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Insights on the Proteolytic and Biological Properties of Floral Honeys with Various Botanical Sources

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ABSTRACT

Honey is an apiary product with health-promoting properties.However, its biological activities can be affected by its composition of the bioactive molecules. Thus, the study aims to determine the total concentration of proteins, phenolics, and carotenoids in the aqueous extracts of Manuka honey (HM), Sidr honey (HS), Egyptian honey (HE), and mountain honey (HH), in addition to the honey's biological potential and proteolytic activity. Utilizing microscopic methods, the pollen grains analysis was examined. The results demonstrated the anti-hemolytic effect of about 95%, the antioxidant effects of 37.18%, 78%, 88.84%, and 70.29%, and the anti-inflammatory activity of 73.9, 93.8, 95.21, and 87.4 of the honey extracts from HH, HE, HS, and HM, respectively. The Pearson correlation coefficient of the total phenolic content was used to connect and correlate these various bioactivities. Nonetheless, there is a noteworthy correlation between total phenols and biological activity that is almost 0.9 (R^2). Notably, casein was used as a substrate to test the proteolytic activity of the chosen honey extracts. The results showed that the proteases in the extracts had different catalytic efficiencies and kinetic characteristics (V max, Km). The catalytic efficiencies of the HH, HE, HS, and HM extracts were 10, 658, 139, and 55, respectively. The therapeutic proteases treated with honey extracts showed non-competitive inhibition of trypsin and activation of papain and chymotrypsin. Further studies are recommended to separate the bioactive compounds in honey samples and ascertain their botanical origins.

Keywords: Phenolic compounds, Antioxidant, Anti-inflammatory, Proteolytic activity.

Introduction

Honey, a naturally occurring substance, is created by honeybees from flower nectar, possessing a sweet taste.[1] Its composition is characterized by a substantial percentage of sugars, comprising 95% of its dry weight, primarily consisting of glucose and fructose. Approximately 20% of its content is water. Additionally, honey contains proteins, amino acids, vitamins, minerals, enzymes, organic acids, and various organic compounds.[2] Honey has been used since ancient times by the Egyptians, Romans, Chinese, Greeks and Indians for its nutritional and therapeutic properties.[3] Studies have shown that it has antimicrobial, anti-cancer, anti-inflammatory, anti-atherosclerosis, anti-clotting, and antioxidative activity properties. It is also used to treat various wounds, burns, eye ulcers, mouth ulcers, and skin diseases.[4] The physical and chemical properties vary depending on the different types of honey due to several reasons, including the plant origin, the geographical location of the beehives, the method of processing the honey, and the harvest seasons.[5]

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Recently, the number of research dealing with the therapeutic methods based on the use of natural materials to enhance human health without side effects has increased, which led to the emergence of the term apitherapy.[6] The therapeutic properties of honey acquired due to its containment of secondary metabolites coming from pollen and flower nectar. So, there are many differences between the types of honey, depending on their botanical origin and the amount and types of these compounds.[7] As a result of the presence of flavonoids and phenols, evidence indicates that honey has many beneficial effects for health, including antioxidants, anti-inflammatory, anti-bacterial, and anti-diabetic, in addition to being beneficial to various human body systems.[8] The compounds present in pollen and flower nectar are transferred to honey, influencing its diverse physical and chemical properties such as flavor, color, taste, and therapeutic benefits.[9] An example is Manuka honey (HM), derived from the Manuka tree (*Leptospermum scoparium*) in New Zealand and Eastern Australia. HM exhibits specific anti-inflammatory effects, notably in ulcerative colitis, and has demonstrated effectiveness in treating leg ulcers, including neuropathic diabetic foot ulcers.[10]

Notably, the bees that feed on the sidr tree nectar which is also referred to as jujube tree are the ones that produces sidr honey, HS is a type of honey that is highly valued for its medicinal properties. The sidr tree is indigenous to the Middle East and the honey extracted from its nectar is regarded as one of the world's most refined and costly honeys.[11] Honey from Egypt (HE) was of importance to the ancient Egyptians as it was their most popular medicine and food. HE honey varies according to its botanical origin and geographical location, but it is distinguished by its high quality, as it is the most common honey in the Arab world.[12] Mountain honey (HH), or wildflower honey, is produced by honeybees that feed on mountain plants. Its taste and colour vary according to the type of plant. It contains sugars, proteins, and various enzymes such as catalase, glucose oxidase, saccharase, invertase, and

diastase.[13] Phenolic compounds are found in honey because bees incorporate them into the honey during its production. Bees mix their body fluids with flower nectar or plant secretions, which contain water, sugars, proteins, and phenolic compounds. Honey consists of a lot of phenolic compounds which give many important properties to honey and thus for human health.[14] Phenolic compounds in honey show extensive diversity, with their composition influenced by the geographical and floral origin of the honey.[15] These compounds possess various properties, including antioxidant, cardioprotective, antiproliferative, anticancer, anti-inflammatory, antiaging, and antimicrobial activities, garnering significant attention from both the scientific community and industry.[16]

Carotenoids are natural pigments produced by plants, algae, and some types of fungi and bacteria. Carotenoids contribute to the flavour, aroma, and vibrant colour of many plants, ranging from yellow and orange to bright red colour. Photosynthesis is a vital metabolite crucial for photosynthesis, plant growth, as well as survival. Prominent examples include β-carotene, lycopene, and lutein, each possessing a distinctive chemical composition and specific functions.[17] Proteases are a group of proteolytic enzymes that break peptide bonds into amino acids. These enzymes act significantly in the physiological functions of living organisms, contributing to programmed cell death, and involvement in protein turnover, decomposing abnormal proteins and facilitating cell signaling.[18] Classifications of protease enzymes are serine proteases, such as chymotrypsin and trypsin, are essential and important in the digestive process, while cysteine proteases, such as papain, contribute to cellular and immune functions. Aspartic proteases, such as pepsin, aid in the digestive process that occurs in the stomach, while metalloproteases, which depend on metal ions, participate in Extracellular matrix remodeling. The protease superfamily includes caspases for programmed cell death as well as immune regulation and matrix metalloproteinases involved in tissue remodeling. Generally, proteases are molecular scissors, which are essential for protein breakdown and regulation of physiological processes.[19] If there is a defect in these enzymes, this leads to the occurrence of immune diseases, cancers, inflammations, and neurodegenerative diseases.

Proteases have a role in the reproduction and continuation of the life cycle of viruses, such as human immunodeficiency virus.[20] Hence the importance of finding inhibitors for these enzymes. Protease inhibitors are compounds that block the activity of proteases, and therefore they can be used in treating various diseases related to the activity of these enzymes. Studies prove that some phenolic compounds and flavonoids are effective in inhibiting the action of proteases such as curcumin and Quercetin.[21] Honey has many inhibitors of proteases, such as the coronavirus protease, and when honey was used, it was observed that there were fewer infections and fewer deaths.[22]

In previous studies, crude honey and its extracts were used to investigate its pharmacological and biological roles. However, only a limited number of studies exist about its proteolytic profiles and bioactive compounds associated with the botanical source. Thus, the purpose of this work was to investigate the biochemical properties of several honey extracts (HM, HS, HE, and HH) that were obtained from distinct geographical locations and plant species. The analysis was centered on total proteins, carotenoids, and phenols determinations as well as investigation of antioxidant, anti-inflammatory, and anti-hemolytic properties. Furthermore, the selected honey extracts' proteolytic efficiency was evaluated.

Materials and Methods

Chemicals and reagents

Chymotrypsin, Trypsin, and Papain were obtained from Genochem World (Spain); Biuret reagent was obtained from Alpha Chemika (India); bovine serum albumin was obtained from TCS Biosciences (Australia); casein and Folin-Ciocalteu phenol reagent were obtained from Sigma-Aldrich(Germany); diclofenac sodium was obtained from Hikma company (Jordan), DPPH was obtained from AK Scientific, Inc. (USA); Gallic acid was obtained from Fragrance & Flavor Chemicals(India). All reagents used were of analytical grade.

Honey samples extract

Four honey samples, collected between March and May of 2023, were used in the study: HM from New Zealand was purchased from i Herb online shopping (Manuka Honey, Liberty Richter, Bloomfield, NJ07003, USA; registration number 0852469004224); HE from Egypt (El-Minya Governorate, Egypt); HH; and HS from Jordan (Wadi Al-Karak, Karak Governorate, Jordan) were obtained from local markets in Amman, Jordan. The honey samples were stored in the dark at 25°C until analysis to preserve their chemical composition. A voucher samples, labeled as HW2023-HM, HW2023-HE, HW2023-HH, and HW2023-HS, were securely stored at the biochemistry laboratory, Mutah University, Jordan. Each honey sample was extracted by diluting it with distilled water in a 1:1 (w/v) ratio at 37°C for 90 minutes. Following this, the mixture was centrifuged at 1500 rpm for 20 minutes and then filtered using filter paper, following the method outlined by Tsiapara et al.[23]

Honey colour assessment

Pfund scale is one of the techniques used to determine the colour of honey (Table 1).[24] This is accomplished through measuring the absorbance of the samples at 635 nm by spectrophotometer (Biotech Engineering Management Co. Ltd., UK) after they have undergone a 50% (w/v) dilution with distilled water, followed by thorough mixing and centrifugation at 3200 rpm for 5 minutes (B. Herml AGD-7209 Gosheim; Germany). Equation (1) was used to acquire the Pfund value measurement.

$$\text{Eq.1} \qquad \text{Pfund} = -38.70 + 371.39 * \text{Absorbance}_{\lambda 635}$$

Pollen grains analysis

Pollen analysis of honey is the basic technique for the determination of its botanical origin, due to factors like shape, size, and outer surface characteristics, pollen grains can vary significantly. Melissopalynological analysis was employed with minor adjustments.[25] The bee products were first diluted with distilled water at a 1:2 (w/v) ratio and then subjected to centrifugation at 3000 rpm for 20 minutes. Subsequently, the resulting precipitate was re-suspended in an equal volume of distilled water and subjected to another round of centrifugation at 3000 rpm for 5 minutes, which was repeated twice. The sediment was collected, and three slides were prepared. These slides were then examined under a microscope at a total magnification of 400x, and photographs of pollen grains were captured using an Eakins camera (1080P 60fps HDMI-Compatible Microscope Camera, China). Atlas of pollen and plants used by bees was used for identification of plant origin of pollen.[26]

Total protein content assay

The content and quantity of proteins were determined using the biuret method. A standard solution of bovine serum albumin (BSA) was prepared at various concentrations ranging from 0.1 to 6.8 mg/mL.[27] 1.5 mL of biuret reagent was combined with 0.5 mL of extracts, which had been previously diluted with distilled water in a 1:10 (v/v) ratio. The mixture was then incubated at 37°C for 15 minutes and the absorbance was measured at 540 nm by UV-Vis spectrophotometer (Biotech Engineering Management Co. Ltd., UK).

Total phenol content assay

The total phenol content was assessed using the Folin-Ciocalteu method with minor adjustments.[28] Initially, 0.1 mL of various extract concentrations was mixed with 0.5 mL of the Folin-Ciocalteu reagent, and the resulting reaction mixture was left at room temperature for five minutes. Following this, 2.5 mL of sodium carbonate (Na_2CO_3) was added, and the reaction mixture was incubated in the dark for 20 minutes. The change in color was subsequently measured at 765 nm.

β-Carotene and Lycopene Content Assay

The honey was diluted with distilled water to 50% (w/v). Then, 10 mL of a hexane-acetone mixture in a 6:4 (v/v) ratio was added to 2.0 mL of the diluted solution, and the mixture was thoroughly mixed for ten minutes. Subsequently, the mixture was filtered, and its absorbance was measured at wavelengths of 663 nm, 505 nm, and 453 nm using a spectrophotometer, with deionized water (DI) as blank.[29]

Table 1: Pfund scale of honey colour

Colour Name	Pfund Scale (millimeters)	Optical Density
Water White	<9	0.0945
Extra White	9 - 17	0.189
White	18 - 34	0.378
Extra Light Amber	35 - 50	0.595
Light Amber	51 - 85	1.389
Amber	86 - 114	3.008
Dark Amber	>114	-

The concentrations of β-carotene and lycopene were determined using equation 2 and 3, respectively.

Eq.2 $\beta - \text{carotene (mg / 100mL)} = 0.216 * A\_\lambda 635 - 0.304 * A\_\lambda 505 + 0.452 * A\_\lambda 453$

Eq.3 $\text{Lycopene (mg / 100mL)} = 0.0458 * A_{\lambda 635} + 0.372 * A_{\lambda 505} + 0.452 * A_{\lambda 453}$

Where A is the absorbance.

Antioxidant activity assay

The radical scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was employed to assess the extracts' capability to neutralize free radicals. The reaction mixture comprised 1.0 mL of a DPPH solution (60 μM in methanol) combined with 1.0 mL of different concentrations of extracts (0.1-1.0 mg/mL). This mixture was then incubated in the dark for 30 minutes. The absorption value was measured at 715 nm by spectrophotometer. The linear regression equation was established using gallic acid as the standard, and the DPPH solution serving as the control.[30] Equation 4, which follows, was used to calculate the percentage of antioxidant activity.

Eq.4 $\textbf{Percentage of antioxidant activity}\ (\%) = \frac{\textbf{Control absorbance} - \textbf{Sample absorbance}}{\textbf{Control absorbance}} * \mathbf{100}$

Inhibition concentration assessment (IC_{50})

It represents the degree of effectiveness in inhibiting a particular biological or biochemical activity by half. The IC_{50} value was calculated using IC_{50} calculator by applying the AAT Bioquest software.

Anti-inflammatory activity assay

The assessment of anti-inflammatory activity was determined using previously reported method.[31] In the test solution, 0.45 mL of BSA was combined with 0.05 mL of extracts at various concentrations (0.1-1.0 mg/mL). The control test contained distilled water instead of extract, while the product control substituted distilled water for BSA. These solutions were incubated at 37 °C for 20 minutes and then at 70 °C for 10 minutes. After adding 2.5 mL of phosphate buffer, the absorbance was measured at 660 nm. Equation 5 (below) was used to compute the proportion of anti-inflammatory action.

Eq.5 $\text{Percentage of anti-inflammatory activity}\ (\%) = 100 - \left(\text{AT} - \frac{\text{AP}}{\text{AC}}\right) * 100$

Where AT is the absorbance of test solution; AP is the absorbance product control and AC is the absorbance test control.

Anti-hemolytic activity assay

The anti-hemolytic activity was assessed using the hydrogen peroxide (H_2O_2) method. Blood samples were obtained through EDTA tubes (Ethylene Diamine Tetra acetic acid), followed by centrifugation at 1500 rpm for 5 minutes and three subsequent washes with normal saline. The method described by Nabavi et al.,[28] with slight modifications was used for estimation of the anti-hemolytic activity. The reaction mixture comprised 0.5 mL of RBCs, 0.125 mL of the extract (0.1-1.0 mg/mL), and 0.625 mL of normal saline. The negative control consisted of 0.5 mL of RBCs and 0.75 mL of normal saline, while the positive control replaced 0.625 mL of normal saline with distilled water. All tubes were then incubated at 37 °C for 10 minutes. Following this, 0.125 mL of H_2O_2 was added to the test and control tubes, and they were incubated at room temperature for 2 hours before being centrifuged at 3000 rpm for 10 minutes. Using equation (6), the proportion of anti-hemolytic activity was determined.

Eq.6 $\text{Percentage of Anti-hemolytic activity}\ (\%) = \left(Ap - \frac{\text{As}-\text{Ac}}{\text{Ap}}\right) * 100\%$

Where Ap is the control positive absorbance; Ac is the control negative absorbance and As is the sample absorbance.

Enzyme activity and kinetic assays

The proteolytic activity was determined by Fahmy method using casein as a substrate and tyrosine as standard.[32] Furthermore, the Lineweaver-Burk plot analysis was used to determine both the Michaelis constant (Km) and the maximum rate of the reaction (Vmax) using Various concentrations of casein.[33] The reaction mixture consisted of 0.5 mL of casein, 0.25 mL of extract, and 0.25 mL of enzyme, then it was incubated at 37 °C for a duration of 30 minutes. Subsequently, 0.5 mL of TCA was introduced into the mixture, followed by centrifugation at 3000 rpm for 10 minutes. From the resulting solution, 0.25 mL of the supernatant was extracted, and to this, 1.0 mL of sodium carbonate (Na_2CO_3) and 0.2 mL of Folin-Ciocalteu reagent were added. The resulting mixture underwent incubation at 37°C for 20 minutes, after which the absorbance was measured at 750 nm, as described in a study by Atrooz and Al-Maitah.[34] The enzyme kinetic parameters were determined to assess the effects of extract on the trypsin, chymotrypsin and papain enzymes.

Statistical analysis

The data was presented as the mean value along with the standard deviation, derived from a triple analysis. Microsoft Excel version 365 was employed for result analysis and for the correlation between phenolic compounds and biological activities.

Results and Discussion

Honey has a variety of nutritional and therapeutic benefits, due to its contents and components.[1] Honey has an important role in preventing different diseases and it has the ability to heal diseases from burns to various types of cancer.[35]

Regarding plant diversity, environmental impact, diverse geographic regions, flower sources, and chemical compositions, the finding of the selected honey extracts were found to exhibit different physic-chemical and biological properties that match the conclusion of Becerril-Sánchez et al. study.[14]

Honey colour

The colour of honey varied from water white to amber depending on the Pfund scale. HE had the lowest Pfund value (1.0 mm), while MH had the highest Pfund value (111.7 mm) (Table 2).

The results of the four selected honey extracts were different, they ranged from water white to dark amber colour. Our findings indicated that HE was water white, Smetanska study indicates that HE was white; this difference is due to several factors, which reflects the difference in the chemical components and compositions.[36] HS was extra light amber; this is consistent with what was mentioned in Hegazi study,[11] that HS ranges in colour from light white to dark black. The HM was the darkest; HM is known to have a dark colour, and this is attributed to its components, this is consistent with the results of Alvarez-Suarez study,[37] which prove that HM has a dark colour, related to the phenolic content and plant origin.

Table 2: The Pfund value and colour of honey samples

Honey	Pfund value (mm)	Colour
HM	111.7	Amber
HE	1.0	Water white
HS	37.8	Extra Light Amber
HH	18.5	White

Plant origin of bee products

The presence of pollen in honey can be used as a biomarker of authenticity. All samples show the presence of pollen in varying proportions, indicating a diverse plant origin. Pollens were found to have a diversity of 30 species in HH, while HE sample contained 25 species. On the other hand, HS contained 23 types of pollens, while in the HM sample had only three types. The most common plant species in each honey sample are shown in Table 3.

Studying the pollen grains in honey extracts explains the differences between different types of honey in their properties. All honey samples were multifloral because each contained more than one pollen grain from different plants. The difference in plant origin for each type of honey is considered the main factor in the difference between each sample in its physical and chemical characteristics from other samples. Knowing the plant origin of each sample gives the possibility of knowing the source of the compounds and chemical components in honey.

Table 3: Most common plant species in the honey samples

Honey types	Plant species
HH	*Echium* sp.*; Stachytarpheta cayennensis; Typhaceae* sp.
HS	*Helianthus annuus; Matricaria chamomilla; Stachytarpheta cayennensis.*
HE	*Cereus spegazzinii.; Helianthus annuus; Cereus hexagonus.*
HM	*Leptospermum scoparium; Kunzea ericoides; Bejaria resinosa.*

The content of total protein, total phenols and carotenoids in honey samples

The biuret method was used to determine the protein content of the extracts, and BSA was used as a standard. The extracts contained different amount of protein, as demonstrated by the following values: HM extract was 4.149 ± 0.013 mg/ml, HE extract was 3.928 ± 0.043 mg/ml, HS extract was 3.510 ± 0.024 mg/ml, and HH extract was 3.128 ± 0.021 mg/ml (Table 4).

The results showed that honey extracts contain a significant phenolic content, as demonstrated by the following values: HH extract was 1.387 ± 0.020 mg/ml, HS extract was 1.326 ± 0.010 mg/ml, HM extract was 1.217 ± 0.007 mg/ml and HE extract was 0.769 ± 0.010 mg/ml (Table 4). Honey samples carotenoid content was variable. The lowest values of β-carotene and lycopene (mg/Kg) were 4.3 and 7.3 mg/Kg in HH, respectively. However, the highest values were 92.1 and 216.8 mg/Kg in HS, respectively. Values of β-carotene and lycopene are presented in Table (4).

β-carotene and lycopene have distinct nutritional importance. β-carotene contributes to enhancing vision and immune health. On the contrary, due to its antioxidant properties, lycopene provides protective benefits against oxidative stress, thus protecting against heart disease and cancer. The β-carotene content of the extracts ranged from 4.3 to 93.1 mg/kg, while the lycopene content of the extracts ranged from 7.3 to 216.8 mg/kg. The highest content of them was for HS and the lowest for HH. So, honey is considered as source for them, and this is consistent with the results of Smetanska et al. results.[36]

The results obtained regarding protein levels provide the nutritional compositions of the tested extracts. Measuring protein content serves as an indicator of nutritional value and potential applications. The high protein levels in the extracts indicate that the samples are a source of amino acids, which contribute to their overall nutritional quality. These findings hold implications for the food, pharmaceutical, and dietary supplement industry. HM contained a significant amount of protein, and this is consistent with Johnston results.[38] Honey is one of the foods known for containing a large amount of proteins. So, it is a product with high nutritional value.

Phenolic compounds, such as flavonoids, phenolic acids, and other polyphenols, are not only capable of giving honey flavor and aroma, but also give it properties that are beneficial to health, such that they play a pivotal role in biological activities. These phenols in honey come from plants, so here lies the importance of studying the phenolic content and knowing their source, to know the possibility of using honey or the plant source of phenols in research specialized in the nutritional and medical fields. The highest phenolic content was for HH, and its IC_{50} µg/mL value was low 0.4002. HH has a large quantity of phenolic compounds and that is described its biological activities. Our results are consistent with the Nedić et al. results, which describe the phenolic content of HH and the relationship between the phenolic content and the its biological activities.[39] The phenolic content in their study ranged from 307.01 ± 4.77 to 1273.75 ± 6.25 mg/kg, and this difference from our findings due to different circumstances, the most important of which is the plant origin. Due to different in plant origin, HS, HM, HE have different phenolic amounts as illustrated in Table 4.

Antioxidant activity

The DPPH radical scavenging method was used to determine the ability of the extracts to eliminate free radicals, and gallic acid was used as a standard. All extracts have antioxidant activity, as demonstrated by the following values: HH extract was 88.84% ± 0.562, HE extract was 78.00% ± 0.572, HS extract was 70.29% ± 0.358 and HM extract was 37.18% ± 0.221 (Table 5). The IC_{50} values (µg/mL) for HM, HE, HS, HH honey were 0.8013, 0.2313, 0.7412 and 0.9486, respectively (Table 5). The antioxidant activity was evaluated by DPPH method. In this study, results indicated that the tested honey extracts possess significantly anti-oxidative activity. The honey extracts were found to exhibit a promised activity in scavenging free radicals, the result ranged from 37.18- 88.84% and the highest percentage was found in the HH extract, and its IC_{50} value was 0.9486 µg/mL. The diversity between the results refers to the amount of phenolic compounds and the type of them because each phenolic compound has a specific role. Results of Almasaudi et al. study revealed little activity of antioxidant of HM.[40]

Table 4. The content of total protein, total phenols and carotenoids in honey samples.

Bee product types	Total protein (mg/ml)	Total phenol (mg/ml)	*β*-carotene (mg/kg)	Lycopene (mg/kg)
HM	4.149 ± 0.013	1.217 ± 0.007	9.6 ± 0.011	20.7 ± 0.017
HE	3.928 ± 0.043	0.769 ± 0.010	37.4 ± 0.01	75.4 ± 0.012
HS	3.510 ± 0.024	1.326 ± 0.010	92.1 ± 0.03	216.8 ± 0.033
HH	3.128 ± 0.021	1.387 ± 0.020	4.3 ± 0.027	7.3 ± 0.015

Mean ±SD, n=3.

Many factors other than plant origin and geographical location, such as honey processing methods and harvesting time, mainly affect the components of honey and thus its activity.

Anti-inflammatory activity

The percentage of anti-inflammatory for extracts was determined by using diclofenac standard. All extracts have anti-inflammatory activity, as demonstrated by the following values: HH extract was 95.21% ± 0.455, HE extract was 93.80% ± 0.383, HS extract was 87.40% ± 0.581 and HM extract was 73.90% ± 0.749 (Table 5). The IC_{50} values (µg/mL) for HM, HE, HS, HH were 0.0925, 0.3785, 0.8311 and 0.0942, respectively (Table 5). Anti-inflammatory activity is usually tested to determine the effect of substances on symptoms, markers, or pathways of inflammation. The results obtained show a significant activity of the extracts as anti-inflammatory. These results highlight the potential therapeutic implications of honey extracts in the development of anti-inflammatory drugs. The percentages of anti-inflammatory potentials revealed high activity ranged from 73.90- 95.21%, the highest percentage was found in the HH extract. HM had the least percentage (73.90%) and matched with the results of Almasaudi study.[40] Flavonoids play a vital role in preventing inflammation and their quantity plays a different point between the honey samples which reflects the diversity in their ability. In addition, honey has the ability to alleviate and reduce the symptoms of inflammation, so it is a good product for treating many diseases.

Table 5: The activity (%) and IC_{50} (µg/mL) of biological activities of the honey extracts

Biological activity	HM		HS	HE	HH
Antioxidant	DPPH(%)	37.18% ± 0.221	70.29± 0.358	78.00± 0.572	88.84± 0.562
	IC_{50}(µg/mL)	0.8013	0.7412	0.2313	0.9486
Anti-inflammatory	Activity(%)	73.90 ± 0.749	87.40 ± 0.581	93.80 ± 0.383	95.21 ± 0.455
	IC_{50}(µg/mL)	0.0925	0.8311	0.3785	0.0942
Anti-hemolytic	Activity(%)	95.09 ± 0.021	95.20 ± 0.012	96.01 ± 0.030	96.20 ± 0.009
	IC_{50}(µg/mL)	0.7926	0.0516	0.0559	0.5689

Mean ± SD.

Anti-hemolytic activity

All extracts have anti-hemolytic activity, as demonstrated by the following values: HH was 96.20% ± 0.009, HE extract was 96.01% ± 0.030, HS extract was 95.20% ± 0.012 and HM extract was 95.09% ± 0.021(Table 5). The IC_{50} values (µg/mL) for HM, HE, HS, HH were values of 0.7926, 0.0559, 0.0516 and 0.5689 µg/mL, respectively (Table 5). The anti-hemolytic activity results indicate a significant reduction in RBCs hemolysis, indicating the ability of honey extracts to mitigate or inhibit the harmful effects of hydrogen peroxide on red blood cells. Ibrahim's study proved that honey has anti-hemolytic properties, and it is consistent with our results.[41] Regarding our findings of HH extract, it was found to exhibit a high activity value of 96.20% of anti-hemolytic activity and phenolic content and it is considered as the best honey sample to do many biological activities and that refer to the type of the mountain plants which are known for their diversity, often less impacted by pollution and their ability to adapt against the environmental conditions.

A linear relationship between phenolic content and biological activities was found. The relationship values showed that there is a direct and strong relationship between them as presented in Table (6). Phenolic compounds are considered biological materials known for their diverse biological activities, which makes them an important point in many researches. Pearson's coefficient findings between total phenol and antioxidant, anti-inflammatory and anti-hemolytic activities revealed a strong relationship and consistent with Jiang study.[42] So, the phenolic content is responsible for these biological activities, and here lies the importance of phenolic compounds in protecting against many diseases.

Proteases

The honey extracts displayed activity on casein with different catalytic activities (Figure 1). The honey extracts have clear effects on the enzyme kinetic parameters (Vmax and Km) of trypsin, chymotrypsin, and papain enzymes. The four honey extracts illustrate a non-competitive inhibitor on trypsin, but illustrate an activation effect on chymotrypsin and papain. A summary of the enzymes kinetic is displayed in Table (7). Proteolytic activity is an important enzymatic process within honey that plays a vital role in the quality and health-related properties of honey. This process involves breaking down proteins into amino acids due to protease enzymes. Proteolytic activity is important in honey because of its effect on the nutritional and therapeutic values of honey. Understanding the proteolytic activity of honey is important to evaluate honey quality and correlate the differences between honey types depending on their source and environmental factors. A high ratio of Vmax/Km indicates the catalytic efficiency. All honey extracts demonstrated the presence of proteolytic enzymes. The highest catalytic efficiency was observed in the HE extract (658), followed by the HS extract (139), HM extract (55), and HH extract (10). According to Alaerjani study,[43] honey is recognized for containing biological molecules, including enzymes derived from various sources like nectar, bee secretions, or microorganisms.

Table 6: Pearson correlation coefficient between total phenolic content and different biological activities

		TPC	DPPH	Anti-inflammatory	Anti-hemolytic
HM	TPC	1			
	DPPH	0.930	1		
	Anti-inflammatory	0.950	0.950	1	
	Anti-hemolytic	0.793	0.862	0.719	1
HE	TPC	1			
	DPPH	0.934	1		
	Anti-inflammatory	0.791	0.951	1	
	Anti-hemolytic	0.746	0.947	0.904	1
HS	TPC	1			
	DPPH	0.938	1		
	Anti-inflammatory	0.99	0.962	1	
	Anti-hemolytic	0.906	0.944	0.921	1
HH	TPC	1			
	DPPH	0.916	1		
	Anti-inflammatory	0.929	0.989	1	
	Anti-hemolytic	0.919	0.976	0.946	1

Table 7. The proteolytic activity of different honey extracts and kinetic parameters values associated with trypsin, chymotrypsin, and papain enzymes

Extract types	MH		HS	HE	HH
Extract	Vmax	0.2025	0.278	0.1973	0.0617
	Km	0.0037	0.002	0.0003	0.0063
Trypsin enzyme	Vmax	0.319	0.319	0.319	0.319
	Km	0.018	0.018	0.018	0.018
Trypsin enzyme with extracts	Vmax	0.245	0.22	0.178	0.243
	Km	0.002	0.0004	0.002	0.001
The type of effect		NCI	NCI	NCI	NCI
Chymotrypsin enzyme	Vmax	0.134	0.134	0.134	0.134
	Km	0.003	0.003	0.003	0.003
Chymotrypsin enzyme with extracts	Vmax	0.233	0.224	0.209	0.215
	Km	0.0003	0.001	0.002	0.001
The type of effect		Activator	Activator	Activator	Activator
Papain enzyme	Vmax	0.125	0.125	0.125	0.125
	Km	0.004	0.004	0.004	0.004
Papain enzyme with extracts	Vmax	0.277	0.156	0.222	0.247
	Km	0.0003	0.0005	0.002	0.001
The type of effect		Activator	Activator	Activator	Activator

Vmax: µmol/min^{-1}, Km: mM^{-1}, NCI: Non-competitive inhibitor.

Inhibitors are substances or molecules that restrict the activity of enzymes. They can bind to enzymes, changing their function or structure, and thus preventing them from carrying out their reactions. They can be classified as competitive, uncompetitive, and non-competitive inhibitors. they play an important role in regulating biological pathways, and they are considered as an essential tool in understanding cellular processes and tool for developing drugs in various medical treatments.[44] All the selected honey extracts have the ability to activate chymotrypsin and papain because the Vmax was increased and that is very good to enhance the activity and efficiency for both enzymes. Nevertheless, the honey extracts exhibited a non-competitive inhibitor for trypsin enzyme, an important result in which they can be used to inhibit the trypsin and other proteases enzyme so decrease the symptoms and prevent the virus's infection because some viruses depend on these enzymes to complete their life cycle.[21,45] Proteases are vital enzymes in the life cycle of viruses and are responsible for breaking down large viral proteins into small proteins necessary for different stages of replication and development. Inhibiting the activity of these enzymes disrupts the life of viruses, making it a target for antiviral therapies.[45]

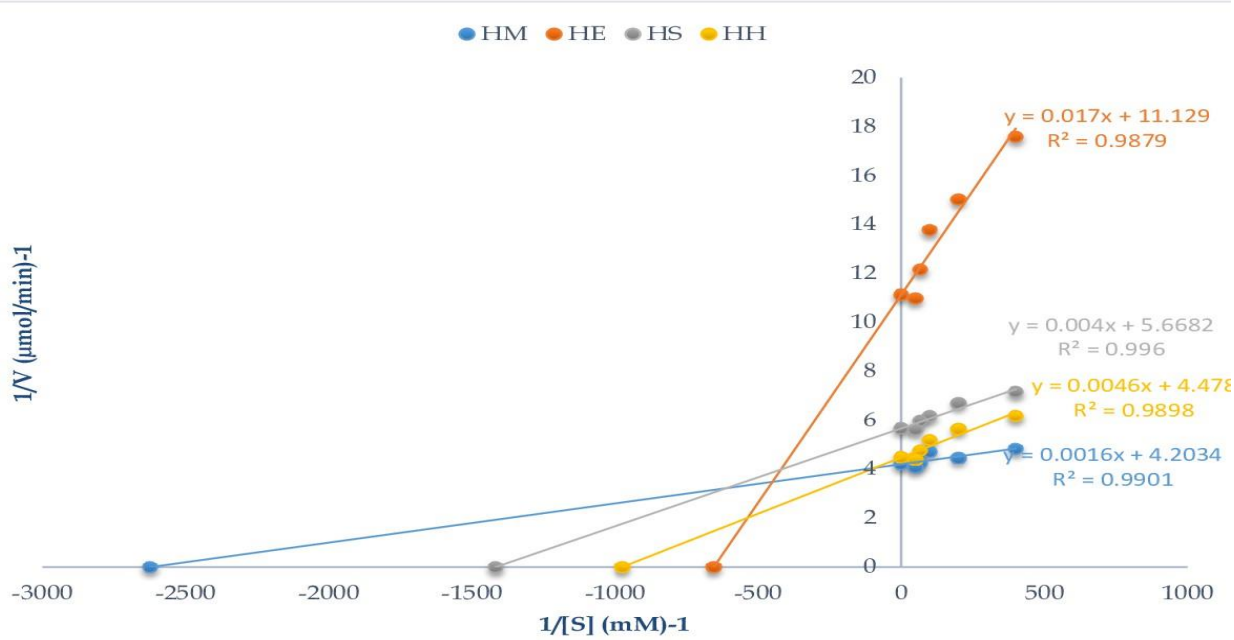

Figure 1. Lineweaver-Burk reciprocal plot for determination of the Km and Vmax values for protease enzymes in the crude honey extracts of HM, HE, HS, and HH using casein as a substrate

Conclusion

Ancient civilizations used and relied on honey for its wonderful properties, which prompted a study to be conducted on different types of honey, such as HM, HS, and HE and HH. The selected honey extracts were characterized by a high protein content and presence of beneficial compounds such as carotenoids and phenols. These components have many health benefits, including antioxidant, anti-inflammatory, and antihemolytic properties, along with notable proteolytic activity. Furthermore, these honey extracts contain inhibitors for trypsin enzyme.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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