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Research Paper

Potent Antibacterial Activity of Proteins Isolated from Acacia Honey

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ABSTRACT

Antibacterial resistance to antibiotics has become a global health issue. Therefore, alternative antimicrobial strategies are urgently needed. The use of alternative remedies such as plant-based products might be effective. Natural honey has been used as an antibacterial agent against many bacterial species. In this study, the antibacterial activities of natural honey and honey proteins were tested against six pathogenic bacteria. Honey proteins were extracted by ammonium sulfate and acetone followed by protein estimation the Lowry method. SDS-PAGE was used to determine the molecular weight of honey proteins. Extracted honey proteins were separated by Gel Filtration Chromatography using HPLC. Antibacterial activity of the total honey protein was performed by broth microdilution method and IC_{50} was determined. The Acacia honey showed potent antibacterial activity with IC_{50} of 45 ng/μL to 55 ng/μL against bacterial isolates. The GFC fractionated protein (peak 3) of Acacia honey inhibited the growth of *E. coli* (86%)*, P. aeruginosa* (79%)*, S. typhi* (88%), and *S. aureus* (87%).

KEYWORDS: Antibacterial activity; Honey protein; HPLC; Gel Filtration Chromatography

INTRODUCTION

Antibiotic resistance is increasing day by day and has become an alarming issue. Resistance occurs when bacteria or other organisms develop the capability to fight against antibiotics. In this way, bacteria evolve the mechanism which keeps them to grow and continuing to multiply in spite of the presence of the drugs and their killing effects. Therefore, many bacteria have become resistant to many of the existing antibiotics. This phenomenon is known as Anti-Microbial Resistance (AMR) which is a burning issue of the time and leads to ineffective treatment of bacterial infections. Therefore, this issue has remained an extensive research focus so far. Because of increased drug resistance, the interest in using alternative therapies and natural remedies for bacterial infections has rapidly increased. Current research proved that antimicrobial resistance (AMR) is increasing due to the evolution and spread of resistance genes. Many of the bacterial isolates including *E. coli, Klebsiella pneumonia,* and *Staphylococcus aureus* exhibited antibacterial resistance against all classes of antibiotics that is 47.1%, 51.0%, and 45.2% respectively. Another study also support the resistance of bacteria that is 75.8% in gram-positive bacteria and 74.2% in gram-negative bacteria. According to WHO approximately one third of the annual deaths worldwide are attributed to infectious diseases [1,2]. While in Pakistan reported the antibacterial resistance in healthcare settings and found resistance in gram-negative and gram-positive bacteria at 41.6% and 15.4% respectively. Hence research proved that drug-resistant pathogens weakened the progress of antibiotics and only a few pharmaceuticals companies remain active in this field, so it is a big challenge to overcome the increasing resistance against bacteria. Despite all the massive improvements in health care during the last half-century, infectious diseases and antibacterial resistance remain a public threat and cause of mortality worldwide. And pathogenic and antibiotic-resistant bacteria pose a very serious threat to public health which makes them the main cause of mortality and morbidity in hospitals and communities as well [3].

Noticing all these resistance patterns of bacterial isolates it is a need of time to reduce the use of antibiotics and we have to search for treatment options in natural remedies. The introduction of new drugs is considered as last resort option and used only under the most severe circumstances when all alternatives fail. The failure of these antibiotics has resulted to search for more effective sources of natural products from plants and others. Therefore in this scenario natural "honey" has been proven as an antibacterial agent internationally and the World Health Organization (WHO) also appreciates the rationale use of plant-based alternative medicines so honey has been declared as a natural treatment option to reduce the burden of antibiotic resistance. Honey was proved as an antibacterial candidate by in 1892 [4]. Although it has limited use in modern medicine due to a lack of scientific support but proven as a natural substance and appreciated for its therapeutic abilities since antique times and has a key role in human health because of its constituents which are flavonoids, phenolic acids, and other compounds. In addition, its antioxidant and anti-inflammatory properties also have been reported [5,6].

Honey is viscous and a liquid-like substance with a light brown or dark golden color and sweet in taste having a particular aroma. It is produced by honey bees from the nectar of flowers. Its color and flavor are only because of the flower from which the nectar is collected. The whole process of making honey is all about gathering juices from flowers, its refining and making is being done in the second stomach of the honey bee called as honey sac [7].

Honey is composed of proteins, amino acids, organic acids, vitamins, minerals, polyphenols, and volatile compounds. The predominant sugars in honey are fructose (38%) and glucose (31%). The physicochemical characteristics of honey including taste, color, viscosity, and solubility are significantly influenced by its moisture content. It is characterized by low water activity, low humidity (<18%), and high acidity. The physicochemical and biological properties of honey depend on its botanical and geographical origin. Other components are commonly referred to as non-sugar constituents and comprise of flavonoids, phenolic acids, proteins (enzymes), amino acids, minerals including Calcium (Ca^{2+}), Copper (Cu^{2+}), Iron (Fe^{2+}), Magnesium (Mg^{2+}), Manganese (Mn^{2+}), Phosphorous (P^{3+}), Potassium (K^{+}), Sodium (Na^{+}) and Zinc (Zn^{2+}). As per natural honey also consisting a range of vitamins including vitamin C, vitamin B, thiamine, niacin, riboflavin, and pantothenic acid) which adds to the nutritional value of honey [8]. Also, it is composed of proteins, enzymes, and non-essential amino acids. These enzymes are responsible for its antibacterial properties. The glycoproteins present in honey has growth-inhibitory and bactericidal properties against bacterial isolates. And these glycoproteins exhibited sequence uniqueness with the Major Royal Jelly Protein-1 (MRJP-1) [9,10]. Another research suggested that all the honey samples can be eminent from non-Leptospermum honeys by the existence of Methylglyoxyl, which facilitates its exclusive antibacterial activity. Also stated by it has unique antibacterial activity because of methylglyoxal (MGO). Another important compound of honey is MRJP, as described by the purified MRJP consists of predominantly β-sheets and random coil in the native conformation which also contributes physiologically and functions as physico-chemically [11].

Honey bees provide potential benefit to humans due to their bio actives. Which includes phenolic compounds, Methyl glyoxyl (MGO), oligosaccharides and royal jelly proteins [12] having antimicrobial jelleins, royalisin peptides, and derivatives of hydroxyl-decenoic acid with

antimicrobial, anti-inflammatory, immune-modulatory, neuro-modulatory and anti-aging activities.

Among the honeybees, *Apis mellifera,* the queen bee, produces honey exclusively which is composed of a secretion of the hypo-pharyngeal gland, called as Royal Jelly. And 15% of this secretion is composed of proteins so termed as MRJP. And this protein in honey originates from the traces of pollen in the flower nectar. And these Major Royal Jelly Proteins (MRJP) are considered as the markers of honey authenticity. The Major royal jelly proteins are a family of proteins that mainly consists of different unique protein members including MRJP1, MRJP2, MRJP3, MRJP4, and MRJP5. Major Royal Jelly Protien-1 is the most plentiful protein present in honey. The mechanism of action of MRJP-1 to the killing of bacteria is the bacterial cell wall disruption due to the presence of Jelleins which cause cell wall disintegration and leads to lysis of bacterial cell. Antibacterial activity mainly attributed to proteins present in honey and It is also suggested by that royal jelly (RJ) and worker jelly (WJ) contain identical major proteins and belongs to one protein family that is Major Royal Jelly Proteins having five main members including (MRJP1, MRJP2, MRJP3, MRJP4, MRJP5). The proteins MRJP3 and MRJP5 are polymorphic and account for 82 to 90% of total larval jelly protein with relatively high amounts of essential amino acids. Glucose oxidase is an enzyme that convert glucose to gluconolactone and then to hydrogen peroxide (H_2O_2). This hydrogen peroxide is accountable for the antibacterial effect, successfully eliminating microorganisms but diluting enough therefore not damaging host tissue. The pH of honey is acidic (3.2–4.5), which is enough to inhibit bacterial growth and also helpful for the wound healing process through epithelization. Another characteristic of honey that makes it antibacterial is its osmolarity. Actually honey is one of the supersaturated solutions that inhibit bacterial growth primarily due to its high osmolarity. Its super-saturated sugary nature has a great nutritional value having positive impacts on human health for its antioxidant, antimicrobial, and anti-inflammatory properties [13,14].

The antibacterial activity of honey has been associated with the phenolic compounds and other valuable compounds in honey which have many medicinal properties such as antibacterial activity has been related to high sugar concentration, osmolarity, hydrogen peroxide and low pH. According to its viscosity, osmolality, acidity, bioactive peptides, and most importantly the content of hydrogen peroxide makes it antibacterial with clinical potential because of the enzymatic production of hydrogen peroxide and a varied presence of phytochemical components such as methylglyoxyl (MGO) as well as the activity of bee defensin-1 and other bee-related enzymes. Its high viscosity also helps to provide a protective barrier to prevent bacterial infection. Few studies also relates its antibacterial activity with catalase having non peroxide activity as well as high concentration of MGO, which is derived from di hydroxy acetone, present in large amount in the nectar of *Leptospermum* species [15]. Other factors, such as phytochemical substances and polypeptides like bee-defensin-1, may also contribute to the overall biological effects of honey as well. In general, all types of honey have high sugar content but a low water content and acidity, which is helpful to prevent microbial growth. Research also supports that honey has an inhibitory effect to a lot of bacteria including aerobes and anaerobes and has bactericidal as well as bacteriostatic activity against bacteria that have developed resistance to many antibiotics. Therefore has been used for its healing, nutritional, and therapeutic properties since ancient times. Its

antibacterial potential even against multi-drug resistant bacteria, such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Acinetobacter baumanni* has been proved [16,17].

Precisely antibacterial activity of honey against pathogenic microorganisms has repeatedly been proven. However literature has limited information regarding the effectiveness of honey protein against microbial growth, therefore purpose of this study is to determine the antibacterial activity of honey against bacterial isolates which might have of decrease in medicinal use and may promote the solution of drug resistance in natural sources as an outcome.

MATERIALS AND METHODS

A total of seven honey samples were procured and stored in a clean and dry jar at room temperature. The antibacterial activity of honey samples was also evaluated by Agar well-diffusion assay and zone of growth inhibition was also recorded.

Two different techniques to extract the honey proteins: precipitation by acetone and salting out.

In precipitation by acetone method, the honey sample was diluted in water 50/50% (v/v) and 400 ml chilled acetone was added in 100 ml of the prepared honey sample. After that, The solution was vortex and incubated for 24 hours at -4 C. Afterwards, the mixture was centrifuged at 13000xg for 10 minutes the supernatant was discarded and a pellet of protein was dissolved in 2 ml of Tris-HCL (20mM) buffer and stored at -4 C.

In other method, the 1gm of the honey sample was weighed and dissolved in filtered water to make it 10ml. The mixture was centrifuged at 4,000rpm for 10 min. The supernatant was collected and dissolved in 80% Ammonium Sulfate (5.67gm) and placed in a shaker for an hour. Then samples were again centrifuged at 4,000 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in 0.5 ml Tris buffer.

Estimation of total proteins extracted from different honey samples was done by standardized Folin Lowry Method [18]

The extracted proteins were visualized via SDS PAGE [19].

To fractionate the total honey protein, Gel Filtration Chromatography (GFC) using High-Performance Liquid Chromatography (HPLC) was done with an isocratic system.

Antibacterial activity of total protein and protein fractions extracted from honey samples was done by Broth Micro dilution method using a sterile 96- wells microtiter plate as described by [20,21]

RESULTS

Honey samples were diluted with distilled water at different concentrations and antibacterial activity was tested against bacterial isolates by agar well diffusion assay. The zone of inhibition against each concentration was measured.

Chloramphenicol was used as a control. Inhibition zone (a circular area around the well of the antibiotic in which the bacterial colonies do not grow) of 20 mm was considered as cut off, therefore the concentration of honey at which bacterial isolates showed the zone of inhibition larger than the cut-off was considered as effective while the concentration at which the bacterial isolates showed no zone or <20 mm was considered as having no affectivity against that bacterial isolate. Results showed that honey sample 0829 exhibited a zone of inhibition of 33 mm and 30 mm against *E. coli* and *Bacillus subtilis* with the honey sample while at 50% dilution, it showed a 28 mm zone against these bacteria (Table 1). Similarly, 22 mm and 26 mm zones were observed at 50% and 25% dilution against *S. aureus* and *B. subtilis.*

However, at 10% honey only *Bacillus subtills* were sensitive. The Sample PN at 50% dilution exhibited zones between 12 to 16 mm diameter against *E. coli, P. aeruginosa, S. typhi,* and *S. aureus* (Table 2). The honey samples PO and Ard were less effective at 25%. Whereas *Bacillus subtilis* was sensitive to both honey samples at 50% dilution (Table 3 and 4).

Total protein was extracted from honey samples using Ammonium Sulfate (using 80% saturation at 10 °C) and Acetone method. The overnight chilled Acetone was used for the Acetone method of protein extraction. The honey protein precipitates were stored at –20 °C for further use.

Estimation of protein was done by the Lowry method and absorbance of Bovine Serum Albumin (with known concentrations) and honey protein was measured at 630 nm.

Total honey proteins extracted from three different honey samples were subjected to protein electrophoresis and different protein bands were obtained according to their molecular weight. Monitoring of protein migration during electrophoresis was done and clear visible protein bands were obtained. For Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis, the Protein Marker was used to compare the size of bands. The protein marker covers a wide range of molecular weights from 10 to 245 kilo Daltons. In this experiment, proteins were stained with Coomasie blue. Two different bands (data not shown) of honey proteins at 53 and 41 Kilo Daltons were obtained. Sample P and 1023 showed two bands at 53 KDa and 41 KDa while protein of honey sample UE showed a single band at 53 KDa.

Table 1: Zone of Inhibition against Honey Sample 0829:

No.	Bacterial Isolates	Dilutions of honey sample				
		100%	50%	25%	10%	Chloramphenicol
1-	*Escherichia coli*	33mm	28mm	20mm	10mm	40mm
2-	*Klebsiella pneumonia*	22mm	20mm	20mm	16mm	26mm
3-	*Bacillus subtilis*	30mm	28mm	26mm	26mm	30mm
4-	*Pseudomonas aeruginosa*	20mm	20mm	22mm	20mm	30mm
5-	*Salmonella typhi*	12mm	12mm	NZ*	08mm	25mm
6-	*Staphylococcus aureus*	22mm	22mm	20mm	15mm	25mm

*No Zone of Inhibition

Table 2: Zone of Inhibition against Honey Sample PN:

No.	Bacterial Isolates	Dilutions of honey sample				
		100%	50%	25%	10%	Chloramphenicol 0.5%
1-	*Escherichia coli*	22mm	12mm	10mm	08mm	32mm
2-	*Klebsiella pneumoniae*	16mm	10mm	NZ*	NZ*	24mm
3-	*Bacillus subtilis*	26mm	20mm	20mm	08mm	30mm
4-	*Pseudomonas aeruginosa*	18mm	12mm	12mm	NZ*	30mm
5-	*Salmonella typhi*	20mm	14mm	NZ*	NZ*	26mm
6-	*Staphylococcus aureus*	20mm	16mm	15mm	12mm	28mm

Table 3: Zone of Inhibition against Honey Sample PO:

No.	Bacterial Isolates	Dilutions of honey sample				
		100%	50%	25%	10%	Chloramphenicol 0.5%
1-	*Escherichia coli*	12mm	08mm	NZ*	NZ*	20mm
2-	*Klebsiella pneumonia*	10mm	08mm	NZ*	NZ*	20mm
3-	*Bacillus subtilis*	24mm	20mm	10mm	NZ*	30mm
4-	*Pseudomonas aeruginosa*	18mm	12mm	NZ*	NZ*	26mm
5-	*Salmonella typhi*	10mm	NZ*	NZ*	NZ*	20mm
6-	*Staphylococcus aureus*	16mm	10mm	10mm	NZ*	24mm

Table 4: Zone of Inhibition against Honey Sample Ard:

No.	Bacterial Isolates	Dilutions of honey sample				
		100%	50%	25%	10%	Chloramphenicol 0.5%
1-	*Escherichia coli*	16mm	10mm	10mm	08mm	22mm
2-	*Klebsiella pneumonia*	24mm	20mm	20	NZ*	26mm
3-	*Bacillus subtilis*	20mm	20mm	18mm	10mm	24mm
4-	*Pseudomonas aeruginosa*	18mm	12mm	12mm	NZ*	20mm
5-	*Salmonella typhi*	18mm	20mm	NZ*	NZ*	22mm
6-	*Staphylococcus aureus*	NZ*	NZ*	NZ*	NZ*	16mm

*No Zone of inhibition

Antibacterial assay of total honey protein against bacterial isolates was performed by broth micro dilution method. Total protein of volume 5 µL to 50 µL was added into wells of micro titer plate and bacterial culture was added 50 µL into each well. After incubation, bacterial growth was calculated using absorbance at 630 nm. The IC_{50} was determined by plotting a standard graph between honey protein concentration and percentage inhibition. Honey protein 0829 showed IC_{50} (Figure 1) of 45 ng/µL, 55 ng/µL, 52 ng/µL, and 48 ng/µL against *E. coli*, *K. pneumonia*, *P. aeruginosa,* and *S. aureus* respectively. While IC_{50} of total protein of honey sample PN was 55 ng/µL against *E. coli*. Protein extracted from honey sample PN could not inhibit the growth of other bacterial isolates (Figure 2). Similarly only *P. aeruginosa* was sensitive to total protein of honey sample PO (Figure 3) and Ard (Figure 4) as it gives IC_{50} of 70 ng/µL and 75 ng/µL respectively.

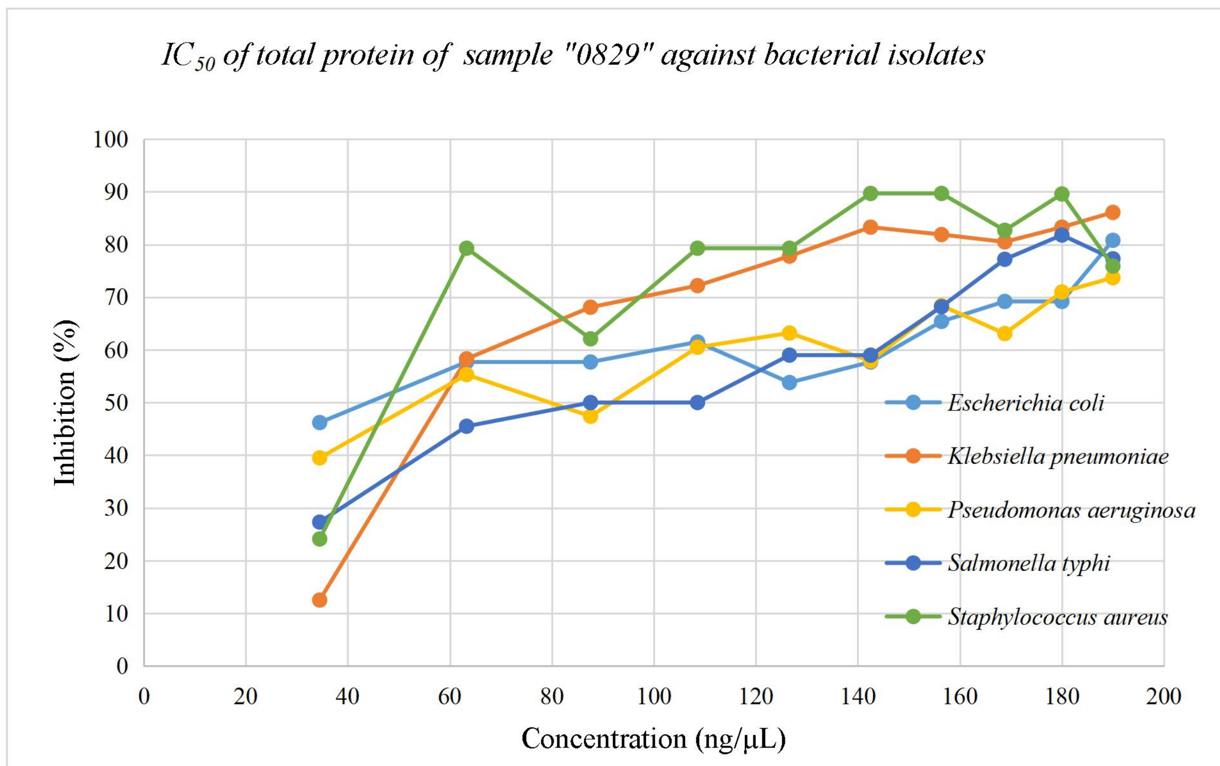

Figure 1: Percentage Inhibition And IC_{50} of Total Honey Protein 0829 against Bacterial Isolates

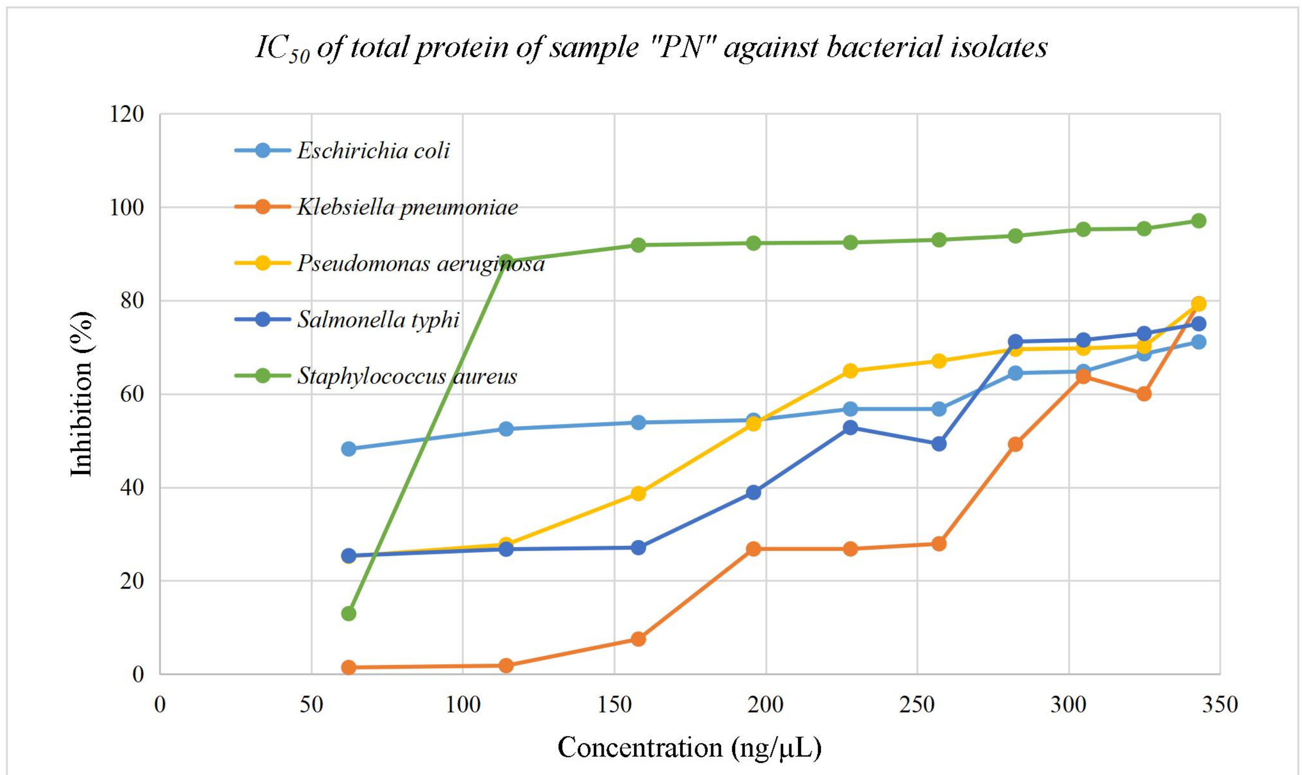

Figure 2: Percentage Inhibition And IC_{50} of Total Honey Protein PN against Bacterial Isolates:

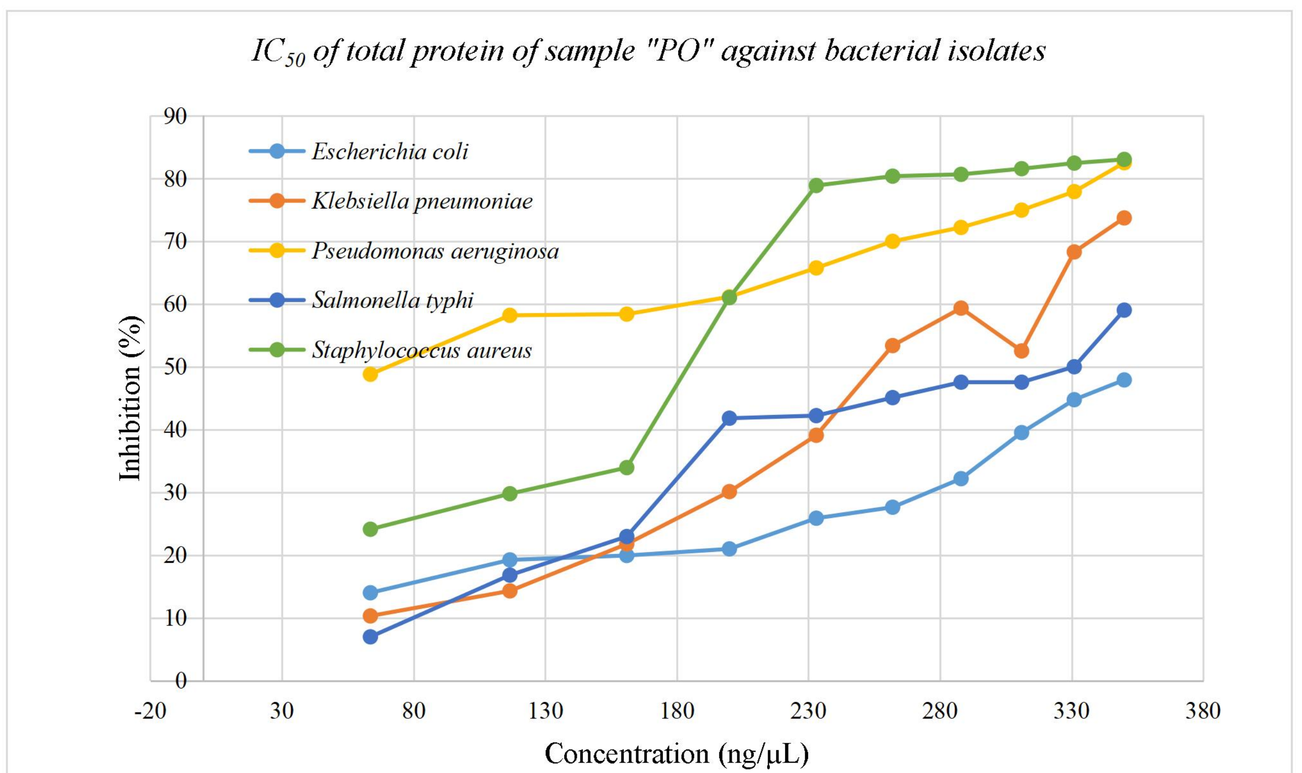

Figure 3: Percentage Inhibition And IC_{50} of Total Honey Protein PO against Bacterial Isolates

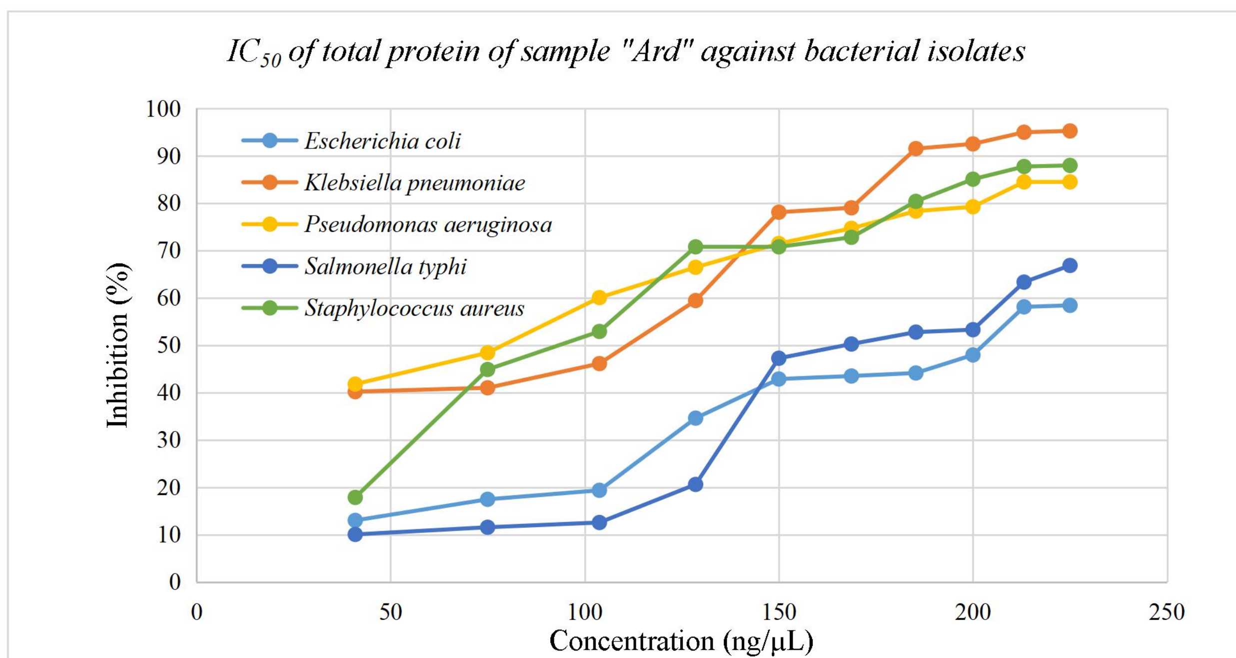

Figure 4: Percentage Inhibition And IC_{50} of Total Honey Protein Ard against Bacterial Isolates.

According to graphical representation of total honey protein concentrations versus percentage inhibition of bacterial isolates, IC_{50} was determined. It was observed that the total protein of honey sample PO and Ard showed IC_{50} of 70 ng/µL and 75 ng/µL respectively against *P. aeruginosa.* And sample PN exhibits IC_{50} of 55 ng/µL and 50 ng/µL against *E. coli* and *B. subtilis* respectively. After testing of all the honey protein samples, it can be dug out that comparatively sample 0829 showed significant IC_{50} which is less than 50 ng/µL against all bacterial isolates except *P. aeruginosa* as shown in figure 5.

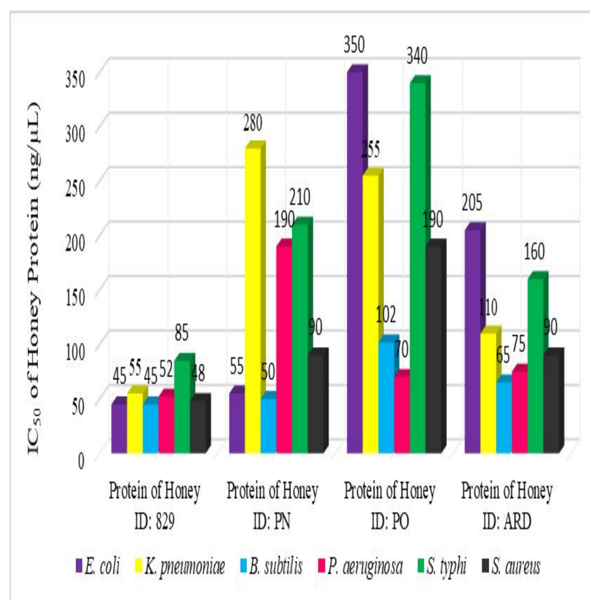

Figure 5: Comparison between IC_{50} of total honey proteins against bacterial isolates.

Gel Filtration Chromatography (GFC) of honey protein by HPLC:

Total protein extracted from honey sample was filtered from 0.45 micron syringe filter and applied on GFC column with dimension of 7.8 mm x 300. Gel Filtration Chromatography was done by HPLC and different peaks of protein were collected. The protein content in all three peaks were estimated using Lowry method followed by antibacterial activity. Total protein of all the honey samples exhibit different peaks on chromatogram at different retention time. Peak 1 elute first containing large sized molecules followed by peak 2 and 3 which elute late from column having small sized molecules as shown in chromatogram of sample P. As chromatogram of honey protein of sample 1023 and UE show peak 3 appears with larger area so it can be interpreted that small sized molecules are more in number figure 6. While honey protein of sample UE exhibit different pattern of peaks as shown in figure 9, which gives six peaks at 5.166, 8.386, 12.511, 13.267, 14.499 and 15.478 minutes respectively. While peak 1 appeared in splitting form at 6.112 minutes.

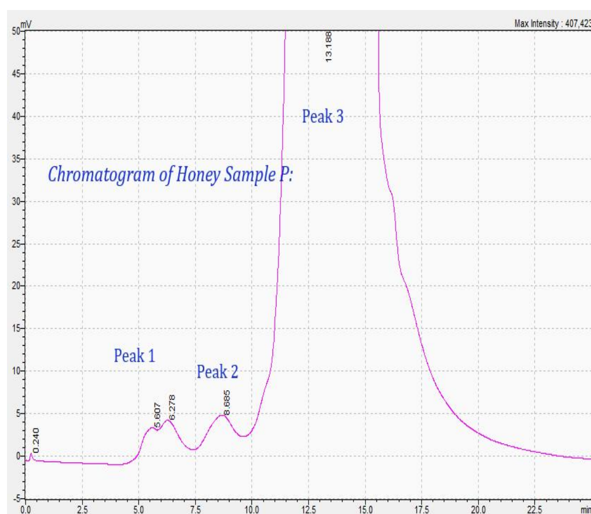

Figure 6: Chromatogram of GFC of total protein of honey sample P:

Similarly protein of same sample (UE) which was extracted by Acetone method presented five clear and sharp peaks at 5.067, 8.389, 10.993, 12.708 and 15.390 minutes. While peak 3 was split at 11.310 minutes as shown in chromatogram (figure 7).

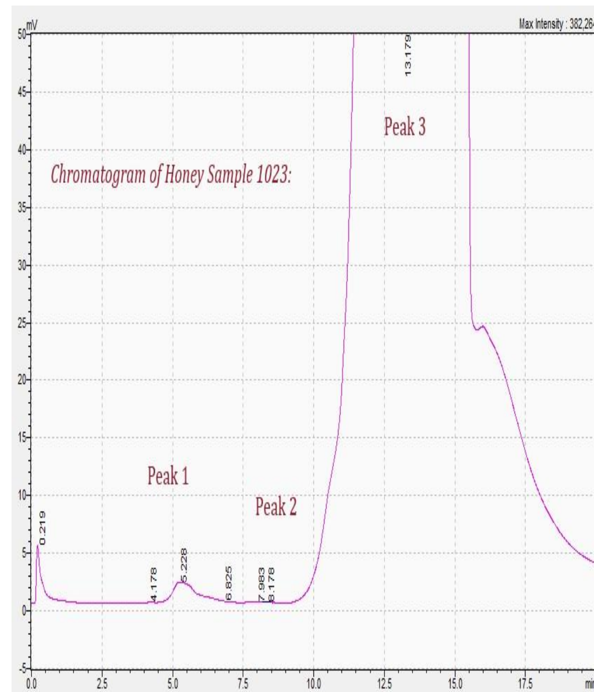

Figure 7: Chromatogram of GFC of total protein of honey sample 1023

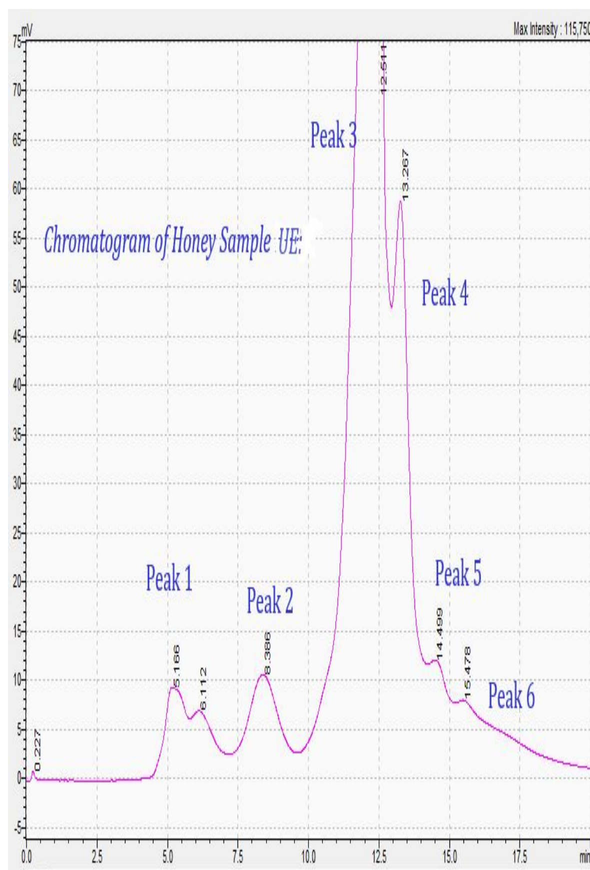

Figure 8: Chromatogram of GFC of total protein of honey sample UE

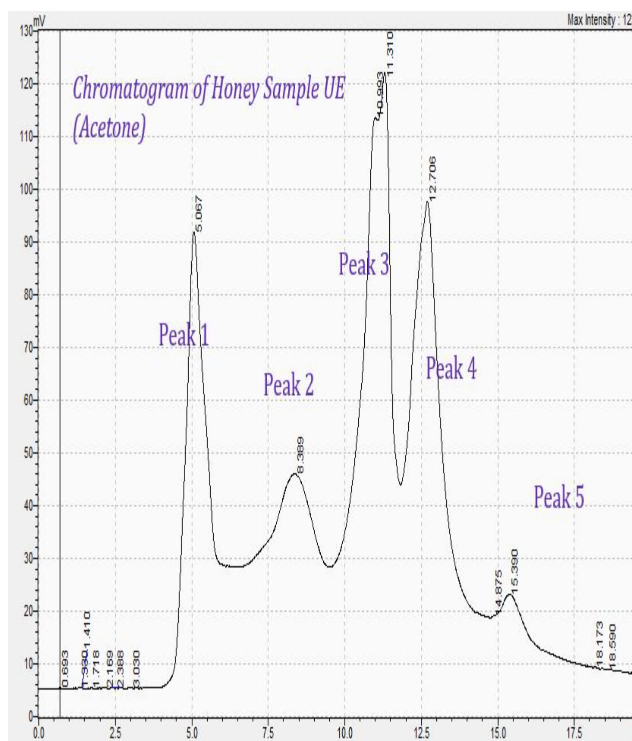

Figure 9: Chromatogram of GFC-peaks of honey UE extracted by Acetone Method

Antibacterial assay of fractionated honey protein was done by broth micro dilution method using 96-wells micro titer plate. Volume of 50 µL of fractionated proteins (peaks) of all the honey proteins was added into wells and same volume (50 µL) of bacterial culture was added into triplet of wells. After incubation at 37 °C for 24 hours, absorbance was measured at 630 nm using 96- wells plate reader. and bacterial growth as well as percentage inhibition was calculated. 20% or more than 20% of percentage inhibition at which fractionated protein inhibit the bacterial growth, was considered as effective. Comparison of antibacterial activity of protein fractions in terms of percentage inhibition described in table 4.16. According to results *E. coli* presented percentage inhibition of 58.82%, 55.08% and 60.43% against all peaks of honey sample P. And fractions of honey sample 1023 inhibit *E. coli* more than 60%. Whereas *S. typhi* showed sensitivity to peak 2 and 3 of sample 1023 and all peaks of sample UE. And all three peaks of sample 1023 inhibit *S. aureus* by 35.49%, 48.44% and 59.33% respectively as described in table 4.16. Fractionated protein of honey sample UE gives 36.35%, 34.87% and 44.46% inhibition of *Klebsiella pneumonia* while *Bacillus subtilis* produced 19.01%, 21.09% and 8.85% against same fractions therefore protein of honey sample UE evidenced as more effective for *Klebsiella pneumonia* as compared to *Bacillus subtilis*. It is also observed that peak 3 of honey sample UE appeared as more effective to maximum of bacterial isolates as it gives more than 80% of percentage inhibition against *E. coli,* (86.36%) *Pseudomonas aeruginosa,* (79.02%) *Salmonella typhi,* (88.25%) and *Staphylococcus aureus* (87.31%).

DISCUSSION

Antimicrobial agents are essentially important in reducing the global burden of infectious diseases. As research proved that resistant pathogens are increasing day by day, the effectiveness of antibiotics is reduced. This type of bacterial resistance to antimicrobial agents poses a very serious threat to public health. Therefore, alternative antimicrobial strategies are urgently needed, so the use of ancient remedies might be effective, such as plants and plant-based products, including honey, which can be used for the treatment of several human ailments. As stated its antibacterial activity is also because of the presence of an extensive variety of bioactive compounds [22,23]. Another research of proving that there is a synergistic effect amongst the sugars, proteins and lipid that makes the honey unique than other [24]. Published data indicate that natural honey have many ingredients and mainly composed of carbohydrates, proteins, minerals, phytochemicals and antioxidants and these components are responsible for medical and biological activities of honey in the treatment of many bacterial infections. Its antibacterial activity against various bacteria may because of its natural ingredients, weather or the source from where the bees were raised, or on the natural structure of the blossom nectar. Its effectiveness and potency may also be depends upon bee type and its botanical origin.

Honey is a nutritious and energy-rich complex having different components and all of its constituents are present at different concentrations. These components work synergistically, allowing the honey to be potent against a variety of microorganisms including multidrug-resistant bacteria. It is also been used in the treatment of wounds and burns when applied with antibiotics and have the ability to promote fast healing. This study focused on antibacterial activity of honey protein and comprises on two phases that are antibacterial activity of total honey protein as well as antibacterial activity of fractionated (GFC-peaks) honey proteins against bacterial isolates. In this study total seven different types of honey samples were used and tested for their antibacterial activity against six bacterial isolates including *Escherichia coli, Klebsiella pneumonia, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella typhi and Staphylococcus aureus.* The antibacterial activity of natural honey was evaluated using it’s dilutions, total extracted protein and fractionated protein by agar well diffusion method and broth micro dilution method in terms of zone of inhibition and percentage inhibition. It was observed that different types of honey retain different effectiveness against bacterial isolates [25,26].

According to results of Agar well diffusion assay, honey samples were diluted with distilled water to 50%, 25% and 10% and tested for antibacterial activity. Chloramphenicol was also used as control. A cut off value of 20 mm zone of inhibition was considered as effective. Zone of inhibition against each well of honey dilution was measured which was varied from 10 mm to 40 mm in diameter against bacterial isolates. According to results ,honey sample- 0829 exhibit 28 mm and 22 mm zone of inhibition at 50% dilution against *E. coli* and *S. aureus* respectively. And at 25% the same sample gave 26 mm and 22 mm zone of inhibition against *Bacillus subtilis* and *P. aeruginosa*. Similarly *Bacillus subtilis* appeared as sensitive to 10% of honey as it gives 26 mm zone on inhibition. Other honey dilutions observed as less effective against bacterial isolates which gives less than 20 mm zone or no zone of inhibition. This assay proved that 50% diluted honey sample (0829) show 28 mm zone of inhibition in comparison of 30 mm zone of chloramphenicol against *Bacillus subtilis.* Similarly other samples at

different dilutions also found effective against other bacterial isolates as described in results, table 4.2, 4.3 and 4.4. And there is no zone of inhibition appeared around 10% honey sample- PO. While 10% honey sample- Ard presented 8 mm and 10 mm zone on inhibition against *E. coli* and *B. subtilis* which is less than 20 mm, further it gives no zone of inhibition against others bacteria at 25% dilution therefore considered as less effective. Hence it can be supposed that honey sample 0829 have more potent antibacterial activity at 50% against *E. coli* (28 mm) and 25% against *B. subtilis* (26 mm) in comparison to other isolates. But the honey samples which gives no zone or the zone less than 20 mm, evidenced as non-effective against those particular bacterial isolate. In comparison to international research of different honey samples exhibit zone around 24 mm against *S. aureus* and *P. aeruginosa,* while *E. coli.* And *S. typhi* produce around 20 mm zones of inhibition [27,28,29].

Subsequently total protein was extracted from honey samples using Ammonium sulfate precipitation method and Acetone method. Then protein estimation was done by a standard protocol of protein estimation known as Lowry method. The standard curve between absorbance and concentration of BSA was constructed and concentration of total honey protein was determined using known concentration of Bovine Serum Albumin (BSA). Different amount of total protein was found in different honey samples. Amount of protein content in two different samples of honey (PN) and (PO) was found as 0.7 µg/ uL while in honey sample (0829) protein content was found as 0.38 µg/ µL, and total protein in another sample (Ard) estimated as 0.45 µg / µL (table 4.5). In comparison of previous research of (Maria da Conceicao Tavares Cavalcanti Liberato., et al., 2013) highest protein content was reported as 1121.00 µg/g (0.001121 µg / µL) was reported in honey sample named as Anacardium occidentale among the samples analyzed. Another study of (Badiaa Lyoussi., et al., 2022) suggested that protein content in two different honey samples have found in ranged between 212 µ g / g and 4121.2 µ g / g and between 27.4 µ g / g and 790.82 µ g / g respectively. In comparison to another international study, Monuka honey sample contained <0.5 µg / µL protein content. Different sample from different localities have almost similar amount of protein but may vary on the basis of their floral source [30,31].

Molecular weight of total honey protein was determined using SDS-PAGE by visualizing protein bands appeared on gel. A couple of bands of total protein of honey sample (P) and (1023) were appeared on gel whereas honey sample UE exhibit a single band of total protein. In contrast to ladder, it is observed that the total proteins extracted from sample (P) that is “Acacia” honey sample from Kohat and sample (1023) that is also acacia honey sample obtained from Iran, presented the two different protein bands having molecular weight of 53 K Da and 41 K Da (Kilo Dalton). Another protein from Kohat honey sample (UE) produced a single band at 53 K Da, in parallel to ladder as described in table 4.6. Therefore it can be analyzed that different honey samples either they obtained from different region may have almost similar molecular weight of total proteins.

Antibacterial activity was done by broth micro dilution method. Which is the most fundamental and important antimicrobial susceptibility testing methods for natural products including honey against bacteria. The same volume of honey proteins from 5µL to 50µL was applied on each bacteria and amount of total honey protein applied on bacterial suspension in a single well was calculated as described in table 4.7. Bacterial growth and percentage inhibition was calculated using absorbance of

particular bacteria. In case of *Bacillis subtilis* all the test honey proteins expressed inhibition percentage of more than 80% at same volume. Therefore the volume of protein was decreased and evaluated again with volume of 1 µL to 10 µL gradually. Finally IC_{50} of total proteins against all bacterial isolates was determined by standard graphs as shown in figure 5 and table 4.13. The IC_{50} is the inhibitory concentration of honey protein at which half (50%) of the bacterial growth can be inhibited. According to results, a honey Sample (0829) exhibited IC_{50} against *Klebsiella pneumonia, E. coli, Bacillus subtilis, Pseudomonas aeruginosa, Salmonella, Staphylococcus aureus* that is 55 ng/µL, 45 ng/µL, 45 ng/µL, 52 ng/µL, 85 ng/µL and 48 ng/µL respectively as described in table 4.13. If any compound or test sample of honey has shown the higher IC_{50} value means that is less effective against bacterial growth and having low antibacterial activity against that particular bacteria. Similarly if IC_{50} is lower, the higher will be the affectivity. As per standard research parameters, IC_{50} value category is powerful if the IC_{50} value <10 µ g/mL, strong if the IC_{50} value is between 10 and 50 µ g/mL, mild if the IC_{50} value is between 50 and 100 µ g/mL, weak if the IC_{50} value is between 100 and 250 µ g /mL and not active if IC_{50} is above 250 µ g/mL. Therefore standard graph between inhibition percentage of bacterial growth and concentration of total honey protein was constructed and IC_{50} was determined. In this study protein of honey sample 0829 exhibited IC_{50} of 45 ng / µL against *E. coli* as well as *B. subtilis*. While 55 ng / µL IC_{50} was observed against *K. pneumonia* by sample 0829 and PN. But other two honey sample's protein which showed IC_{50} more than 100, did not considered as effective against those particular bacterial isolates. Results proved that total protein of honey sample 0829, which exhibited IC_{50} less than 50 ng / µL against maximum bacterial isolates therefore it can be comprehend that it has better antibacterial activity as compared to other honey samples. In comparison with the study of extracted protein from *Melipona beecheii* honey had possessed highest antimicrobial activity against *S. aureus*, *L. monocytogenes*, *S. typhimurium, E.coli* and *P. aeruginosa* with IC_{50} of 2.4 ± 0.4 µg/µL. Another research of (Abdul Rahman S. Bazaid., 2022) also support the statement and stated that Sumra honey exhibited strong antioxidant activity and reported 7.7 mg / mL of IC_{50}. In spite of it, total protein form honey sample PO and Ard found less effective as they exhibit higher IC_{50} [32,33]

Total protein was then fractionated by Gel Filtration Chromatography (GFC) using High Performance Liquid Chromatography by (Schimadzu LC-20AT- HPLC). The total honey protein was filtered by 0.45 µ (micron) syringe filter and applied on GFC column having silica beads with dimensions of 7.8 x 300 mm. Different peaks of each protein sample (extracted from honey) were collected almost at similar retention time. These peaks were collected individually at 280 nm using tris buffer with Sodium Azide and NaCl (pH 7.3). The cycle was run for 30 minutes and all the peaks of proteins fractions were separately collected. Honey sample "P" having total protein content of 1.2 µg/uL expressed molecular weight of 53 K Da and 41 K Da. While 1.3 µg/uL and 1.1 µg/uL of protein content was found in sample 1023 and UE respectively. The extracted total protein with known concentration was fractionated through Gel Filtration Chromatography using HPLC. And different peaks were collected. Total protein (extracted by Ammonium Sulfate precipitation method) of sample "P" fractionated as first peak at 5.607 minutes, second peaks at 8.685 minutes and third peaks at 13.188 minutes (figure 6) as shown in chromatogram. First two peaks were produced smaller in size having smaller area

as compare to third peak that is produced at 13.188 minutes with larger peak size. The protein content in peaks of honey sample P were estimated as 0.4 µg /µL, 0.72 µg /µL and 0.62 µg /µL respectively. The chromatogram expressed that there is non-protein part included in third peak while in first peak there is MRJP present. As (Stefan Alberta., et al., 2003) and (Carmen Ioana Muresan., et al., 2022) stated that MRJP is the 80-90 % of total protein content having large molecular size that eluted first from column. Similarly protein of another honey sample (1023) was precipitated out by same method of extraction (Ammonium Sulfate method) also fractionated from GFC and three different peaks having protein concentration of 0.44 µg /µL, 1.32 µg /µL and 0.88 µg /µL (table 4.14) were collected. As chromatogram of honey sample 1023 show the peaks 1 and 3 appeared at 5.228 and 13.179 minutes with larger area of third peak having slighter splitting may be because of any nearest sized molecules which passed through column. Another honey protein sample that is "UE" was fractionated in to three peaks at different retention time (figure 9) expressed different pattern with total six peaks appeared at 5.166 minutes, 8.386 minutes and 12.511 minutes. While peak 1 and 3 also appeared as in split form at 6.112 minutes and 13.267 minutes. And two small peaks 5 and 6 also appeared at 14.499 minutes and 15.478 minutes, which may because of similar molecular sized protein which eluted one after the other. While second peaks show normal pattern and appeared at 8.386 minutes. While the chromatogram clearly express the splitted peaks of fractions in third peak at 12.511 and 13.267 minutes of retention time. Peak 3 also split at ends with two more smaller peaks 5 at 14.499 minutes and peak 6 at 15.478 minutes.

In comparison of other protein's fractions including sample "P" and "1023" the sample UE expressed first and second peaks larger at same retention time. The protein of same sample "UE" was also extracted by Acetone method and a clear difference was observed in between both the precipitation methods, the protein precipitates from Ammonium sulfate method could not produced clear and larger peaks but protein precipitates obtained from Acetone method, produced larger and vibrant peaks as compare to other. The first and second peaks was obtained at 5.067 and 8.389 minutes respectively. These both have protein content of 0.26 µg / µL with MRJP found in abundant quantity and may have molecular mass of ~260 k D and ~160 k D comprised of 'major royal jelly protein-1'-containing complexes [36,37].While third peak appeared at 11.310 minutes and split at 12.706 minutes. Results show that acetone method is comparatively better method to extract protein form honey sample. The chromatogram of UE honey protein show the protein part having MRJP in first peak with large molecular size. While peptides and other compounds or non-protein part of small sized molecules eluted in last from column having smaller molecular size with concentration of 0.56 µg/µL protein.

There is a pulsating difference was found between Acetone method and Ammonium Sulfate method of protein extraction and a foremost difference was observed in the chromatogram of proteins by their extraction method. The total protein of the "UE" sample which was extracted by the acetone method, has fractionated differently with heightened first peak at 5.067 minutes, second peak at 8.389 minutes while the third peak was obtained in the vibrant split form at 11.310, and 12.706 minutes at 280 nm. All the fractions were diligently gathered in clean eppendorffs with appropriate specimen identification marks and stored at -20 C for further use. After the collection of all GFC- fractions (peaks), their protein was quantified by the Lowry Method of protein estimation. An antibacterial assay for each

fraction against all bacterial isolates was performed and the growth percentage in wells of microtiter plate was measured in terms of absorbance and inhibition percentage was calculated. According to results *E. coli* show sensitivity to all three peaks sample "P" that is 58.52%, 55.08%, and 60.43%. Whereas *Pseudomonas aeruginosa* does not show any sensitivity. And peak 3 shows 47.66% and 24.14% inhibition against *Bacillus subtilis* and *Salmonella typhi.* Fractionated protein peak 3 of sample "1023" show affectivity and exhibits percent inhibition 70.59%, 70.94% and 59.53% against *E. coli*, *S. typhi* and *S. aureus* respectively. While peak 2 could not show a significant effect against *Klebsiella pneumonia.* Similarly Fractionated protein of honey sample "UE" exhibits a different pattern against bacterial isolates, its peak 3 show better antibacterial activity and exhibits an inhibitory effect of 86.36%, 79.02%, 88.25% and 87.31% against *E. coli, Pseudomonas aeruginosa, Salmonella typhi,* and *Staphylococcus aureus* respectively.

In general, the use of honey as a natural remedy may reduce economic loss and may provide benefits by lowering direct costs and using fewer antibiotics. A detailed analysis of honey with its antibacterial activity, chemical composition, and concentrations of synergistic components may become beneficial. Further studies can be helpful to fulfill the need to get more benefits of honey and its potential support to provide an innovative treatment should not be overlooked.

CONCLUSION

In this study, the antibacterial activities of natural honey and honey proteins were tested against six pathogenic bacteria. Honey proteins were extracted by Ammonium Sulfate and Acetone methods followed by protein estimation the Lowry method. SDS-PAGE was used to determine the molecular weight of honey proteins. Extracted honey proteins were separated by Gel Filtration Chromatography using HPLC. The antibacterial activity of total honey protein was performed by broth microdilution method and IC_{50} was determined. The honey sample 0829 showed potent antibacterial activity as compared to other samples with IC_{50} of 45 ng/µL to 55 ng/µL against bacterial isolates. While fractionated honey protein (peak 3) of Acacia honey sample UE inhibited the growth of *E. coli* (86%)*, P. aeruginosa* (79%)*, S. typhi* (88%) and *S. aureus* (87%). It is also noted that Acetone method of protein extraction was comparatively better than Ammonium sulfate method as it gives larger peaks having 1 µM (micromoles) of MRJP present in protein of honey sample. The study concluded that a very minute amount of honey protein is adequate to inhibit bacterial growth up to 80% [38,39].

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