Evaluation of Chemical Composition and Biological Activity of Bee Pollen

Muna A. Al-nimat[1], Enas A. Al-Zeidaneen[2], Omar M. Atrooz[3] *, Ansam O. Atrooz[4]

[1]Department of Chemistry, College of Science, Al-Hussein Bin Talal University, Ma'an, Jordan. E-mail: muna.a.alnimat@ahu.edu.jo. Orcid ID: 0009-0006-9687-730X.
[2]Department of Applied Biology, Faculty of Science, Tafilah Technical University, Tafilah, Jordan. E-mail: enas.13264@gmail.com. Orcid ID: 0009-0002-7200-6607.
[3]Department of Biological Sciences, Mutah University. Mutah, Jordan. E-mail: omihandd@gmail.com. Orcid ID: 0000-0003-0610-5201
[4]Department of Pediatrics, Faculty of Medicine, Mutah University, Al Karak, Jordan.
E-mail: ansamatrooz@mutah.edu.jo. Orcid ID: 0009-0001-8458-9603

Abstract
Bee pollen is a promising natural product with therapeutic potential, yet its chemical-bioactivity relationship remains underexplored. This study aimed to assess the chemical composition and *in vitro* biological activities of bee pollen and examine their relationship to phenolic content. A bee pollen sample collected from the Tafilah region of Jordan was analyzed for its phenolic, protein, and carotenoid content. Its botanical origin was identified using Melissopalynological analysis. Antioxidant, anti-inflammatory, and anti-hemolytic activities were evaluated using standard *in vitro* assays. The sample contained 0.581 mg/mL phenols, 7.939 mg/mL proteins, 138.3 mg/kg β-carotene, and 643.0 mg/kg lycopene. Melissopalynological analysis revealed a wide variety of botanical sources. The extract demonstrated antioxidant, anti-inflammatory, and anti-hemolytic activities of 48.7%, 56.74%, and 71.24%, respectively. A positive correlation was observed between total phenolic content and biological activities. This finding validates the fact that bee pollen is biologically active with great biological potential related to phenolic content and therefore has potential as apitherapeutic agent and potential development of functional natural products.

Keywords: Antioxidant activity, Apitherapy, Bee Pollen, Biological Activity, Phenolic contents

Introduction

Apitherapy refers to a class of alternative medicine that treats a wide range of illnesses by using various bee products. The rise in autoimmune disorders, cancer cases, and chronic illnesses prompted researchers to look for novel approaches based on natural therapeutic items that are highly effective, safe, and will not promote microbial resistance (Bava et al., 2023).

The angiosperm anther produces tiny particles known as pollen grains. Using the comb and the hair on their legs, the bees gather them, and then they add enzymes and secretions to them to create bee pollen. Bee-collected pollen (BP), sometimes referred to as apicultural or corbicular pollen, is a highly nutritious food source since it includes lipids, proteins, and other elements that are essential for honeybee health. The bee colony harvests between 50 and 250 g daily, or 15 to 40 kg annually. Pollen can have a variety of hues, including white, yellow, orange, red, gray, green, or brown, depending on the type of plant, bee behavior, location, weather, and soil type. These differences impact the chemical, physical, nutritional, and functional composition of the pollen (Thakur & Nanda, 2020; Algethami et al., 2022). Because BP is regarded as a nutritious and beneficial supplement, the ancient Egyptians called it "the life-giving dust" (Khalifa et al., 2021). Bee pollen is composed of 13–55 percent carbohydrates, 10–40% proteins, 1–13 percent lipids, 0.3–20 percent crude fiber, and 2–6% ash. Furthermore, it contains minerals, vitamins (mostly B-complex), carotenoids, phenols, and flavonoids in addition to all essential fatty and amino acids (Thakur & Nanda, 2020). Depending on the type of plant, the percentage of protein varies, but all essential amino acids, including tryptophan, leucine, lysine, methionine, and phenylalanine, are present. These amino acids are necessary for human health and growth because they engage in the processes of gene expression, digestion, and nutrient absorption (Khalifa et al., 2021).

Many benefits, including anti-inflammatory, antioxidant, anti-microbial, anti-allergic, anti-aging, anti-diarrheal, anti-carcinogenic, and anti-atherosclerosis properties, are demonstrated by bee pollen. Furthermore, according to Abdelnour et al. (2019), it enhances immunity, wound healing, fertility, and the health of the liver, kidney, gut, muscles, skin, and prostate. Due to its high vitamin and mineral content which the ancient Chinese employed for cosmetic purposes it is utilized as a natural product in the cosmetics. It brightens, hydrates, and shields the skin against aging, dryness, UV radiation, and inflammatory substances that harm human skin (Kebede et al., 2024).

This research was aimed at determining the nutritional, chemical and biological characteristics of a bee pollen extract in terms of total protein, total phenolic content, total carotenoids and the antioxidant, anti-inflammatory, and anti-hemolytic properties. Additionally, the correlation between the total phenolic content and biological activity was studied.

Materials and Methods

Bee pollen extract

A sample bee pollen was collected from honeybees whose colonies are dispersed over Tafilah region. The following bee species were used to gather the sample in the spring: Buckfast, Italian honeybees, Kona queen, and Carniola. It was extracted three times separately by adding 10.0 g of BP to 75.0 mL of methanol and letting it sit at room temperature for an hour (Mărghitaş et al., 2009). Following that, the sample was centrifuged for 10 min, 3500 rpm and the sample filtered with filter paper. After removal of the supernatant by evaporation, the yield (%) (w/w) was calculated by the following formula (Al-Zeidaneen and Atrooz, 2025):

Extract recovery = (A/B) *100%, where A is the extract's weight after evaporation and B is the sample's weight before evaporation.

Melissopalynological investigation

Through interpretation of the properties of the pollens, Melissopalynological analysis can recognize pollen in a sample of bee pollen and establish its botanical source. The sample was evaluated based on the methodology by Bodó et al. (2020). Part of the procedure was done using the sample diluted with distilled water in ratio of 2:1 (w/v). The precipitate which was obtained upon centrifuge twice of the diluted sample in 20 min at 3000 rpm in a centrifuge (B. Hermle, Germany) was deposited on 3 slides. The slides were examined under a 400x compound microscope. Identification of pollen is conducted according to the Atlas of Pollen and Plant utilized by the bees utilizing an Eakins camera in order to photograph the pollen (Cláudia et al., 2020).

Carotenoid content assessment

Natural pigments and carotenoids provide the plant with aroma, flavor, and color. Carotenoid composition (β-carotene and lycopene) was determined based on Hunter et al. method (2021). Bee pollen diluted 50% (w/v) was shaken with 10 mL hexane-acetone (6:4 v/v) for 10 min, after which the mixture was filtered. In comparison to deionized water, the absorbance's' at 453 nm, 505 nm, and 663 nm were measured. The following equations were validated for the calculations of the concentrations of lycopene and β-carotene:

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

Lycopene (mg/100 mL) = $0.0458 * Abs_{663} - 0.372 * Abs_{505} + 0.452 * Abs_{453}$, where Abs is the absorbance.

Total protein content assessment

Quantification of the protein content of the BP extract was achieved utilizing the biuret assay. Bovine serum albumin (BSA) was used as a standard at concentrations ranging from 0.1 to 1.8 mg/mL to attempt to produce a linear regression equation. Denholm et al. (2021) method, with minor adjustments, was used to quantify the total protein: 1.5 mL biuret reagent was combined with 0.5 mL 1:10 (v/v) diluted extract in distilled water. This mixture was incubated for 15 min at 37 °C, and 540 nm absorbance was quantified by a spectrophotometer (Biotech Engineering Management Company, UK).

Total phenolic content assessment

The total phenolic content (TPC) was determined based on gallic acid as a standard and Folin-Ciocalteu (F-C) reagent to produce the value of TPC in the extract. By making slight modifications, Khatri & Chhetri (2020) methodology was employed in calculating total phenolic content: 0.5 mL F-C reagent was added to 0.1 mL of extract at given concentrations and reacted with it with 5 min standing period at ambient temperature followed by addition of 2.5 mL sodium carbonate (Na_2CO_3). The absorbance of the reaction mixture at 765 nm was measured after 20 min of incubation in darkness.

Antioxidant activity assay

To determine the resistance and elimination of free radicals of the extract, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity test was used. According to the procedure described by Baliyan et al. (2022), 1 mL of a DPPH solution (1349 μM in methanol) was added to 1 mL of extract in different concentrations. The reaction mixture obtained was allowed to incubate in the dark in darkness, which lasted half an hour. An absorbance was then measured at 517 nm. The calculation of the ratio involved the use of DPPH solution as a control. The following equation was used to determine the antioxidant activity percentage:

Percentage of Antioxidant Activity (%) = $(C_{Abs} - S_{Abs} / C_{Abs}) * 100\%$, Where C_{Abs} is the absorbance of the control, S_{Abs} is the absorbance of the extract.

Inhibition concentration assessment

Inhibition concentration (IC_{50}) is a scale utilized in pharmacology, and other medication studies, in order to mark the level at which a medication confines biological or biochemical action by 50%. The IC_{50} calculator provided by the AAT Bioquest platform (https://www.aatbio.com/tools/ic50-calculator) is one of the tools that are used to calculate the IC_{50} values with precision and within a short time.

Anti-inflammatory activity assay

The anti-inflammatory activity was quantified using the Borah et al. (2019) method. 0.05 mL of extract in varying concentrations and 0.45 mL of BSA formed the test solution. While for the product control test, distilled water was employed as a substitute for BSA, in the control test, it was substituted for the extract. The solutions were incubated for 20 min at 37°C followed by 10 min at 70°C. Then, 2.5 mL of phosphate buffer was added and

absorbance at 660 nm was measured. A non-steroidal anti-inflammatory drug diclofenac was used to prepare a linear regression equation. It was intended to calculate the percentage of anti-inflammatory activity using the following equation:
Percentage of Anti-inflammatory Activity (%) = 100 – (T_{Abs} – (P_{Abs} / C_{Abs})) * 100, Where T_{Abs} is the absorbance of test solution; P_{Abs} is the absorbance product control and C_{Abs} is the absorbance test control.

Anti-hemolytic Activity assay
Hydrogen peroxide (H_2O_2) method was used to assess anti-hemolytic activity of the extract. Blood samples were drawn into ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged and washed three times with normal saline. Thereafter, the samples were re-suspended in 50% (v/v) normal saline. Samples were then suspended once more in 50 % (v/v) normal saline. The reaction mixture is a 0.5 mL RBCs, 0.125 mL extract, and 0.625 mL normal saline. The negative control is 0.5 mL of RBCs and 0.75 mL normal saline; the positive control is 0.5 mL RBCs + 0.625 mL distilled water. All the tubes were incubated at 37°C for 10 min. The mixture was placed at room temperature after 0.125 mL of H_2O_2 was added and the mixture was incubated for two hours at room temperature. The absorbance was measured after centrifuging the samples to 10 min at 3000 rpm at 590 nm (Rahmouni et al., 2020). The following equation was used to determine the anti-hemolytic activity percentage:
Anti-hemolytic activity percentage (%)= (P_{Abs} - ((S_{Abs} - C_{Abs}) / P_{Abs}) * 100, In this case, the sample absorbance is S_{Abs}, the control negative absorbance is C_{Abs}, and the control positive absorbance is P_{Abs}.

Statistical Analysis
The outcomes of the data were presented in the form of the mean value of the triple analysis and the standard deviation. Microsoft Excel Version 365 was applied to process and interpret the results and find the relationship between biological activities and phenolic content relying on Pearson correlation coefficient.

Results
Botanical origin of bee pollen
There were fourteen distinct kinds of pollen in the sample collected from bees. The botanical origin of the sample is revealed by examining microscopic images of a few of these grains as illustrated in Fig.1. Using an atlas of pollen and plants that are commonly used for identification, the species of plants were identified (Cláudia et al., 2020). Table 1 provides percentage of various plant species in bee pollen sample.

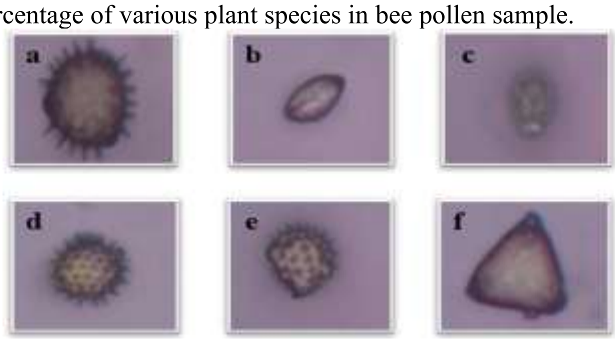

Figure 1. Bee pollen Melissopalynological investigation (400x the total magnification). a) *Helianthus annuus*, b) *Chaenomeles japonica*, c) *Syzygium cumini*, d) *Cereus hexagonus*, e) *Daisies sp.* and f) *Ziziphus spina-christi.*

Bioactive molecules content and extract recovery
According to the results of the Biuret method, bee pollen has a considerable amount of protein (7.939 ± 0.135 mg/mL). By using the Folin-Ciocalteu (F-C) technique, the extract also showed a significant phenol content of 0.581 ± 0.092 mg/mL. Furthermore, the mg/kg levels of lycopene and β-carotene were 643.000 ± 8.967 and 138.300 ± 3.124, respectively. Further, 35% (w/w) of the bee pollen was yielded from extraction using a methanolic extract (Table 2).

Table 1. The prevalence of various plant species in bee pollen, expressed as a percentage.

Plant pollen species (%)			
Cenizos pazotes	3.5%	*Helianthus annuus*	17.8%
Cereus hexagonus	2.3%	*Medicago sp.*	11.4%
Chaenomeles japonica	12.4%	*Olea europaea*	8.6%
Chrysanthemums	4.1%	*Rubus sp.*	1.6%
Cistus sp.	13.0%	*Scoprpiurus muricatus*	5.1%
Cynara scolymus	6.9%	*Syzygium cumini*	2.4%
Daisies sp.	8.0%	*Ziziphus spina-christi*	3.0%

Table 2. Approximate contents of extraction yield and active compounds contents in the bee pollen extract.

Bioactive molecules	Content

Total Protein (mg/mL)	7.939 ± 0.135
TPC (mg/mL)	0.581 ± 0.092
Carotenoids (mg/Kg)	138.300 ± 3.124
Lycopene (mg/Kg)	643.000 ± 8.967
Extract Recovery (%)	35.000

Mean ±SD, n=3.

Biological activities

In vitro tests were conducted to assess the biological capacity of bee pollen extract. The results showed that the extract had an IC_{50} value of 0.6104 (µg/mL) and an antioxidant potential of 48.700% ± 0.155. The anti-inflammatory percentage was 56.730% ± 0.112, and the IC_{50} value was 0.8311 (µg/mL). Furthermore, the extract demonstrated anti-hemolytic potential with an IC_{50} value of 0.0516 (µg/mL) and a percentage of 71.240% ± 0.201 (Table 3). Further, the biological activities were correlated with TPC, a significant positive association was found, as Table 4 illustrates.

Table 3. The biological activities (%) and IC_{50} (µg/mL) for the bee pollen extract.

Biological activity	**Bee Pollen**	
Antioxidant	Activity (%)	48.700 ± 0.155
	IC_{50} (µg/mL)	0.6104
Anti-inflammatory	Activity (%)	56.730 ± 0.112
	IC_{50} (µg/mL)	0.8311
Anti-hemolytic	Activity (%)	71.240 ± 0.201
	IC_{50} (µg/mL)	0.0516

Mean ±SD, n=3.

Table 4. Pearson correlation coefficient between TPC and various biological activity assays.

	TPC	Antioxidant	Anti-inflammatory	Anti-inflammatory
TPC	1			
Antioxidant	0.763	1		
Anti-inflammatory	0.861	0.955	1	
Anti-hemolytic	0.738	0.980	0.917	1

Discussion

Bee pollen that has been harvested from Jordan's Tafilah region is incredibly nutritious and rich in active biomolecules. Because of these qualities, it is a good contender and ingredient to use in pharmaceutical formulations. That is why contemporary medicine finds it useful in therapy and Scientists are interested in natural resources because of the rise in illnesses and injuries.

Examining the pollen sample's botanical origin under a microscope showed that a wide variety of pollen grains, both in size and form, were present, indicating that the sample came from a variety of plant species. The sample's physical, chemical, and biological characteristics are impacted by this enormous diversity. By identifying botanical and thus the geographic origin, this is an excellent way of establishing the credibility and authenticity of honey. The finding, in full concurrence with the data gathered from the beekeepers, showed that pollen from these bees is from plants growing in the Tafilah region and which flower in spring. It has *Medicago sp.*, *Helianthus annuus*, *Cistus sp.*, and *Chaenomeles japonica* pollen. These results are consistent with those reported by Borel et al. (2020).

The sample of bee pollen in this study was characterized by its high carotenoid levels that were detected through the content of β-carotene and lycopene (mg/kg), which were 138.3 and 643 mg/kg, respectively. This information was useful in the nutritional value of this natural product. On close examination of the carotenoids, whose famous antioxidant capacity is made more or less equivalent to its potential health gain, the importance of bee pollen as a dietary supplement is well-defined. According to our research, bee pollen is high in carotenoids, which constitutes the findings in the research by Çobanoğlu et al. (2023). Carotenoids are among the most significant plant pigments that present their consumers with multiple benefits to their health alongside an immunity against a complete list of diseases that include cancer, heart disease, and immune system complications (Li et al., 2024). The results indicate that they could be a significant carotenoids food source applicable to human diets and increase information concerning the bee pollen bioactive compounds. Conduction of additional research on this aspect may allow future use of the bee pollen in the health and nutrition sectors as this would dictate the particular health effects and applications of such compounds.

The sample extract protein level is determined by 7.939 mg/mL, which specifies that the bee pollen is nutritious. Being rich in protein, this fact proves that bee pollen can be a source of amino acids and nutrients. Bee pollen also has protein content, which agrees with the results yielded by the research conducted by Alshallash et al. (2023). The findings contributed to the existing knowledge about the nutritional content of bee pollen and its use as a food supplement. Both qualitative and quantitative analysis of its amino acid content is being investigated to have more complete understanding of its application in human nutrition and health.

A clue to the phenolic content of the extract and its therapeutic value can be derived from quantification of the phenolic content. Phenols were measured, and the content was determined to be 0.581 mg/mL, and this result agrees with Rojo et al. (2023) study. Phenolic compounds have uses in an extensive range of industries and are also reported to have an extremely wide diversity and specialty functions. They also play a central role in ensuring human health. It has a wonderful capability of lessening oxidative stress, improving the symptoms of associated diseases, and preventing numerous diseases. Since it has medicinal qualities, scientists are giving it attention in an attempt to use it to their advantage and include it in the production of drugs and pharmaceuticals.

DPPH procedure was utilized to measure the antioxidant activity of the extract since the free radical scavenging and suppression capability of an extract can be measured using it. That the bee pollen has higher antioxidant activity concurs with the findings of Rojo et al. (2023). Anti-inflammatory activity and percentage analysis of the extract indicated that the extract was determined to be highly effective, and the level of activity noted was 56.738%. The result of the anti-inflammatory activity of bee pollen is in line with Zhang et al., (2023) study. Because inflammation is one of the major causes of the progression of most chronic diseases, this result validates the anti-inflammatory characteristic of the extract and its symptoms. That the bee pollen has anti-inflammatory activity confirms the presence of bioactive molecules with the potential to modulate inflammatory pathways and inhibit inflammatory responses within the body. Flavonoids would constitute the chemical composition of the sample by virtue of having the anti-inflammatory characteristic (Al-Khayri et al., 2022). The H_2O_2 method was utilized in the assessment of anti-hemolytic activity, and the result turned out to be an efficient level. The assay confirms the blood cells are shielded from hemolysis, red blood cell lysis from oxidative stress. Anti-hemolytic activity was observed to be significant, showing the presence of bioactive metabolites that are able to scavenge the reactive oxygen species and ensure the structural stability of the red blood cells. With an IC_{50} value of 0.0516 (µg/mL), the value is low, showing that the extract is highly potent in bringing about this effect.

Positive correlation was determined when Pearson's coefficient was used in the examination of correlation between the phenolic content and biological activity of the extract. The study proves the significance of phenolic compounds in the biological activities of bee pollen previously established, i.e., they can prevent and promote disease and health (Zeng et al., 2024).

In summary, Apitherapy is a form of medical treatment that utilizes an array of bee products for natural treatment. The recent study offers certain conclusive evidence that bioactive compounds are present in the bee pollen and can be a suitable material for the purpose of treatment as well as for producing numerous medicines and other preparations. It is also regarded as a useful dietary supplement. These findings justify why the Egyptians used it and referred to it as "the life-giving dust." According to the findings, it is suggested to carry out further studies to isolate and identify active compounds, with the aim of producing bee pollen from a single botanical source only and in standard techniques and under controlled conditions.

Conflict of Interest

The authors declare that there are no conflicts of interest

Funding Information

Nil.

Author's ontributions

The data collection and experiments were done by Enas Al-Zeidaneen. Enas Al-Zeidaneen, Muna Al-nimat and Ansam Atrooz did the statistical analysis and formulated the ideas, research goals, and aims. Omar Atrooz supervised the research, and prepared, revised, and edited the article.

Ethics

There are no any ethical issues. The authors declare no competing interests.

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