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EVALUATION OF THE PROTEOLYTIC AND BIOLOGICAL ACTIVITIES OF THISTLE HONEY

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ABSTRACT

Functional food plays a vital role in promoting human health. Honey is one of these foods that have high nutritional value and medicinal properties. The main aim of this study is to investigate the total content of proteins, phenols and carotenoids in thistle honey extract and to evaluate the biological activities. In addition, to investigate the proteolytic activity of the extract and whether it contains inhibitors for other proteolytic enzymes. Casein was used as a substrate for the proteolytic analysis and for the determination of kinetic parameters (Km, Vmax) of the extract. Melissopalynological results showed that the honey samples have different plant origins, and this explains the different chemical contents and biological activities of thistle extract. It was found that the extract contained considerable amounts of carotenoids, proteins, and phenolic compounds. In addition, the extract exhibited 72.33%, 54.422%, and 63.599% of antioxidant, anti-inflammatory, anti-hemolytic potentials, respectively. Furthermore, results revealed that the extract of thistle honey had proteolytic activity using casein as a substrate and performed an activation effect on the therapeutic enzymes, namely, trypsin, chymotrypsin, and papain. Thistle honey extract was of high nutritional and therapeutic value. This attribute grants it suitability for inclusion in a wide array of pharmaceutical formulations and for the prophylaxis of numerous autoimmune and chronic disorders.

Keywords: Thistle honey, Phenolic compounds, Biological activities, Proteolytic activity

INTRODUCTION

Functional foods, commonly denoted as nutraceuticals, are foods that reduce the risk of certain diseases and improve body functions because they contain bioactive constituents. It was found that it has the ability to boost the immune system and protect the body from free radicals, inflammation and microbes **(Mustafa *et al*., 2020)**.
Phenolic compounds are the most common plant-derived secondary metabolites consisting of one or more aromatic rings bonded to one or more hydroxyl groups **(Singh & Yadav, 2022)**. They are transmitted through nectar to honey. They are one of the most studied bioactive molecules, due to their beneficial properties biologically and environmentally for the plant and therefore for the human consumer **(Albuquerque *et al*., 2021)**. They have received attention for their ease of access, long administration, acceptability and low cost. Moreover, they have properties that make them anti-inflammatory, antioxidant, anti-microbial, anti-mutagenic, antilipidemic and anti-depressant **(Singh & Yadav, 2022)**.
Phenolic compounds have the ability to scavenge free radicals, thus reducing oxidative stress and the potential to hinder the progression of various diseases. In addition, it has the ability to reduce and prevent inflammation, this is to reduce tissue damage and pain resulting from inflammation **(Rakotondrabe *et al*., 2023)**. In the case of chronic inflammation, the inflammatory mediators are secreted for a long time, and the harmful signal transduction pathways are activated, which leads to various diseases such as allergies, cancers and arthritis **(Nunes *et al*., 2020)**. Furthermore, phenolic compounds may carry out many interactions to protect the membrane of erythrocytes from destruction. Some phenolic compounds and flavonoids have proven their effectiveness as protease inhibitors **(Culp & Wright, 2017)**. Proteases or peptidases a group of hydrolytic enzymes that play a significant role in the cell's functions of growth, adaptation, regulation, and death **(Ward, 2011)**. Moreover, protease activity has been associated with several diseases like AIDS because the human immunodeficiency virus (HIV) uses a protease enzyme to break down viral proteins into functional components for the virus to infect new cells and replicate. Protease inhibitors are used in several medicines to reduce symptoms of several diseases and stop diseases progression, such as cancers, neurodegenerative diseases, blood clotting and viral infections such as HIV **(Culp & Wright, 2017)**.
Honey is a natural sweetener produced by honeybees from the nectar of flowers and the secretions from living parts of plants. It was used in bygone eras by the Greeks, Romans, Chinese, Egyptians, and Babylonians due to its nutritional and therapeutic benefit, as they are reliable sources because they maintain their components and properties over time **(Mohamadzade Namin *et al*., 2023)**. Honey consists of sugars (about 80%), water (about 17%), and about 3% of other compounds, which include amino acids, proteins, vitamins, organic acids, minerals, phenolic and volatile compounds. Fructose and glucose are the predominant sugars in honey, with insignificant amounts of sucrose, maltose, isomaltose, maltulose and turanose. There are about 320 types of honey, they differed in the nectar of flowers which are mainly responsible for the components of honey and its characteristics of color, flavor, odor and texture **(Meo *et al*., 2017; Mohamadzade Namin *et al*., 2023).**
Thistle honey is one of the common types of honey in the Mediterranean regions. Its botanical origin is thistle plants and wild herbal plants, but it is produced in a small amount, so it is rarely obtained, due to its botanical origin. It is characterized by a slightly sour and bitter taste, medium sweetness and intense aroma. At the start of its production, it smells like fruit, then later turns into a strong animal smell. Its color ranges from extra white to dark amber **(Mouffok, 2020)**. Several scientific papers have characterized thistle honey for its phenolic, flavonoid, protein and mineral contents and the antioxidant, antibiotic, antitumor and anti-inflammatory activities **(Mouffok, 2020)**. The current study was conducted to investigate the proteolytic activity of the thistle honey extract collected from Tafilah district and to illustrate its effect on therapeutic enzymes like trypsin, chymotrypsin and papain. Additionally, total proteins, phenols, and carotenoids contents and the activities of antioxidant, anti-inflammatory, and anti-hemolytic in the extract were investigated.

MATERIAL AND METHODS

Honey extract

Thistle honey obtained from beekeepers in the Tafilah district, Jordan (Latitude 30.834586 and longitude 35.615856). The types of bees that were responsible for the thistle honey production: Carniolan, Buckfast, Kona queen and Italian honeybee. The Beehive's location is on the highest mountain of Tafila, and the months of bee pasture are from June to September 2022. The thistle honey sample was stored in the dark at 4 °C until analysis to preserve its chemical composition. A voucher sample was stored and kept at the biochemistry laboratory, Mutah

University at number H2022. Thistle honey was extracted by diluting them with distilled water in a ratio of 1:1 (w/v) at 37 °C for 90 minutes, then centrifuged at 1500 rpm for 20 min and filtered by filter paper **(Anna *et al*., 2009)**.

Honey color estimation

Pfund scale 'Pfund colorimeter' is one of the methods used to determine the color intensity of honey according to the amber scale **(Bodor *et al*., 2021)** (Table 1). This is done by measuring the absorbance of the samples at 635 nm after it has been diluted with distilled water at 50% (w/v) then mixed and centrifuged at 3200 rpm for 5 min. The Pfund was measured according to the following formula:

$$\textbf{Pfund} = -38.70 + 371.39 * \textbf{Absorbance}_{\lambda 635}$$

Table 1 Pfund scale of honey color, United States standards for grades of extracted honey. USDA, Agricultural Marketing Service.

Color 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	-

Melissopalynological analysis

Melissopalynological analysis is a microscopic analysis of bee products to determine the pollen content, thus knowing the botanical origin of the products, depending on the different pollen grains in shape, size and outer surface. Moreover, it is one of the methods used to determine authentication **(Bodó *et al*., 2020)**. The method of **Bodó *et al*. (2020)** was adopted with slight modifications for Melissopalynological. The bee products were diluted with distilled water at a ratio of 1:2 (w/v) before centrifuged at 3000 rpm for 20 min. Then the precipitate was dispersed with same amount of distilled water and centrifuged at 3000 rpm for 5 min, this was repeated twice. The sediment was taken, and three slides were prepared. Further, the slides were read and adopted regarding the Atlas of Pollen and Plant used by bees **(Cláudia *et al*., 2020)** by using a compound microscope with a total magnification of 400x, and pictures of pollen grains were taken using an Eakins camera.

Total protein content estimation

The presence and quantity of proteins can be detected by the biuret test because it is an affordable method. Bovine serum albumin (BSA) is considered as the best standard, so it was used as standard at different concentrations (0.1–1.8 mg/mL) to know the linear regression equation. Total protein was determined according to the method of **Denholm *et al*. (2021)** with slight modifications, 1.5 mL from biuret reagent was added on 0.5 mL from extract which was diluted with distilled water in a ratio of 1:10 (v/v) at 37 °C for 15 min. The absorbance was measured using a spectrophotometer (Biotech Engineering Management CO. LTD.; UK) at 540 nm.

Total phenol content estimation

To determine the total phenolic content (TPC) of the extract, the Folin-Ciocalteu (F-C) method was used. It is a colorimetric test that depends on the use of F-C reagent. Gallic acid is considered as the standard, so it was used at different concentrations.
Total phenols were determined according to **Khatri & Chhetri (2020)** method with slight modifications, 0.5 mL of the F-C reagent was added to 0.1 mL of different concentrations from extract and reaction mixture kept for five minutes at room temperature, then 2.5 mL of sodium carbonate (Na_2CO_3) was added. The reaction mixture was incubated in a dark condition for 20 minutes. The change in color is measured by a spectrophotometer at 765 nm.

Carotenoid content estimation

Carotenoids are natural pigments produced by photosynthetic and non-photosynthetic organisms, and they give several plants flavor, aroma, and bright colors. The carotenoid content (β-carotene and lycopene) was determined according to the method described by **Hunter *et al*. (2021)**. The bee products were diluted with distilled water by 50% (w/v), 10 mL hexane-acetone mixture 6:4 (v/v) was added to 2.0 mL of the solution, mixed for ten minutes, and the mixture was filtered. The absorbance was measured against deionized water (DI) by a spectrophotometer at 663 nm, 505 nm and 453 nm. The concentrations of β-carotene and lycopene were calculated according to the following formulas:

$$\textbf{β-Carotene (mg/100mL)} = 0.216 * A_{\lambda 663} - 0.304 * A_{\lambda 505} + 0.452 * A_{\lambda 453}$$

$$\textbf{Lycopene (mg/100mL)} = 0.0458 * A_{\lambda 663} - 0.372 * A_{\lambda 505} + 0.452 * A_{\lambda 453}$$

Where A is the absorbance.

Antioxidant activity assay

2,2-Diphenyl-1-picrylhydrazylradical (DPPH) radical scavenging activity was used to determine the ability of the extract to scavenge free radicals. DPPH radical scavenging activity was measured according to **Baliyan *et al*. (2022)**, in which, 1.0 mL of DPPH solution (60 μM in methanol) was added to 1 mL of different concentrations of extract, and the reaction mixture was incubated in a dark condition for half an hour. The absorption value was measured at 515 nm by spectrophotometer. The linear regression equation is based on gallic acid which was used as standard and DPPH solution was used as a control. The percentage of antioxidant activity was measured according to the following formula:
Percentage of Antioxidant Activity (%) = (Ac – As / Ac) * 100%, Where Ac is control absorbance, As is sample absorbance.

Inhibition concentration estimation (IC_{50})

It is a measure that indicates how effective a substance is in inhibiting a specific biochemical or biological activity by half. A IC_{50} Calculator/AAT Bioquest is a special calculator that was used to calculate IC_{50} extracts online (https://www.aatbio.com/tools/ic50-calculator).

Anti-inflammatory activity assay

Anti-inflammatory activity was determined according to the method of **Borah *et al*. (2019)** with slight modifications, the test solution contained 0.45 mL of BSA and 0.05 mL of extract with different concentration, the control test contained distilled water instead of extract, but the product control contained distilled water instead of BSA. These solutions were incubated at 37 °C for 20 min and then at 70 °C for 10 min. Before reading the absorbance at 660 nm, 2.5 mL from phosphate buffer was added. Diclofenac, a non-steroidal anti-inflammatory drug was used in different concentrations to find the linear regression equation. The percentage of anti-inflammatory activity was measured according to the following formula:
Percentage of Anti-inflammatory Activity (%) = 100 – (AT – (AP / AC)) * 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 hydrogen peroxide (H_2O_2) method was used to determine the anti-hemolytic activity. Blood samples were collected by EDTA (Ethylene Diamine Tetra acetic acid) tubes. The samples were centrifuged at 1500 rpm for 5 min and washed three times with normal saline. The blood samples were re-suspended with normal saline at 50% (v/v), according to the method used by **Kumari *et al*. (2015)** with slight modifications. The reaction mixture contained 0.5 mL of RBCs, 0.125 mL of extract and 0.625 mL of normal saline 0.25 mL. The negative control contained 0.5 mL of RBC and 0.75 mL of normal saline, but the positive control contained 0.625 of distilled water instead of normal saline. All tubes were incubated at 37 °C for 10 min. 0.125 mL of H_2O_2 was added to sample test and control tubes, then they were incubated at room temperature for 2 hours before they centrifuged at 3000 rpm for 10 min. The absorption value was measured at 590 nm by spectrophotometer. The percentage of anti-hemolytic activity was measured by the following formula:

Percentage of Anti-hemolytic Activity (%) = (AP – ((AS - AC) / AP) * 100,

Where AP is the control positive absorbance; AC is the control negative absorbance and AS is the sample absorbance.

Enzyme assay

Different concentrations of casein were used to determine Michaelis constant (Km) and maximum rate of the reaction (Vmax) using Lineweaver-Burk plot. The reaction mixture contained 0.5 mL of casein, 0.25 mL of extract and 0.25 of enzyme, and was incubated at 37 °C for 30 minutes. 0.5 mL of TCA was added to the mixture and then centrifuged at 3000 rpm for 10 min. 0.25 mL of the supernatant was taken and 1.0 mL of sodium carbonate (Na_2CO_3) and 0.2 mL of Folin-Ciocalteu reagent were added. It was incubated at 37 °C for 20 minutes, then the absorbance is measured at 750 nm **(Afoshin *et al*., 2023).**

Statistical analysis

The results were expressed as the mean ± standard deviation from triplicate analysis. Microsoft Excel version 365 was used to analyze results, draw curves and find correlations.

RESULTS

Honey color

The Pfund value for the thistle honey sample was 15.894 mm, so the color is extra white regarding to the Pfund of honey color of United States standards for grades of extracted honey.

Botanical origin of Bee products

The existence of pollen grains in honey can be used as a crucial indicator of authenticity. There were eleven varieties of pollen grain found in thistle honey. Figure 1 shows some pollen grains on microscopic slides that are used for plant identification. The atlas of pollen and plants used by bees was used for species identification **(Cláudia *et al.*, 2020)**, the percentage of each plant species is illustrated in Figure 2.

Figure 1 Melissopalynological analysis of thistle honey (400x the total magnification). a) *Porter weeds sp.*, b) *Cynara sp.*, c) *Cotton thistles sp.*, d) *Cirsium vulgare*, e) *Silybum marianum* and f) *Galactites sp.*.

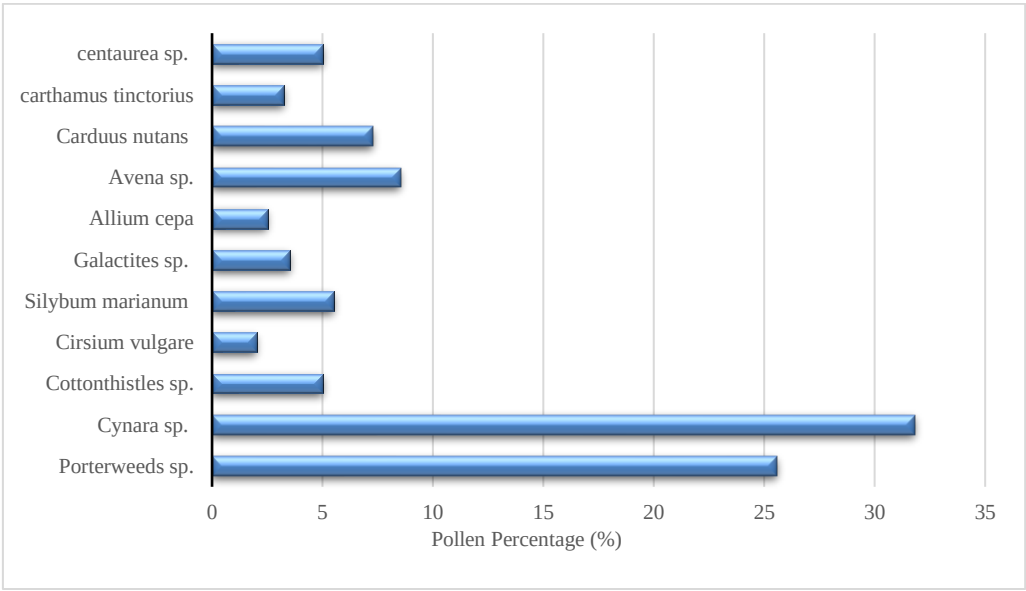

Figure 2 Relative occurrence (%) of plant species in the thistle honey.

Protein content

The protein content was determined by Biuret method and measured using BSA as the standard. According to the findings, thistle honey extract contains a significant quantity of protein. The protein content of thistle honey extract was 7.124 ± 0.006 mg/mL (Table 2).

Total phenols

The phenol content was quantified by employing gallic acid as the standard. Based on the results, it was observed that thistle honey extract possesses a considerable amount of phenol, as indicated by the following value 0.739 ± 0.002 mg/mL (Table 2). IC_{50} Regression results of total phenol and biological activity for samples are illustrated in Figure 4.

Carotenoid content

The β-carotene and lycopene value (mg/Kg) of thistle honey extract was 2.892 and 17.901, respectively (Table 2).

Table 2 Approximate contents of extraction yield, total proteins, and total phenolic contents

Extract	Total protein (mg/mL)	Total Phenols (mg/mL)	Carotenoids (mg/Kg)	Lycopene (mg/Kg)
Thistle honey	7.124 ± 0.006	0.739 ± 0.002	2.892± 0.004	17.901± 0.323

Mean ±SD, n=3.

Biological activities

In the in *vitro* assay, the extract had the potential to be antioxidant by using the DPPH radical scavenging method. Gallic acid was used as the standard. The percentage of antioxidant activity obtained demonstrates that thistle honey extract has 72.330% ± 0.195 (Figure 3) with IC_{50} values 0.3662 (µg/mL) (Figure 4).

In the in *vitro* assay, diclofenac was used as the standard to measure the percentage of anti-inflammatory. The extract had the potential to be anti-inflammatory. The percentage of anti-inflammatory activity of thistle honey extract has 54.422% ± 0.271 (Figure 3) with IC_{50} values 0.3785 (µg/mL) (Figure 4).
In the in *vitro* assay, the extract had the potential to be anti-hemolytic. Our findings illustrate that thistle honey extract was 63.599% ± 0.233 (Figure 3) with IC_{50} values 0.0559 (µg/mL) (Figure 4).

Correlation between total phenol and biological activities

The assessment was conducted on the linear correlation between TPC and diverse biological activities, which exhibited a prominent and affirmative correlation between the variables as illustrated in Table 3.

Protease activity

The investigation was conducted to ascertain the proteolytic activity of extract on casein and the influence of the thistle honey extract on the kinetic parameters of trypsin, chymotrypsin and papain in the presence and absence of the extract. The initial reaction rates were assessed at varying concentrations of the substrate casein (0.0025 – 0.02 mM). The thistle honey extract displayed significant activity on casein, with a catalytic activity (kat) value of (0.0091 mol.min^{-}1).

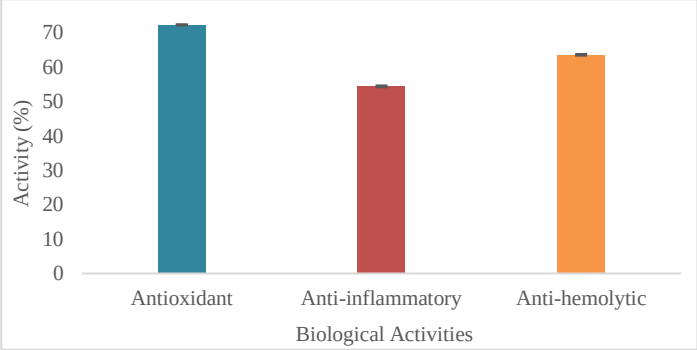

Figure 3 The biological activities (%) of thistle honey extract. Mean ±SD, n=3.

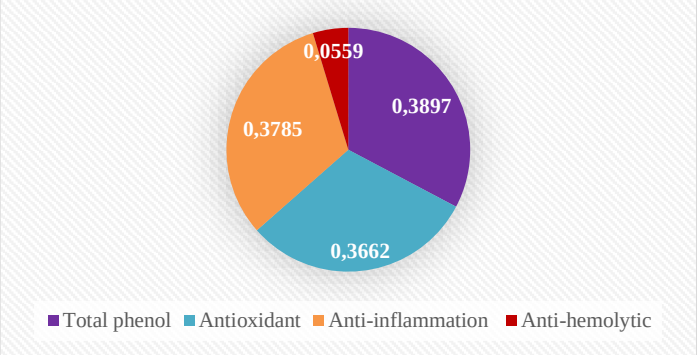

Figure 4 IC_{50} values (µg/mL) of Total phenol, Antioxidant, Anti-inflammatory and Anti-hemolytic.

Table 3 The linear correlation between total phenolic content (TPC) and various biological activity assays.

	TPC	DPPH	Anti-inflammatory	Anti-hemolytic
TPC	1			
DPPH	0.973	1		
Anti-inflammatory	0.999	0.989	1	
Anti-hemolytic	0.929	0.974	0.931	1

Thistle honey extract contained activators for the three enzymes, as Vmax increased in the presence of the extract (Figure 5,6,7). A summary of the enzymes' Km and Vmax values is available in Table 4.

DISCUSSION

The thistle honey produced by the honeybees in Tafilah district has high nutritional value and contains effective biomolecules that exert its effects on the selected biological activities, so, they can be used in the pharmaceutical industry. Previous report illustrated that honey has a role in medicines production and is considered apitherapeutic agents for many diseases by strengthening the immune system.
The importance of using Pfund as a comprehensive method to evaluate the quality and origin of honey. Our findings of the thistle honey had extra white color with a Pfund value of 15.89 mm. **Mouffok (2020)** reported that thistle honey varies in color from extra white to dark amber, which is consistent with our results. Further, the thistle honey exhibited the pronounced floral and fruity aromas, and this is corroborated with **Bambina *et al*. (2023)**.

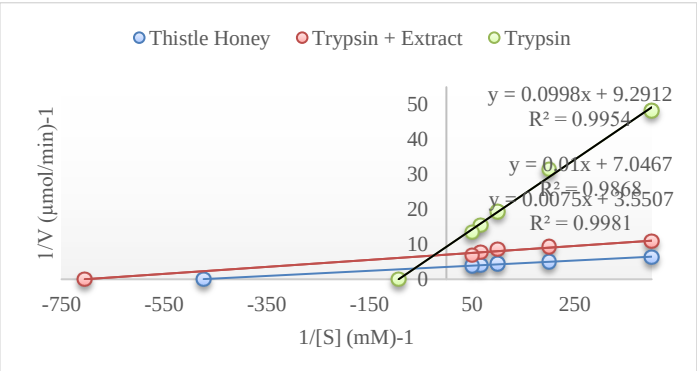

Figure 5. Lineweaver-Burk reciprocal plot for the determination of Km and Vmax values for the trypsin enzyme in the presence and absence of thistle honey extract. Mean ± SD, n = 3.

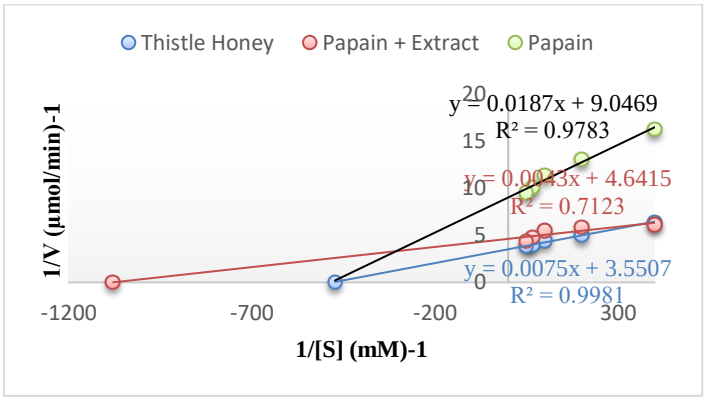

Figure 6 Lineweaver-Burk reciprocal plot for the determination of Km and Vmax values for the papain enzyme in the presence and absence of thistle honey extract. Mean ± SD, n = 3.

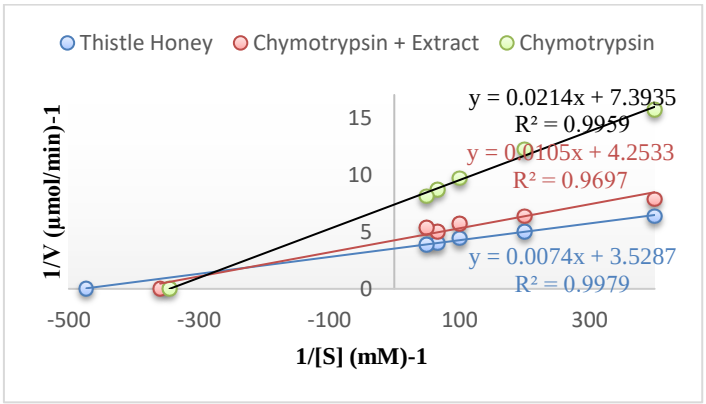

Figure 7 Lineweaver-Burk reciprocal plot for the determination of Km and Vmax values for the chymotrypsin enzyme in the presence and absence of thistle honey extract. Mean ± SD, n = 3.

Table 4 A summary of the proteolytic activity of thistle honey extract and kinetic parameters values associated with trypsin, chymotrypsin, and papain enzymes.

	Vmax	Km
Extract	0.278	0.002
Trypsin	0.1076	0.0107
Trypsin with extract	0.1419	0.0014
Chymotrypsin	0.1353	0.0029
Chymotrypsin with extract	0.2613	0.0036
Papain	0.1106	0.0021
Papain with extract	0.2156	0.0009

Vmax: µmol/min^{-1}, Km: mM^{-1}

Honey may originate from a single plant species, known as unifloral, or from multiple plant species, hence referred to as multifloral **(Scripcă & Amariei, 2021)**. Results of pollen identification revealed a diverse of plant species, which reflected on its other characteristics, and its botanical origin was found the thistle plants like *Cirsium vulgare* and *Silybum marianum*. Melissopalynology is a method to determine the botanical and geographical origin of honey and the extent of the honey's credibility **(Bodó *et al*., 2020)**. According to the results, honey is highly dependable because it contains pollen belonging to thistle plant and this is consistent with the results of the Pfund examination. *Cynara sp.* and *Porterweeds sp.* had the highest presence in the honey, at more than 50%, and this may be an indication that the benefits and activities of honey are attributed to the previous two plant species.
Carotenoids, pivotal plant metabolites, aid in growth and survival by supporting photosynthesis and acting as free radical scavengers. Their advantages transcend plants, impacting human health significantly. Serving as a precursor to vitamin A, they support immune, reproductive, and visual functions. Moreover, their antioxidant properties potentially lower the risk of cardiovascular diseases, eye diseases, and certain cancers **(Hermanns *et al*., 2020)**. Carotenoids are a valuable indicator of honey sources and potential health benefits, making knowing their content a key aspect of our analysis. Our findings show that thistle honey extract has a high content of carotenoids, and this is consistent with the results of **Özcan & Uslu's (2023)** study. The presence of these compounds gives honey many properties, as they are considered antioxidant and anti-inflammatory agents and promote healthy eyes, skin, and immune system **(Smetanska *et al*., 2021)**.
The protein content of the extract of sample was high amount. Proteins are one of the essential nutrients for the body, as they are one of its components and are

responsible for many physiological processes like enzymatic reactions and signal transduction **(Rivero Meza *et al*., 2023)**. Therefore, the protein content gives bee products a high nutritional value.

Phenolic compounds play a significant role in protecting human health and their main source is the secondary metabolites in plants. In the context of honey analysis, understanding the phenolic content is of paramount importance to gain insights into the role of phenolic compounds in honey's overall composition and its potential contributions to its nutritional and therapeutic value. The thistle honey extract had a high amount of phenolic compounds with low IC_{50} value. The effectiveness of a substance can be determined by its IC_{50} value, where a lower value indicates a higher efficacy **(Borah *et al*. 2019)**.

The DPPH assay is a pivotal tool for assessing the antioxidant capacity of honey, which is essential for both quality evaluation and understanding its potential health benefits. In the present study, it was observed that thistle honey extract exhibited high antioxidant activity, as demonstrated by its higher capacity for scavenging the DPPH radical with a value 72.33% and it had 0.3662 µg/value. Therefore, low concentrations of thistle honey contain high phenolic compounds with high antioxidant activity. Thistle honey has been shown to possess potent antioxidant activity (16.57 ± 0.05 µmol TE/100g), so it neutralizes free radicals and prevents oxidative damage to cells **(Bambina *et al*., 2023)**.

In addition to its ability to scavenge free radicals, thistle honey extract's inherent anti-inflammatory properties may also lead to a reduction in inflammation. The study of honey's anti-inflammatory ability holds substantial implications for both its therapeutic potential and its place in the realm of natural remedies, making it an essential aspect of our investigation. As per the findings of the present study, the extract of thistle honey demonstrated a moderate anti-inflammatory activity with 54.422%. Flavonoids such as chrysin, apigenin, and kaempferol can reduce inflammation and its symptoms if they are used in the manufacture of anti-inflammatory drugs. Various honey types contain flavonoids and thus acquire therapeutic properties **(Kurek-Górecka *et al*., 2020)**.

The evaluation of anti-hemolytic properties in natural products, particularly in honey, is essential in understanding its potential therapeutic applications. The study revealed that thistle honey extract had 63.599% anti-hemolytic activity, as evidenced by the inhibition of RBCs hemolysis. The result indicated that the extract was highly effective in preventing the breakdown of erythrocytes. This is because the extracts contain antioxidants that scavenge free radicals that may target the erythrocyte membrane as illustrated **Ousaaid *et al*. (2022)**. **Graça Miguel (2022)** asserted various honey types possess factors that can prevent the degradation of erythrocytes.

A strong correlation was observed between the total phenolic content (TPC) and biological activities of thistle honey extract. The Pearson correlation coefficient values are high as shown in table 3. By assessing the phenolic composition and conducting a comprehensive analysis of biological activities, we aim to elucidate the extent to which phenolic compounds contribute to the health-related attributes of honey, the reason is that phenolic compounds have many benefits to enhance human health and immune system. So, thistle honey extract possesses biologically active molecules, which can be used in the treatment of many diseases such as immune and neurological diseases and cancer **(Jiang *et al*., 2021)**.

The parameters used to describe enzyme kinetic include Vmax, which is the rate at which an enzyme-catalyzed reaction occurs when the enzyme is saturated with substrate, and Km, which is the substrate concentration at which the enzyme activity is half of its maximum. A high ratio of Vmax/Km indicates that the enzyme has a higher catalytic efficiency, which means that it is more efficient at converting substrate into product **(Liu *et al*., 2022).**

Casein was used as the substrate to evaluate the proteases activity of thistle honey extract. The extract contained proteases enzymes. Therefore, the extract has proteolytic activity, and this is due to their content of protease enzymes. A group of studies showed that honey possesses proteolytic activity **(Darwish *et al*., 2023)**. Because of the benefits of proteases enzymes for the body, honey samples are a reliable source for helping physiological processes in the human body, such as: apoptosis, protein turnover and cell signaling **(Bond, 2019)**. Furthermore, thistle honey extract was used to study its effect on the kinetic parameters of therapeutic enzymes like trypsin, chymotrypsin and papain. All the enzymes had a proteolytic activity on casein, and it was found that papain had the highest activity. In the presence of the extract of thistle honey the value of Vmax increased, and this indicates that this extract contained activator for the three enzymes. So, this extract contains compounds that increase the effectiveness and activity of enzymes **(An *et al*., 2020)**.

CONCLUSION

Function food, like honey, has a high nutritional value and is targeted by consumers, but attention is paid to the color and flavor of the products to ensure their credibility. Experiments were conducted to describe some of the physical and chemical properties of this product and the ability of their compounds to perform several biological activities. Thistle honey from Tafilah district has been found to have numerous potential health benefits due to their rich composition of nutrients, including proteins, phenolic and carotenoids. Studies have shown that thistle honey has antioxidants, anti-inflammatory and anti-hemolytic agents. Experiments revealed that the thistle honey has a proteolytic activity. All these characteristics support the idea that honey is one of the sources for disease prevention and strengthening human immunity.

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Authors' contributions: Prof. Omar Atrooz designed, supervised, and structured the project. Enas A. Al-Zeidaneen performed all the experiments related to biological activities. Prof. Omar Atrooz performed the data analysis. All authors drafted the paper together. All authors read and approved the final manuscript.

Conflict of Interest: The authors declare that there is no conflict of interest.

Compliance with Ethical Standards: This article does not contain any studies involving human or animal subjects.

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