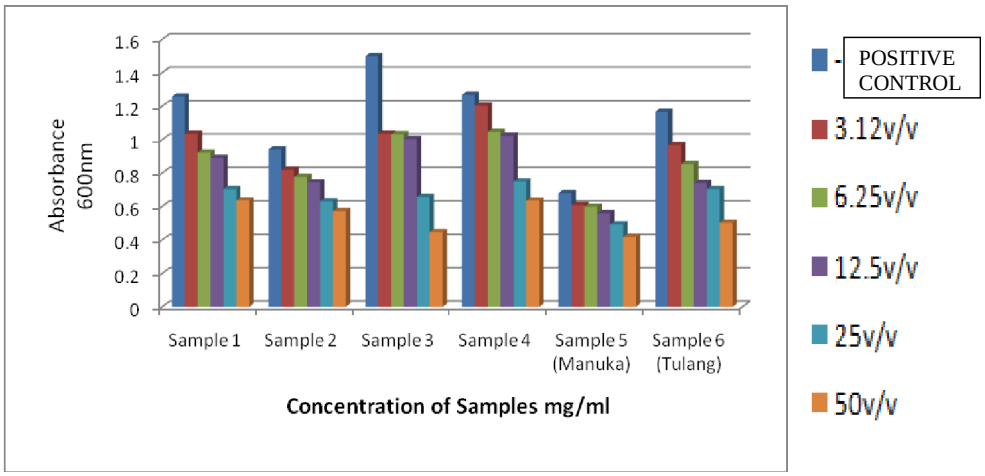

Fig. 3. Distribution of bacteriostatic activity (MIC) among 6 honey samples against *Staphylococcus* spp.

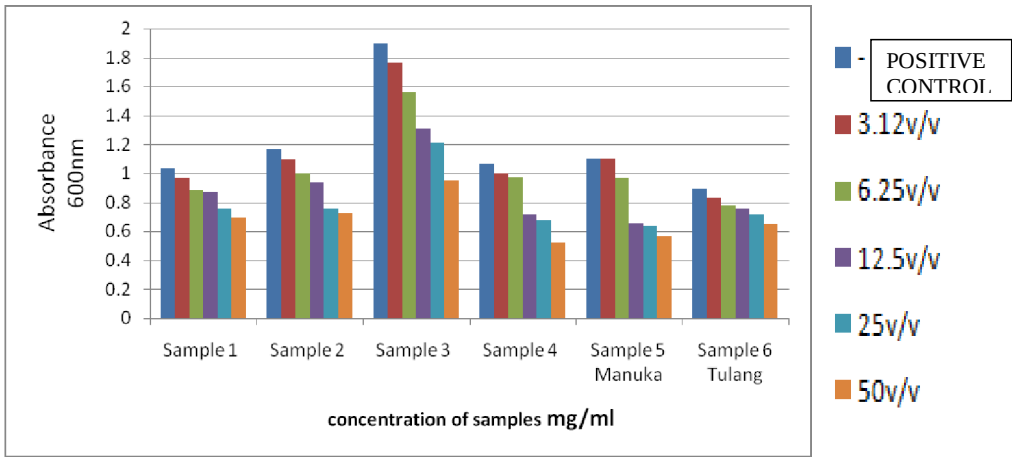

Fig. 4. Distribution of bacteriostatic activity (MIC) among 6 honey samples against *Eshcherichia coli*

and a sterile glass spreader was used for even distribution over the media.Five wells were made using a sterile cork borer and each well was filled with different concentrations (25, 50, 75 and 100v/v) of the honey .The plates were incubated at 37°C for 24hrs and observed for zone of inhibitions.

The antibacterial activity was expressed as the mean diameter of inhibition zone (mm) produced by the honey[3].

4. *Determination of Total Phenolic content:*

By using the FolinCiocalteu method: -

Honey solutions were prepared at a concentration of 0.1 g/ml. An aliquot of 0.5 ml of the stock solution were mixed with 0.3 ml

of the Folin–Ciocalteu reagent and 2 ml of a 15% sodium carbonate solution.Distilled water was added to a final volume of 5 ml. Incubation for 2 h at room temperature the absorbance of the reaction mixture were measured at 798 nm against a blank of distilled water. Total phenolic content was expressed as mg gallic acid equivalents/ml of honey.[3]

5. *Determination of flavonoids:*

By using aluminum chloride method:-

The honey solutions were prepared at the concentration of 0.2 g/ml.Two milliliters (2 ml) of the stock solution were mixed with 3 ml of a 5% aluminum chloride solution. Incubation for 30 min, the absorbance of the reaction mixture was measured at 437 nm against a methanol blank.The total flavonoid content was expressed as quercetin mg/ml of honey.[5]

6. *Radical scavenging activity: DPPH assay:*

The scavenging activity against 1,1-diphenyl-2-picrylhydrazil (DPPH) radical was used in this study.

0.75ml of the honey solution (0.1g/ml) in warm water was mixed with 1.5ml of 0.09mg/ml DPPH in methanol .The mixture was then incubated at 25°C in a water bath for 5 mins after which the absorbance was measured at 517nm against a blank sample consisting of honey solution with distilled water.The absorbance of a radical blank was also measured using 0.75ml of distilled water.The radical scavenging activity (RSA) of honey was expressed in terms of percentage inhibition of DPPH radical by honey and was calculated as follows:

$$RSA\ (DPPH\cdot\ Inhibition, \%)=[(A_B+-A_T)/A_B]\times 100$$

Where,

A_B = Absorbance of radical blank (DPPH without honey)

A_T = Absorbance of test sample (DPPH with honey).

The concentration of honey sample required to scavenge 50% of DPPH (IC50) was determined based on the ascorbic acid calibration curve (0–10mg/L).

7. DNA Degradation Assay:-

puc 19 2µl + 10ml of 50% honey sample + 8µl of milipore grade water were added on test tube. Then Incubated for 5 hours at 37^0C. Then load in Agarose Gel Electrophoresis with standard plasmid.[1]

Table-1. Catalase assay (CAT activity – Catalase activity)

Samples	Test	Blank 1	Blank 2	Blank3	CAT activity
1	0.339	0.436	0.281	0.018	97.56
2	0.358	0.613	0.327	0.001	111.11
3	0.301	0.360	0.460	0.001	27.1
4	0.294	0.374	0.235	0.040	211.3
5(Manuka)	0.315	0.340	0.244	0.005	27.1
6(Tulang)	0.330	0.367	0.263	0.015	37.94

Table-2. Phenolic content
Galic acid- Standard to all phenolic content

Sample	Concentration (gallic Acid mg/ml)
1	0.22
2	0.195
3	0.233
4	0.0051
5(Manuka)	0.18
6 (Tulang)	0.07

Table-3. Flavonoids (Quercitin- Standard to all flavonoids)

Sample	Concentration Quercitin(mg/ml)
1	0.0158
2	0.0020
3	0.0215
4	0.0075
5(Manuka)	0.0122
6(Tulang)	0.0075

Table-4. Radical scavenging activity (DPPH) ICD50-The amount of oxidant necessary to half the initial DPPH concentration.

Samples	IC 50 Values µgm/ml
1	21
2	18.5
3	29
4	10
5 (Manuka)	16
6 (Tulang)	16

8. *Statistical analysis :*

The extraction of phenolic acids and flavonoids were performed. The results were reported as a mean ±standard deviation from the triplicate analysis.

From our results of Catalase we got the highest value of catalase in sample No. 2 and the least result in the Sample 3. (Table-1). From comparing with.[3] The minimum bactericidal activity of different concentration against *Staphylococcus spp., Bacillus spp. Escherichia coli, Salmonella spp.* From results the highly sensitive organism was *Escherichia coli* and *Salmonella spp.* Giving higher zone of inhibition this was due to sensitivity of these organism to the hydrogen peroxide which represented an inhibine factor in honey and *Staphylococcus spp., Bacillus spp*. Was found to be more resistant to honey. The minimum inhibitory concentration of honey sample 3 found to be high MIC value compare to the rest. (Fig. 2,3,4,5).

From comparing with[3] the dark honey sample i.e sample 3 were richer in phenolic and flavones and showed higher antioxidant activity.

Based on the IC_{50} values the honey sample 3 showed antioxidant potential. Comparing with[2] the incubation of honey with puc 19 plasmid and *E.coli* DNA produced a visible conversion of supercoiled circular DNA to nicked open circular DNA and linear DNA indicate that the strand break formation included both the single and double stranded being cut.

No DNA degradation was observed in *E.coli* DNA treated with the sample 4.

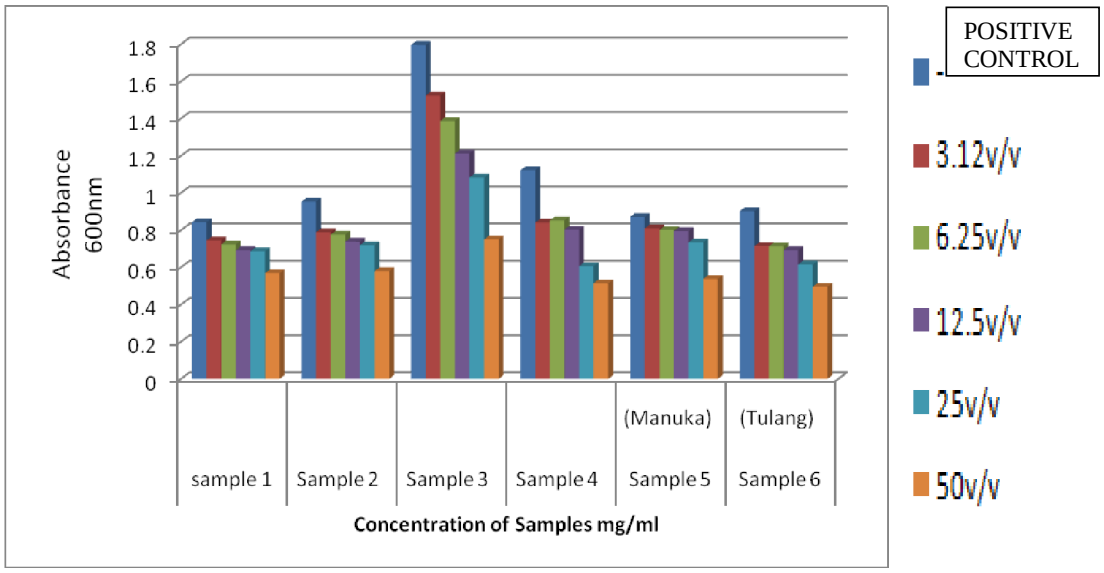

Fig. 5. Distribution of bacteriostatic activity (MIC) among 6 honey samples against *Salmonella spp.*

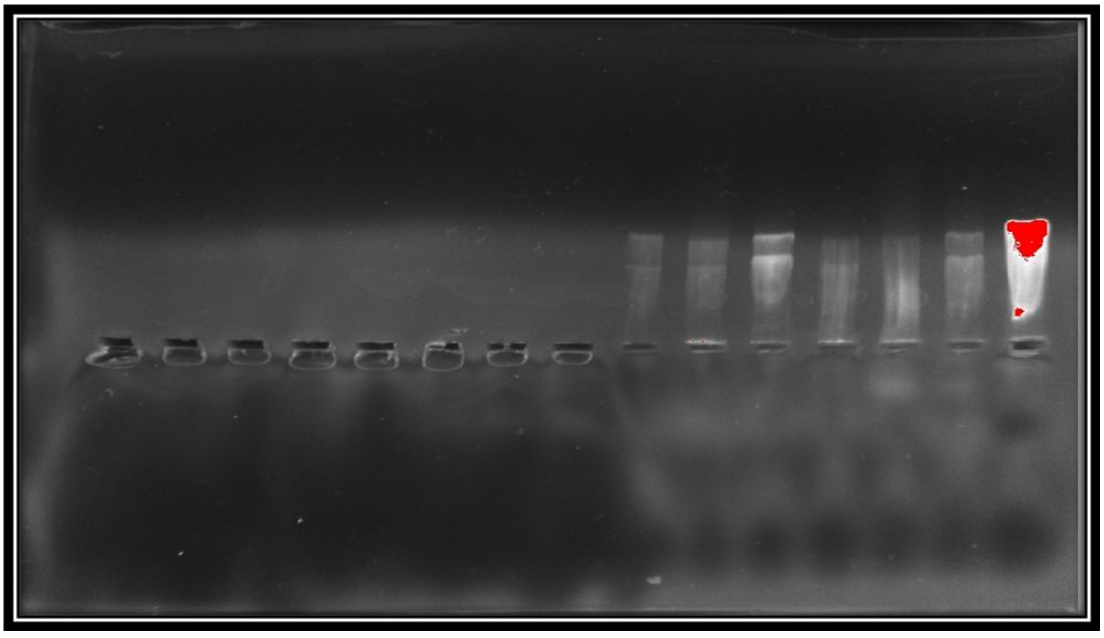

Fig. 6. Extent of DNA degradation by different honey samples. Agarose gel electrophoresis ; puc 19:-Standard Plasmid also served as control Sample 1-6:- Honey sample

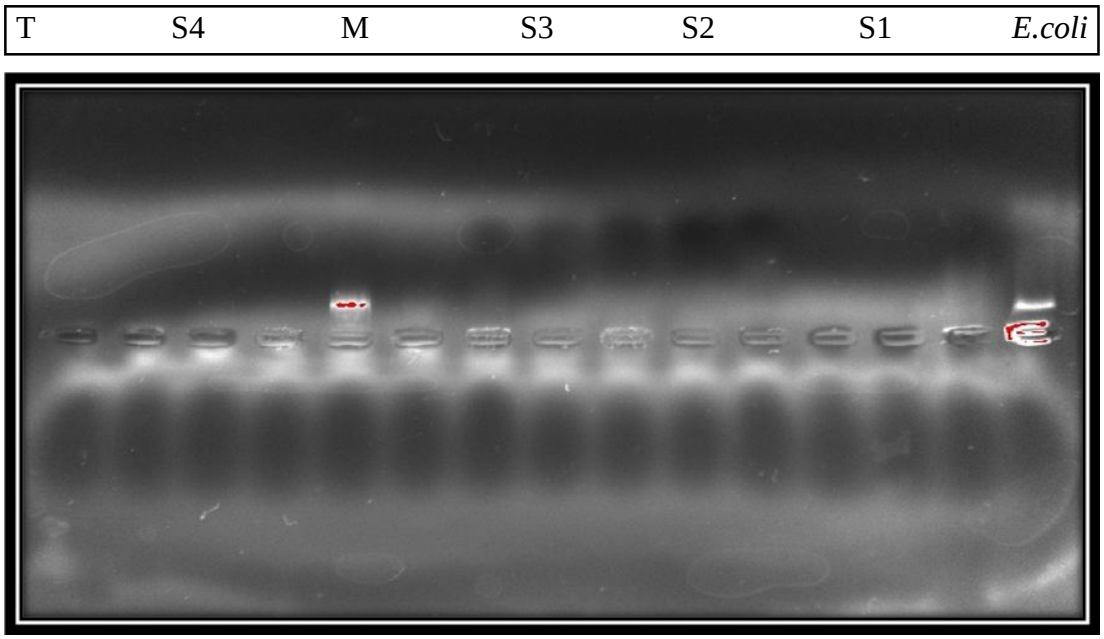

Fig. 7. Extent of DNA degradation by different honeysamples . Agarose gel electrophoresis; *Escherichia coli*-Standard plasmid. T-Tulang honey M- Manuka Honey.

In this study MIC of six honey samples were performed and all six samples gave the successful results and showed inhibitory effect to the microorganisms such as *Bacillus spp. Staphylococcus spp., Salmonella spp., Escherichia coli* due to bioactive components in honey. MBC of all six samples of honey were performed and from that *Salmonella spp*. And *Escherichia coli* showed the inhibitory zones around the wells.Sample 3 of honey exhibited variable activities against many different microorganisms. In some cases it showed equivalent or better activities than other samples of honey, especially against *Salmonellaspp., Escherichia coli*.

The study established a correlation between the color, flavone, flavonol, and phenolic contents, and antioxidant capacity of the honey samples evaluated. Darker honey samples had higher amounts of phenolic compounds, flavones, and flavonols and increased antioxidant activity.

We tried unraveled here that the coupling chemistry between polyphenols and H_2O_2 that resulted in phenolic auto-oxidation and a generation of radical species was the mechanism responsible for DNA degradation by honey. Polyphenols exhibiting antioxidant activity have the potential to become powerful prooxidants in the presence of oxygen sources, such as O_2 or H_2O_2. Polyphenol auto-oxidation generates H_2O_2. via the Fenton type of reaction H_2O_2 is converted to hydroxyl radicals which have been shown to induce strand-

breaks in DNA. The polyphenol autooxidation can be inhibited by catalase and catalase-treatment prevented DNA degradation.Our observations that H_2O_2 alone did not affect DNA integrity but its removal by catalase from honeys had a protective effect on DNA provided a support for the postulated by view that the interaction between honey polyphenols and hydrogen peroxide is responsible for DNA degradation.These results demonstrate a critical role of antioxidant/pro-oxidant activities of honey polyphenols in bacterial growth inhibition and DNA degradation.

References :

1. Brudzynski, K., K. Abubaker and A. Castle (2011). *Frontiers in microbiology*, *2*: 213.
2. Brudzynski, K., K. Abubaker and D. Miotto (2012). *Food Chemistry*, *133*(2): 329-336.
3. Gambo, S.B., M. Ali, S.U. Diso and N.S. Abubakar (2018). *Pharmacological and Pharmaceutical Reports*, *1*(3): 13-21.
4. Goth, L. (1991). *Clinica chimica acta*, *196*(2-3): 143-151.
5. Pontis, J. A., L.A.M.A.D. Costa, S.J.R.D. Silva and A. Flach (2014). *Food Science and Technology*, *34*(1): 69-73.