ESTIMATION OF CHEMICAL COMPOSITION BY LC-ESI-MS/MS METHOD AND THE ANTI-HEXOKINASE ACTIVITY OF ALGERIAN BEE POLLEN

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Abstract. Few studies aim to evaluate the chemical composition and the effects of algerian bee pollen on antioxidant potential, total phenolics, total flavonoids, and the antihexokinase effect. Also, the algerian bee venom (ABV) possesses potent natural properties with antimicrobial effects. The aim of the study is to investigate the antibacterial effect of the ABV and antidiabetic activity of Algerian bee pollen (ABP). The analyses were performed with ABP extract, which was obtained using aceton 70% as the solvent for the extract. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was used to assess the antioxidant activity. *In vitro* anti-diabetic effects of the isolate of bee pollen from Algeria were evaluated. In this study, phenolic identification, Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometric (LC-ESI-MS/MS) were used. Using the method of disk diffusion to evalute the antimicrobial activity of ABV. Our results showed that the highest yield was detected with 70% acetone (72.44%). The phenolic and flavonoid contents of ABP were 54.92 mg GAE/g ± 0.48 and 6.18 mg QE/g ± 1.17, respectively. The half maximal inhibition concentration (IC50) values of ABP for DPPH scavenging activity were found to be 0.19±0.004 mg/mL. The LC-ESI-MS/MS analysis led to the identification of phenolic compounds; seventeen compounds in the extract of ABP were detected. The Algerian bee pollen isolate was assessed at 126 µg/mL concentration for the *in vitro* antidiabetic effect, yielding a 97.7% HK (Hexokinase) inhibition value. The algerian bee pollen has a very important effect as an anti-diabetic agent. The Algerian bee venom has an antimicrobial effect that is very interesting.

Key words: Algerian bee pollen; Algerian bee venom; antimicrobial activity; antioxidant activity; LC-ESI-MS/MS.

INTRODUCTION

Clinicians treat diabetes mellitus by prescribing insulin in addition to synthetic anti-diabetic medications like metformin and glipizide, but these medications have side effects. To eliminate these irritating side effects, scientists are actively studying herbal metabolites, in particular, as potential for new anti-diabetic medications. The abnormal increases in HK2 and the unscheduled glycolysis play an important role in impaired glycolysis control, as suggested by recent research. In this case, the key players in the tissue-specific chronic pathogenesis of hyperglycemia associated with diabetes are abnormal increases in G6P and downstream glycolysis intermediates [43].

The pollen of bees is created from agglutination by the nectar and bee enzymes, i.e., amylases and catalases, which are found in flower pollen that has been collected by bees. The pollen grains are supplied by bees with small amounts of saliva, nectar, and honey. The pollen of bees has been called the only perfectly complete food, as it contains all the essential amino acids that a human organism needs [39]. The bee pollen composition and antioxidative effect are species-specific, dependent very much on the type of plant source coupled with biogeographic regional origin, environmental habitat, season, or entomology [11, 12, 39]. In the case of this natural product, it is a relatively recent evolution, depending mainly on pollen being removed from bee legs as they go into their hives [39]. Proteins (5.6–60%), lipids (4.7–7%), reducing and non-reducing sugars (13–55%), crude fibers (0.3–20%), vitamins, enzymes, and phenolic compounds, primarily flavonoids, are all present in bee pollen [32].

Polyphenols found in medicinal plants and the human diet, including phenolic acids, flavonoids, and coumarins, are a significant group of secondary metabolites. They play a role in the antioxidant properties of plants and foods [7]. Depending on their chemical structures and the number of hydroxyl groups, phenolic compounds can serve as hydrogen or electron donors, reducing agents, and metal ion chelators [35].

Several studies have examined the natural antioxidant compounds found in medicinal bee products. because, in addition to their use as preservatives in food by replacing synthetic antioxidants, they are involved in the treatment of hyperglycemia. Thus, the study of the extraction of phenolic compounds by aceton 70% aims to reach the most efficient active molecules that have antioxidant and anti-diabtic effects to find a natural source to reduce blood glucose.

Additionally, BV has been shown to exhibit antimicrobial effects against both Gram-negative and Gram-positive bacteria, such as *Escherichia coli* and *Salmonella spp*. [20, 25], as well as *Enterobacter cloacae*, *E. coli*, and *Citrobacter freundii* [8], and *Staphylococcus aureus*, Coagulase-negative *Staphylococcus*, and *E. coli* [38]. The objective of this study was to assess the antimicrobial properties of Algerian bee venom against specific strains of medically significant Gram-positive and Gram-negative bacteria and fungi, to be used for sterilization against microbes as a natural source.

MATERIALS AND METHODS

Collection of the samples

The Algerian bee pollen was collected from a multiflowring plant in Blida (Algeria) in 2022.

The Algerian bee venom was collected from El-Medea (Algeria) in 2023.

Extraction. Acetonic extract of Algerian bee pollen in room temperature

A mixture of 5 g of crude bee pollen and 50 mL of aceton 70% was stirred for a full day (24 h). Then the mixture was filtrated through filter paper. The extraction was repeated three times with the residue.

The solvent was evaporated by rotavapor machine at 40°C and stored in steamroom at 60°C for 6 days. After that, the sample extract was stored at -20 °C.

Determination of the Total Phenol Content

Total polyphenol content in samples was determined by the Folin-Ciocalteau colorimetric method [6]. 100 µL of sample extracts (1 mg/mL of distilled water) were mixed with 500 µL of the folin-ciocalteau (10%) reagent, and 400 µL of Na_2CO_3 (75g/L) were added in a 1.5 mL Eppendorf tube. and the absorbance was measured at 735 nm after 5 min of incubation at 40°C in the dark using a UV-VIS spectro-photometer (JP Selecta UV-2005). Total polyphenol content was expressed as mg/g (gallic acid equivalents) (Fig. 1), (Y= 0.0122x – 0.0052 R^2=0.9995). Triplicate measurements were taken for all samples.

Total flavonoids content

Total flavonoid content in samples was determined by the method of Zhishen *et al.* (1999), with modifications [52]. In a 1.5 mL Eppendorf tube, 200 µL of samples were mixed with 60 µL of $NaNO_2$ (5%). After 5 min, 60 µL of $AlCl_3$ (10%) was added, and the mixture was vigorously mixed. After 6 min, a volume of 400 µL of NaOH 1M was added. The absorbance was read immediately at 510 nm against the control by UV-VIS spectrophotometer. A solution of quercetin has been prepared. For determining the calibration curve, solutions prepared from a stock solution at various concentrations of 0.1 to 1000 g/mL were applied (Fig. 2), (Y= 0.0018x + 0.0357, R^2=0.9991). Triplicate measurements were taken for all samples.

LC-ESI-MS/MS

The phenolics were quantitatively analyzed using an Agilent Technologies 1260 Infinity II, 6460 Triple Quad Mass Spectrometer. There was one Poroshell 120 SB-C18 (3.0 × 100 mm, I.D., 2.7 µm) column used [15]. In a 2 mL Eppendorf tube, 50 mg of the samples were taken and dissolved by adding 1 mL methanol. The resulting extract was extracted with 1 mL of hexane. It was centrifuged at 9000 rpm for 10 minutes. Finally, 100 µL of the methanol phase was taken and diluted with 900 µL as 450 (water)/450 (methanol). In the last step, this mixture was introduced into the instrument after being filtered through a 0.22 µm filter. 5.12 µL was the injection volume, and 0.40 mL/min was the adjusted flow rate. The mobile phase contained ammonium formate (5.0 mM) and formic acid (0.1%) in methanol B and ammonium formate (0.1%) in water A. For the B mobile phase, the gradient program was adjusted to 25% for 1-3 minutes, 50% for 4-12 minutes, 90% for 13-21 minutes, and 3% for 22-25 minutes. The temperature in the column was 40°C. The capillary voltage was 4000 V, the gas temperature was 300°C, the nebulizing gas (N2) flow rate was 11 L/min, and the pressure was 15 psi.

DPPH

DPPH free radical scavenging test: The antioxidant test was performed using the DPPH method [37].

ABP extract was added to 1.0 mL, 0.26 mM of DPPH solution at 20, 40, and 80 µL concentrations for 30 minutes at room temperature. A UV-VIS spectrophotometer measuring at 517 nm was used to measure the absorbance; a lower absorbance of the reaction product indicated a higher activity. Equation (1) was used to calculate the DPPH scavenging activity. We used equation (2) from figure (Fig.3) to calculate the IC50.

$$I\% = ((A1 - A2)/ A2) \times 100 \quad (1)$$

$$Y = 0.7079x + 14.604 \quad (2)$$

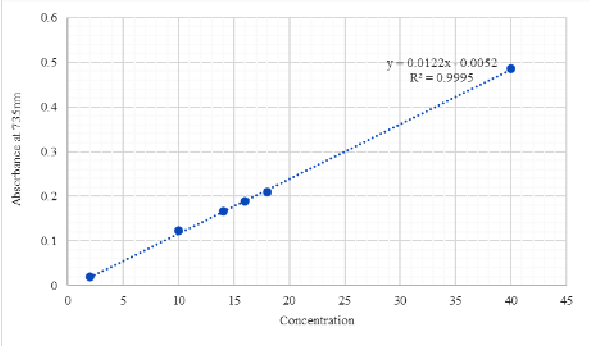

Figure 1. Total polyphenols calibration curve

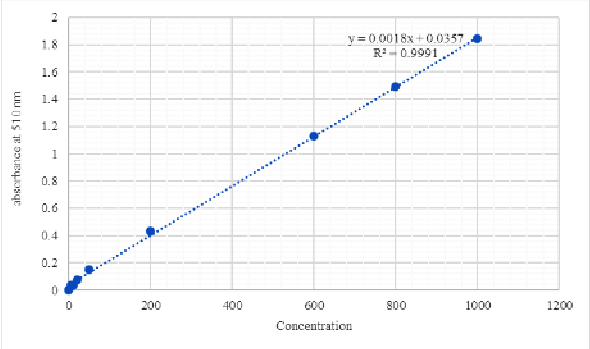

Figure 2. Total flavonoids calibration curve

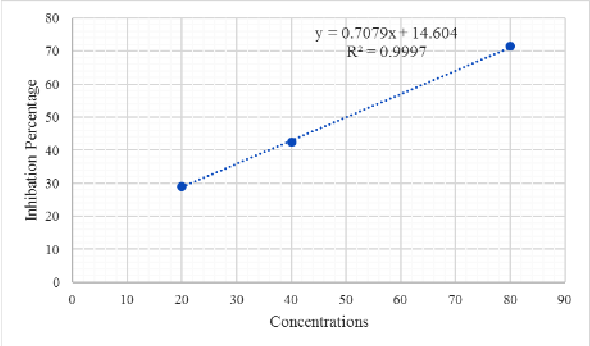

Figure 3. DPPH calibration curve

Hexokinase (HK) activity

HK activity was assayed spectrophotometrically through a coupled reaction with glucose-6-phosphate dehydrogenase (G6PD), following the NADP reduction at 340 nm [29]. The mix contained: R1 (MES buffer: 5.0 mmol/L, pH 6.0; Mg^{2+}: 24 mmol/L; ATP: ≥ 4.5 mmol/L; NADP: ≥ 7.0 mmol/L; preservative) and R2 (HEPES buffer: 200 mmol/L, pH 8.0; Mg^{2+}: 4 mmol/L; HK (yeast): ≥ 300 µkat/L; G-6-PDH (*E. coli*): ≥ 300 µkat/L; preservative). The assay was made on yeast HK isoforms (GLUC3, Roche/Hitachi Cobas) as well as on untreated medium. The enzyme was incubated with compounds (126 µg/mL) for 10 min before the assay.

Antimicrobial activity of algerian bee venom

We conducted an agar disk diffusion method with a modification [24]. We assessed the antimicrobial activity of bee venom against microbial and fungic strains using plates containing Mueller-Hinton agar pre-inoculated with a standardized microbial suspension at 10^8 CFU/mL. Whatman paper disks saturated with 15 µL of bee venom were positioned on the agar surface, with each disk having a 6 mm diameter. Following a 24-hour incubation at 37 °C, we interpreted the results by measuring the diameter of inhibition zones in millimeters (mm). All experiments were carried out in triplicate.

Statistical analysis

Tests were executed in triplicate and expressed as mean ± standard error (SE) with $p < 0.05$ as the level of significance by EXCEL 2016. The effect of enzyme inhibitor was analyzed by one-way analysis of variance (ANOVA), by using the SPSS 17.0 software.

RESULTS

Extraction yields

The principal step to evaluate the phytochemical activities of the sample is the extraction method of polyphenols and flavonoids molecules. The extraction with acetone 70% showed a higher yield of bee pollen extract with a value of 72.44%, which indicates the strong concentration and large quantity of extracted polyphenols and flavonoids.

Estimation of concentration of polyphenols and flavonoids molecules

The total phenolic content (TPC) and the total flavonoid content were evaluated for Algerian bee pollen. In this study, 54.92 mg GAE/g ± 0.48 and 6.18 mg QE/g ± 1.17 were calculated.

LC-ESI-MS/MS

The major organic components in algerian bee pollen preparation were identified by the LC-ESI-MS/MS method. 45 phenolic compounds were used as standards (Table 1) (Figure. 4).

Table 1. Esmation of chemical composition of Algerian bee pollen by LC-ESI-MS/MS

Compounds	Analyte	Rt	Phenolics (µg analyte/ g Bee pollen)
0	1	2	3
1	Shikimic acid	1.46	ND
2	Gallic acid	3.23	5.02
3	Protocatechuic acid	5.42	175.16
4	Epigallocatechin	6.82	ND
5	Catechin	6.86	ND
6	Chlorogenic acid	7.38	352.26
7	Hydroxyben zaldeyde	7.63	5.74
8	Vanillic acid	7.73	ND
9	Caffeic Acid	7.81	29.40
10	Syringic acid	8.35	ND
11	Caffein	8.34	1.27
12	Vanillin	8.59	19.55
13	o-coumaric acid	9.39	3.48
14	Salicylic Acid	9.73	20.45
15	Taxifolin	9.68	ND
16	Resveratrol	9.74	ND
17	Polydatine	9.87	ND
18	Trans-ferulic acid	10.05	ND
19	Sinapic acid	10.33	ND
20	Scutellarin	11.16	2.12
21	p-coumaric acid	11.57	ND
22	Coumarin	11.56	1.85
23	Protocatehuic ethyl ester	11.68	ND
24	Hesperidin	11.72	ND
25	Isoquercitrin	11.74	36.05
26	Rutin	12.21	15.91
27	Quarcetin-3-Ksilozid	12.41	ND
28	Kaempferol-3-glucoside	13.07	39.20
29	Fisetin	13.34	2.00
30	Baicalin	13.78	ND
31	Chrysin	14.04	ND
32	Daidzein	14.18	ND
33	Trans-cinnamic acid	14.27	ND
34	Quercetin	14.76	ND
35	Naringenin	14.86	44.54

0	1	2	3
36	Silibinin	15.60	ND
37	Hesperetin	15.83	ND
38	Morin	15.70	ND
39	Kaempferol	16.36	ND
40	Baicalein	17.07	ND
41	Luteolin	17.78	44.57
42	Biochanin A	17.90	ND
43	Capcaicin	18.17	ND
44	Dihydrocapcaicin	18.58	ND
45	Diosgenin	23.53	ND

Rt: Retention time. **ND:** not detected

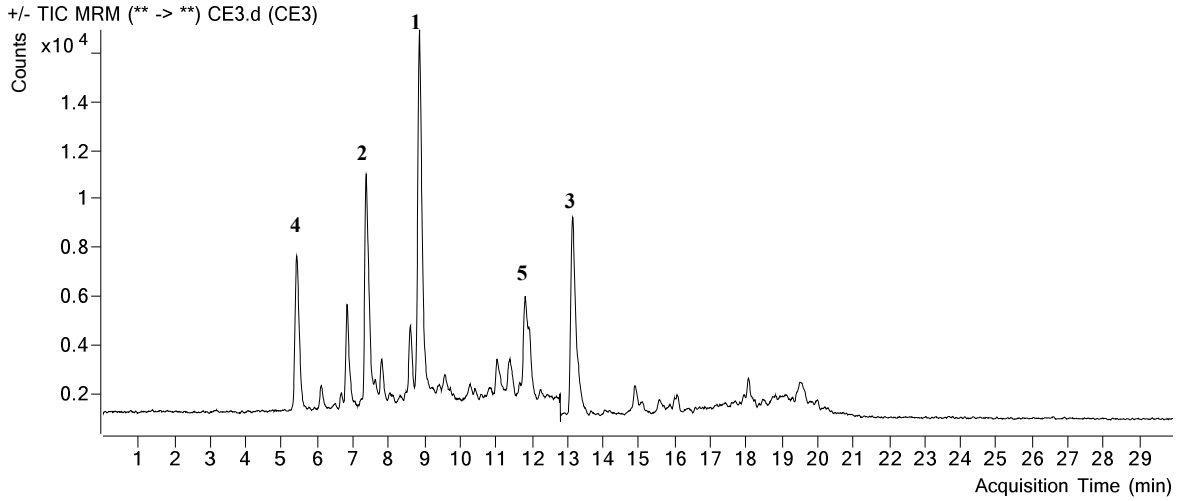

Figure 4. LC-ESI-MS/MS chromatograms of Algerian Bee Pollen; where: **1 -** Vanillin, **2 -** Chlorogenic acid, **3 -** Kaempferol-3-glucoside, **4 -** Protocatechuic acid, **5 -** Isoquercitrin

Antioxidant activity

The results of the half maximal inhibition concentration (IC50) values of Algerian bee pollen, BHA, BHT, and Trolox measured by the DPPH scavenging activity method are represented in Table 2.

Hexokinase (HK) Inhibitory Assay

In vitro anti-diabetic effects of the isolate of bee pollen from Algeria were evaluated, with a 97.7% HK inhibition value of the isolate at a concentration of 126 µg/mL. The HK inhibitory assay was used to determine the algerian bee pollen isolate's anti-diabetic activity. Consequently, it was discovered that the isolate of algerian bee pollen had high anti-diabetic activity.

Antimicrobial activity of bee venom

The results of the antimicrobial activity (mm) of Algerian bee venom are represented in Table 3 (Fig. 5).

DISCUSSION

Natural sources of nutrients and phytochemicals, specifically polyphenols, can be found in bee pollen. These substances have a variety of biological activities, which make them beneficial to human health. The aim of this study was to investigate some natural products that are traditionally used to treat multiple diseases.

Table 2. The DPPH radical-scavenging activity of Algerian bee pollen.

Antioxidant activity	DPPH scavenging IC50 (µg/mL)	R^2
BHA	4.39	0.04
BHT	10.86	0.06
Trolox	4.95	0.03
Bee pollen	191.64	4.05

Table 3. Results of the antimicrobial activity of Algerian bee venom.

Strain	SAµ *Staphylococcus aureus*	E C *Escherichia coli*	K P *Klebsiella pneumoniae*	CA *Candida albicans*	SA33 *Staphylococcus aureus*
ATCC	29213	25922	70603	10231	33862
Gram	positif	Negatif	negatif	fungi	Positif
Bee venom (100 mg/mL)	13±1	14.66±1.15	10±0	13±1.7	14±1

ATCC: American Type Culture Collection

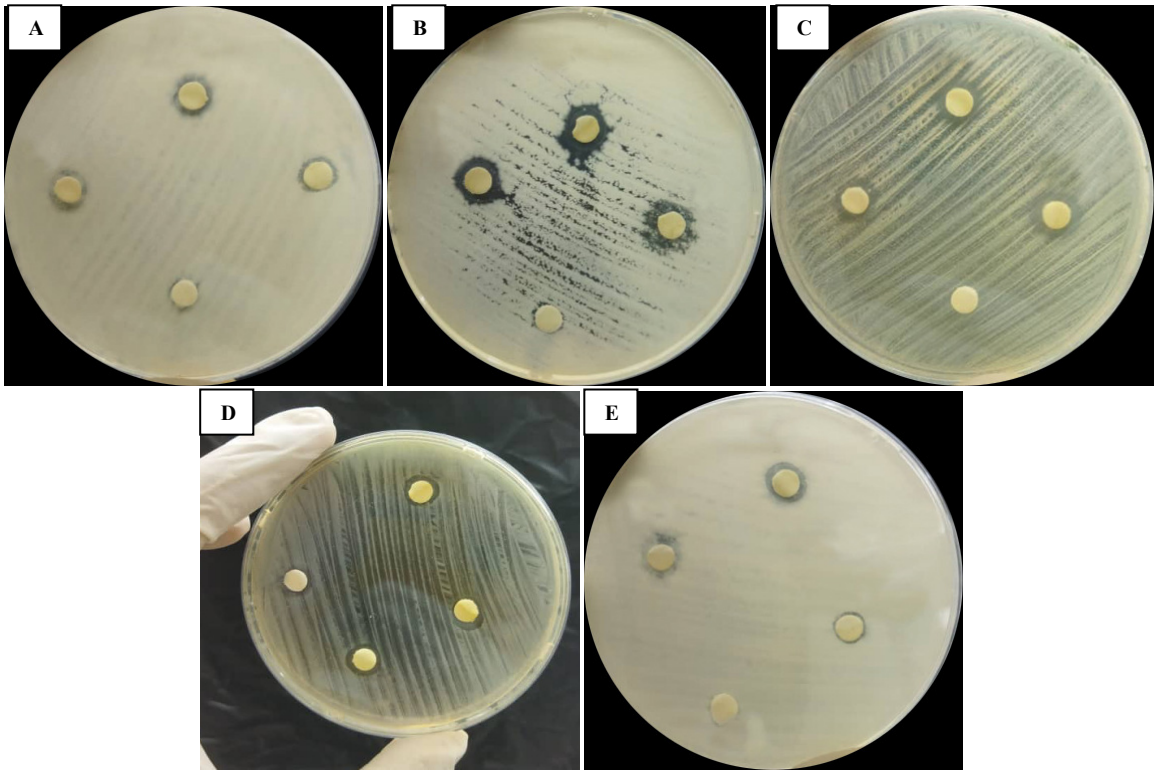

Figure 5. Results of the antimicrobial activity of Algerian bee venom, where: A – *Staphylococcus aureus* SAμ; B – *Escherichia coli* E C; C – *Klebsiella pneumoniae* K P; D – *Candida albicans* C A; E – *Staphylococcus aureus* SA33.

The results demonstrated greater extraction yields, total flavonoid and phenolic content, and antioxidant activity. The most notable characteristic of polyphenols is their highly unequal distribution throughout the various biological materials, which can be attributed to a variety of physiological evolution stages as well as extreme environmental conditions (high salinity, high temperature) [16, 44]. However, when compared to methanol and ethanol in all of the study's analyses, the extraction using 70% acetone produced the best results, with the exception of antioxidant activity, where ethanol performed better [44]. Many researchers proved to the point that acetone extracts antioxidant phenolics better than other organic solvents [18, 19, 22, 31, 34, 42, 44]. Of which the most phenolics have been found in acetone has the lowest polarity [1, 44, 50].

The results about the yield showed that we have a great yield as compared with anouther study [45], which used methanol as a solvent for extraction. These results confirmed that the extraction with aceton gives greater yields than methanol. The extraction yield is dependent on a number of variables, including the extraction method's solvent, temperature, and duration [23, 44, 48].

Significant results were obtained from the quantitative estimation of phenolics and flavonoids in the extracts of the tested algerian bee pollen presented the most important amounts of phenolic compounds (54.92 mg GAE/g ± 0.48) than determined by Rebiai and Lanez (2012) [45], and lower in flavonoids (6.18 mg QE/g ± 1.17) than Rebiai and Lanez (2012) [45], in the three samples of Blida and Boumerdes, but greatest with the other samples. The reason for this is the exceptionally high temperatures and high salinity experienced in Blida in 2010 and 2021.

The research about Algerian bee pollen compounds is very limited. Seventeen phenolic compounds were determinad from algerian bee pollen by the LC-ESI-MS/MS analyses (Table 1). Quantitative analysis (Figure 4) revealed that chlorogenic acid was the major compound with a value of 352.26 (μg/g extract). Moreover, the protocatechuic acid 175.16 (μg/g extract), luteolin 44.57 (μg/g extract), naringenin 44.54 (μg/g extract), kaempferol-3-glucoside 39.20 (μg/g extract), isoquercitrin 36.05 (μg/g extract), caffeic acid 29.40 (μg/g extract), salicylic acid 20.45 (μg/g extract), vanillin 19.55 (μg/g extract), rutin 15.91 (μg/g extract), hydroxyben zaldeyde 5.74 (μg/g extract), gallic acid 5.02 (μg/g extract), o-coumaric acid 3.48 (μg/g extract), Scutellarin 2.12 (μg/g extract), fisetin 2 (μg/g extract), coumarin 1.85 (μg/g extract), caffein 1.27 (μg/g extract) were determined in Algerian bee pollen extract. In another study, thirteen compounds were determinad from Chile bee pollen by HAPLC-DAD [9]. Bee pollen collected in Brazil contains twelve compounds, as shown by the HPLC [46]. In this context, we can say that the time and region of collection of bee pollen (geographic conditions), the extraction method, the solvant of extraction and also the technique used for the esmation of compounds can alter the composition of the samples.

The results of the DPPH test for free radical scavenging were represented as IC50 (mg/mL). According to the test, ABP's DPPH scavenging capacity was lower than that of BHA, BHT, and trolox (Table 2). But it’s higher (0.19±0.004 mg/mL) than

that found in other studies (Table 4) [2, 5, 14, 33, 51]. This finding is consistent with the results of previous studies and could be explained by the nature, quantity, and structure of the synthesized polyphenols [44]. The antioxidant's spatial structure determines the mechanism of the reaction between it and the DPPH radical [27, 44]. In antioxidant activity, the extraction with ethanol was more effective than acetone [44], but in our results, extraction with aceton is more powerful than ethanol as compared with the chine results [51]. This may be explained by the major compound in our sample « Cholorogenic acid » because it has an antioxidant effect [36].

Table 4. IC50 of antioxidant activity of bee pollen for other studies

IC50	Reference
0.39± 0.13 mg/mL	[14]
1.43± 0.00 mg/g	[2]
233.3± 6.1 µg/mL	[5]
2.36 mg/mL	[33]
1.72 mg/mL	[33]
1.28± 0.03 mg/mL	[51]

For the first time bee pollen is tested for anti hexokinase activity. Results of antihexokinase activity showed that our crude acetonic sample has an interesting inhibitory activity of hexokinase. It's possibly due to the absence of Kaempferol in our composition; the polyphenol that increased hexokinase activity [4, 13]. Fisetin (3,7,3',4'-Tetrahydroxyflavone) reduces the activity of glucose-6-phosphate dehydrogenase [13, 41]. In addition, luteolin is an anti-diabetic agent [3, 49]. Also, fisetin has anti-diabetic effects [10]. Furthermore, hexokinase activity is increased by fisetin's anti-diabetic effects on hepatic enzymes, whereas glucose-6-phosphatase (G6Pase) and glucose-6-phosphate dehydrogenase (G6PD) activity are decreased [3]. On the other hand, protocatechuic acid has antihexokinase activity because it has antihyperglycemia activity [36].

The results from Table 3 indicate that bee venom showed effective antibacterial and antifungal properties against the tested microorganisms. The antimicrobial effects of honeybee venom may be due to the presence of various peptides like melittin, apamin, adolapin, mast cell degranulating peptide, enzymes, biologically active amines, and non-peptide components [28, 30]. Fennel et al. [17] noted that the venom contains melittin, which is more effective against Gram-positive bacteria than Gram-negative ones. These findings are consistent with Kondo and Kanai's [26] observations that *mycobacteria* and *staphylococci* were affected by the bee venom fraction (melittin) but not *E. coli*. However, earlier studies [40, 47] have reported a stronger impact on *E. coli* from bee venom. While Hegazi et al. [21] previously showed that bee products had less effect on *E. coli*, the current study provides evidence that bee venom displays antibacterial activity against both Gram-positive and Gram-negative bacteria, with no significant differences between the two groups.

Finally, this study of the crude extracts for the pollen of Algerian bees by aceton 70% According to the LC-ESI-MS/MS analysis, the major components detected in Algerian bee pollen are protocatechuic acid, chlorogenic acid, kaempferol-3-glucoside, naringenin, and luteolin. The algerian bee pollen aceton extract had increased antioxidant activity, phenolic contents, and inhibited hexokinase activity. In diabetes mellitus treatment, clinicians prescribe insulin together with synthetic anti-diabetic helper drugs such as glipizide and metformin, but they have side effects. Scientists are intensively researching herbal metabolites as new anti-diabetic agents to eliminate these irritating side effects. The results suggested that algerian bee pollen isolate could be used as a natural source of compounds with therapeutic potential for treating disorders associated with diabetes. Given the results of the present study, it could be said that algerian bee pollen can play a beneficial role in the treatment of human health. However, further studies are needed to reach a final conclusion. Additionally, the current discoveries enhance the significance of Algerian bee venom in inhibiting microorganisms. These inhibiting properties have potential for broad use as antiseptics and as distinctive inhibitors in biological research. Further research is needed to standardize their precise composition, assess allergy risks, toxicity, and ensure quality.

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