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ARTICLE

RESEARCH ARTICLE

Characterization and assessment of the antimicrobial efficacy of Algerian propolis against American foulbrood and Nosema disease in honeybee

Caracterización y evaluación de la eficacia antimicrobiana del propóleo argelino contra la loque americana y la nosemosis en las abejas melíferas

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Abstract: *Aim of study:* Like all insects, honeybees (*Apis mellifera*) are vulnerable to infectious diseases. American foulbrood and nosemosis are among the most widespread diseases affecting this species worldwide. The present study aimed to evaluate the efficacy of propolis from different beehives in Algerian apiaries against these major honeybee diseases. *Area of study:* Propolis harvesting was conducted in three regions of M'sila province in Algeria: Bousaada, Ouled Madhi, and Maadid. *Material and methods:* The chemical characterization of Algerian propolis was performed by HPLC-DAD, a simple and highly effective technique for the identification of polyphenolic compounds. *Paenibacillus larvae* was isolated from hives showing clinical signs of infection. The antibacterial activity of ethanolic extracts of Algerian propolis was assessed according to the Mueller-Hinton disc diffusion method, using the antibiotic oxytetracycline (OT) as a positive control. Screening of infected individuals for *Nosema* spp. was conducted and infection intensity was determined by spore quantification. *Main results:* HPLC-DAD analysis of propolis revealed the presence of 13 phytochemical compounds, suggesting a high chemical quality. All tested ethanolic propolis extracts have shown a notable inhibitory activity against diseases affecting *Apis mellifera*. Concerning the antagonistic activity against *Paenobacillus larvae*, we found that the propolis extract from the Bousaada region (EEP3) showed the largest inhibition zones (45.05±3.40 mm) followed by extracts from Ouled Madhi (EEP1; 32.05±7.30 mm) and Maadid (EEP2; 22.16±8.51 mm). Moreover, propolis treatment resulted in a clear reduction on the activity of *Nosema* spp. spore counts, indicating its potential effectiveness against nosemosis. *Research highlights:* The study highlights the chemical composition of propolis and its potential benefits for bee health, aiming to exploit it in natural pest management strategies in apiculture.

Keywords: antibacterial activity; *Apis mellifera*; characterization by HPLC-DAD; *Nosema* spp.; *Paenibacillus larvae*; propolis; scanning electron microscope.

Resumen: *Objetivo del estudio:* Al igual que todos los insectos, las abejas melíferas (*Apis mellifera*) son vulnerables a las enfermedades infecciosas. La loque americana y la nosemosis se encuentran entre las dos enfermedades más conocidas que afectan a esta especie de insecto en todo el mundo. El presente estudio tuvo como objetivo evaluar la eficacia del propóleo procedente de diferentes colmenas de apicultores argelinos contra estas importantes enfermedades de las abejas melíferas. *Área de estudio:* La recolección del propóleo se llevó a cabo en tres regiones de la provincia de M'sila, en Argelia: Bousaada, Ouled Madhi y Maadid. *Material y métodos:* La caracterización química del propóleo argelino se realizó mediante HPLC-DAD, una técnica sencilla y muy eficaz que se ha aplicado para la detección e identificación de compuestos polifenólicos. *Paenibacillus larvae* fue aislado de colmenas que presentaban signos clínicos de infección. La actividad antibacteriana de los extractos etanólicos de propóleo argelino se evaluó según el método de difusión en disco de Mueller-Hinton, utilizando el antibiótico oxitetraciclina (OT) como control positivo. Se realizó el cribado de individuos infectados por *Nosema* spp., y la intensidad de la infección se determinó mediante la cuantificación de esporas. *Resultados principales:* El análisis HPLC-DAD del propóleo reveló la presencia de 13 compuestos fitoquímicos, lo que puede indicar su alta calidad química. Todos los extractos etanólicos de propóleo analizados han mostrado una notable actividad inhibidora contra enfermedades que afectan a *Apis mellifera*. Con respecto a la actividad antagonista contra *Paenibacillus larvae*, encontramos que el extracto de propóleo de la región de Bousaada (EEP3) mostró las mayores zonas de inhibición (45,05 ± 3,40 mm), seguido de los extractos de Ouled Madhi (EEP1; 32,05 ± 7,30 mm) y Maadid (EEP2; 22,16 ± 8,51 mm). Además, el tratamiento con propóleo dio lugar a una clara reducción del efecto del propóleo sobre la actividad de *Nosema* spp. en el recuento de esporas, lo que indica su potencial eficacia contra la nosemosis. *Aspectos destacados de la investigación:* El estudio destaca la composición química del propóleo y sus posibles beneficios para la salud de las abejas, con el objetivo de aprovecharlo en estrategias naturales de control de plagas en la apicultura.

Palabras clave: actividad antibacteriana; *Apis mellifera*; caracterización mediante HPLC-DAD; microscopio electrónico de barrido; *Nosema* spp.; *Paenibacillus larvae*; propóleo.

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Supplementary information ↓

1. INTRODUCTION

Beekeeping is the practice of keeping bees. It has existed since ancient times, and it is still widespread throughout the world. This activity varies according to climate, flora, bee diversity and level of economic development (Abed *et al.*, 2022). As pollinators of many plant species, honey bees are essential to the world's ecological balance and play a vital role in maintaining agricultural systems and biodiversity. They also have other interests, including the production of honey, propolis, royal jelly and wax, which are widely used in the public health and food sectors (Choukri, 2012; Açık *et al.*, 2024).

On the other hand, populations of the honeybee *Apis mellifera* have declined considerably. Loss of habitat, intensive use of pesticides, climate change, nutritional deficiencies, and pathogens are all

responsible for this disappearance (Abban *et al.*, 2024; Açık *et al.*, 2024). These pathogens include a microsporidian fungus *Nosema* sp., causative agent of nosemosis, which spreads in the faecal and infects the epithelial cells of the mid-intestine of young bees, disrupting digestive functions and causing malnutrition, physiological aging, a reduction in hypopharyngeal glands and the premature death of the bees (Domínguez-Rebolledo *et al.*, 2023). It was detected for the first time in *A. mellifera* in 1909 (Rodríguez-García *et al.*, 2021). It can be identified by observing faecal stains containing millions of characteristic infectious spores at the entrance to and inside the hive, which has encouraged the spread of the disease by faecal and oral routes within the colony (Schüler *et al.*, 2023).

Similarly, honeybees face damage caused by *Paenibacillus larvae*, a Gram-positive, rod-shaped, spore-forming bacterium. It is the aetiological agent of the American foulbrood epizootic (AFB), a brood disease that is widespread throughout the world and subject to compulsory declaration in most countries (Poppinga *et al.*, 2012; Ory *et al.*, 2023).

Propolis, also known as bees' glue, is a resinous mixture collected from various trees exudates, such as poplars, birches, willows, and certain conifers (Bouhala, 2012). More than 300 components have been identified in different propolis samples (Šulcerová *et al.*, 2014). Propolis is usually made up of 50% plant resins, 30% waxes, 10% essential and aromatic oils, 5% pollen, and 5% other organic substances (Kalia *et al.*, 2013; Ngenge *et al.*, 2017).

By using this resinous product, bees can reduce the incidence of bacteria and mould inside the hive compared with the external environment (Bilikova *et al.*, 2013). This biological property is linked to its chemical composition, which varies according to plant origin and geographical area (Dos Santos *et al.*, 2022). Several recent studies have demonstrated the antibacterial (Choi *et al.*, 2006; Kalia *et al.*, 2013; Oumani, 2021; Debab & Toumi-Benali, 2022), and antifungal (Koc *et al.*, 2007; Khosravi *et al.*, 2014; Haghdoost *et al.*, 2016; Pratami *et al.*, 2020) properties of propolis.

The aim of this study was to determine the chemical composition and the biological activity of propolis collected from different regions of M'sila province in Algeria. The study compared the bioactive components and biological activities of various ethanolic propolis extracts in response to pathogens affecting bees. It also assessed how characterizing the chemical profile influences these activities.

2. MATERIAL AND METHODS

2.1. Propolis collection from *Apis mellifera* apiaries

The samples were collected during the period 2021-2023. Three samples of Algerian raw propolis were collected from three geographical stations in the M'sila region (located in central Algeria; latitude 35º 40' N, longitude 4º 32' E). The three stations are Ouled Madhi, Maadid, and Bousaada. Sampling in three regions was carried out during the autumn season according to specific criteria. Thus, harvesting propolis using plastic collection frames was performed in the stations located in Ouled Madhi and Bousaada; scarping it from hives was carried out in the station Maadid. It was also harvested according to the types of plants present. These were then kept at (4 °C) in the dark until they were used (Figure 1).

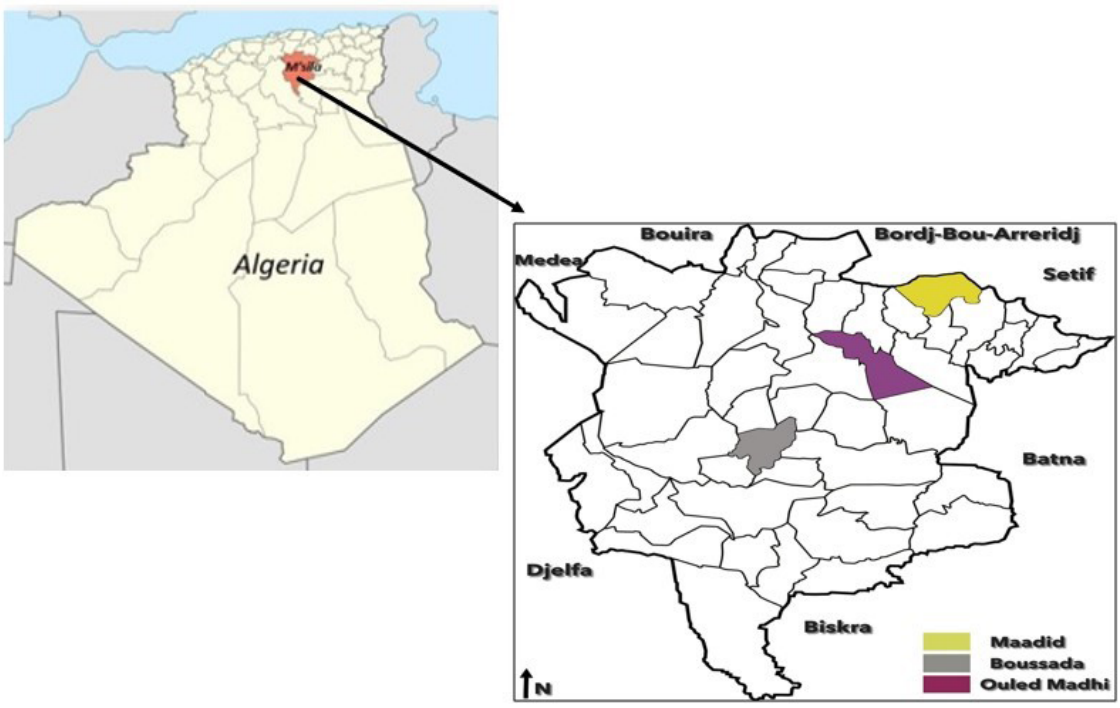

Figure 1. Geographical location of the propolis harvesting areas: M'sila including its three stations of Bousaada, Ouled Madhi, and Maadid, Algeria.

2.2. Propolis extraction

The ethanolic extractions were carried out according to the method of Segueni (2011) with some modifications. Briefly, 10 g of each raw propolis sample was macerated in 100 ml of 70% ethanol at room temperature for one week, with stirring and protection from light. The ethanolic solution was filtered and the ethanolic solvent was removed by evaporation under reduced pressure at a temperature of 50 ºC and then dried in an oven at 40 °C. The dry extracts were named Ouled Madhi (EEP1), Maadid (EEP2) and Bousaada (EEP3).

2.3. Propolis characterization

2.3.1. High-performance liquid chromatography analysis

With some modifications to the protocol described by Pellati *et al.* (2011), reference compounds were used to identify polyphenolic compounds (phenolic and flavonoid) using an HPLC-DAD system, consisting of a vacuum degasser, an auto sampler, and a binary pump with a maximum pressure of 400 bar (Agilent 1260, Agilent technologies, Germany) equipped with a 4.6 × 100 mm reversed-phase C18 analytical column with a particle size of 3.5 µm (Zorbax Eclipse XDB C18). The DAD detector was set to a scan range of 200-400 nm. The column temperature was maintained at 23 °C. The injected sample volume was 2 µl and the flow-rate of mobile phase was 0.4 ml/min. Mobile phase B was milli-Q water consisted of 0.1% formic acid and mobile phase A was acetonitrile. The optimized gradient elution was illustrated as follows: 0-5 min, 10-20% A; 5-10 min, 20-30% A; 10-15 min, 30-50% A; 15-20 min, 50-70% A; 20-25 min, 70-90% A; 25-30 min, 90-50% A; 30-45 min, return to initial conditions. In addition, commercial standards were injected in a range of concentrations for each compound. We then plot calibration curves. Using the quantification equations and the standards' retention times, we were able to identify each molecule.

2.3.2. Scanning Electron Microscope analysis.

The morphological characteristics and size variations of the propolis samples were observed using a scanning electron microscope (SEM), which enables three-dimensional observation of the microstructure. The samples were placed on a base and bombarded with a stream of electrons allowing the structure to be visualized at various magnifications.

2.4. Evaluation of antagonism activities against *Paenibacillus larvae*

Field visits were conducted to detect and isolate the target pathogen, *Paenibacillus larvae*. A contaminated site with *P. larvae* was identified in 2022 at an apiary in the Maadid region (Wilaya of M'sila). Based on the characteristic symptoms of American foulbrood, samples were collected for bacterial isolation.

2.4.1. Isolation and purification

Paenibacillus larvae have been isolated from the pollen (PP) and honey (PM) collected from the infected hives. A sample of 50 g was taken from each hive and stored in sterile bottles marked with the location and date of sampling. Then 10 g of each sample was weighed and dissolved in 90 ml of sterile physiological saline, and the stock solution was autoclaved at 80 ºC to eliminate non-sporulating forms and facilitate purification. Finally, successive dilutions were prepared (Oulebsir-Mohandkaci *et al.*, 2016), and the samples were grown on the selective medium MYPGP (composed of 10 g of Mueller-Hinton broth, 15 g of yeast extract, 3 g of potassium phosphate, 2 g of glucose, 1 g of sodium pyruvate and 20 g of agar) (Genersch *et al.*, 2006; Bilikova *et al.*, 2013). *Paenibacillus larvae* was identified using the protocol of Graaf *et al.* (2013), which strongly emphasizes extending the incubation period for a few more days. This helps to identify the growth of *P. larvae* bacteria in the MYPGP solid medium. This also confirms the basic microbiological techniques and inoculation procedures in the MYPGP solid medium.

2.4.2. Antibacterial activity

The antibacterial activity of the ethanolic extracts of propolis (EEP1, EEP2, and EEP3) was assessed using the Mueller Hinton agar disc diffusion method, the principle of which is to determine the sensitivity or resistance of *P. larvae* to the solutions tested at decreasing concentrations (600 mg/ml, 400 mg/ml, 200 mg/ml). This method consists of using sterile discs. Each disc is impregnated with (10 µl) of the tested EP extract. Oxytetracycline (OT) was selected as a positive control (Murray & Aronstein, 2006; Plavša *et al.*, 2011; Obshta *et al.*, 2023) confirming its efficacy against *P. larvae*, the discs were incubated at 37 ºC for 24 hours. The antimicrobial activity of propolis is assessed by measuring the diameter of the zone of inhibition, which takes the form of a clear halo around the absorbent disc.

2.5. Evaluation of antagonism activities against *Nosema* spp

To detect bees affected by *Nosema* spp., field visits were conducted. The apiary infected with this disease is located in the Meftah region (Wilaya of Blida). Based on signs of infection, samples were collected from affected bees between February and March 2022. The colonies showed visible clinical symptoms of *Nosema* spp*.,* with an average prevalence of 7×10^5 spores per bee.

For this part of the study, modified bread crates (10 × 8.5 × 6 cm) were used to assess the antagonistic effect of the different ethanolic propolis extracts samples (EEP) on the *Nosema* agent. Each treatment consisted of three crates, each containing 20 bees, which were forager honeybees. In addition, the uninfected control bees were collected from a different apiary, after it was confirmed that the bees had not shown symptoms of the disease. These individuals were fed pollen made into balls with sugar syrup, supplied in plastic haemolysis tubes pierced at the bottom. For the treated batches containing diseased bees, the concentrations of EEP. Tested were 20 mg/ml, 40 mg/ml, and 60 mg/ml added to the syrup. Then, to dissolve the propolis and obtain a uniform and homogeneous suspension, the

syrup was stirred for several seconds. Controls containing non-diseased bees were given syrup with no EEP added. A third batch of bees, representing the positive control, was treated with diprim (200 mg sulfamethoxazole + 40 mg trimethoprim TMP-SMX). Antibiotic selection was based on previous studies (Moon & Oberhelman, 2005; Kappagoda *et al.*, 2011; Nsiangani Lusambo *et al.*, 2023). Finally, pollen and syrup consumption were measured daily in treated bees with the EEP and controls for 10 days (Figure 2).

Figure 2. Propolis extracts for *Nosema* spp. treatment.

To assess the efficacy of the propolis tested, *Nosema* spores were extracted daily from treated bees during the 10-day monitoring period of the experiment, and *Nosema* spores were counted using a Malassez cell × 40 light microscope. Our samples were taken exclusively from the abdomen of the foragers since *Nosema* spores only parasitize the bee's intestine. To do this, the abdomens of five workers were taken daily using entomological forceps and ground with 5 ml of distilled water. The suspension obtained was filtered to recover the juice from the crushed abdomens containing *Nosema* spores, and centrifuged at 800 xg, at room temperature for 6 minutes. This process is repeated three times to ensure optimum cleaning of the spores and to remove as much tissue debris as possible (Dadoun, 2021).

2.6.Statistical analysis

To confirm the efficacy of ethanolic extracts of propolis from different regions against the two pathogens, we used the one-way ANOVA test and the mean ± SD by Graph Pad Prism 8.4.3 (686) with Tukey's test. We used univariate generalized linear models and we used negative binomial generalized linear analysis (GLM) and Poisson test.

3. RESULTS

3.1. Profile of phenol compounds in propolis

HPLC analysis was used to characterise the main bioactive compounds in EEP and to determine the differences between samples according to their concentration, then the polyphenols were identified and assigned. The analysis revealed the presence of 13 compounds in the extracts of propolis collected from different regions of M'sila, Algeria. The results are summarised in the following Table 1.

Table 1. Analysis of phenolic compounds by HPLC-DAD of ethanolic extracts of propolis (EEP1, from Ouled Madhi; EEP2, from Maadid; EEP3, from Bousaada) of M'sila, Algeria

		Samples of Propolis									
			EEP1			EEP2			EEP3		
N	**Standards**	**R^2**	**R_T**	**Area %**	**[µg/g EP]**	**R_T**	**Area %**	**[µg/g EP]**	**R_T**	**Area %**	**[µg/g EP]**
1	Gallic acid	0.999	8.95890808	42.8360062	0.018	8.961224556	48.21800995	0.02	8.976020813	180.5948181	0.08
2	Epigallocatechin	0.998	14.5957994	235.425919	0.643	14.59942913	147.5516052	0.401	14.60152531	300.9700012	0.82
3	Epicatechin	0.998	16.6540642	3089.31128	8.5	16.6576996	1685.13672	4.63	16.65615845	3056.462891	8.41
4	Caffeic acid	0.999	17.3507805	91.0633926	0.036	17.4796467	85.057724	4.63	17.48745728	216.4157715	0.089
5	*p*-coumaric acid	0.999	19.2630768	1295.58972	0.377	19.264698	649.669189	0.188	19.27285957	931.6393433	0.27
6	Rutin	0.998	21.1973305	109.345215	0.21	ND	ND	ND	ND	ND	ND
7	Laricitin	0.994	23.2710075	450.781281	0.715	23.2739639	177.449738	0.316	ND	ND	ND
8	Pinobanksin	0.997	23.528616	380.0539246	0.404	23.4992161	201.783127	0.206	23.49507523	447.4087524	0.047
9	Pinocembrin	0.997	25.55077553	116.2926712	0.111	25.5686016	93.49511719	0.085	25.56947327	110.8805237	0.01
10	Apigenin	0.997	25.83195114	54.67795181	0.003	25.83567619	39.00261688	0.007	25.84156227	239.024231	0.144
11	Chrysin	0.994	26.34070969	181.8605957	0.319	26.33722305	51.35354996	0.127	26.34926414	142.0641327	0.26
12	Galangin	0.997	ND	ND	ND	26.85355186	49.64064789	0.036	26.83929634	137.7959442	0.135
13	Quercétine 3.3'	0.998	28.60469055	603.7172852	0.63	28.60931396	374.806488	0.37	28.62710381	784.3408203	0.826

R^2: Coefficient of determination of the calibration curve.

R_T: retention time (minutes)

Area %; Relative percentage of the chromatographic peak area.

µg/g EP: Concentration expressed as micrograms per gram of extracted product.

ND: (not detected)

From the results, we can see that the figures represent chromatograms of propolis extracts where the peaks allowed the identification of thirteen phenolic compounds in propolis sample with different concentrations; (1) gallic acid, (2) epigallocatechin, (3) epicatechin (4) caffeic acid, (5) *p*-coumaric acid, (6) rutin, (7) laricitin, (8) pinobanksin, (9) pinocembrin, (10) apigenin, (11) chyrysin, (12) galangin, and (13) quercétine 3,3' (Figure 3).

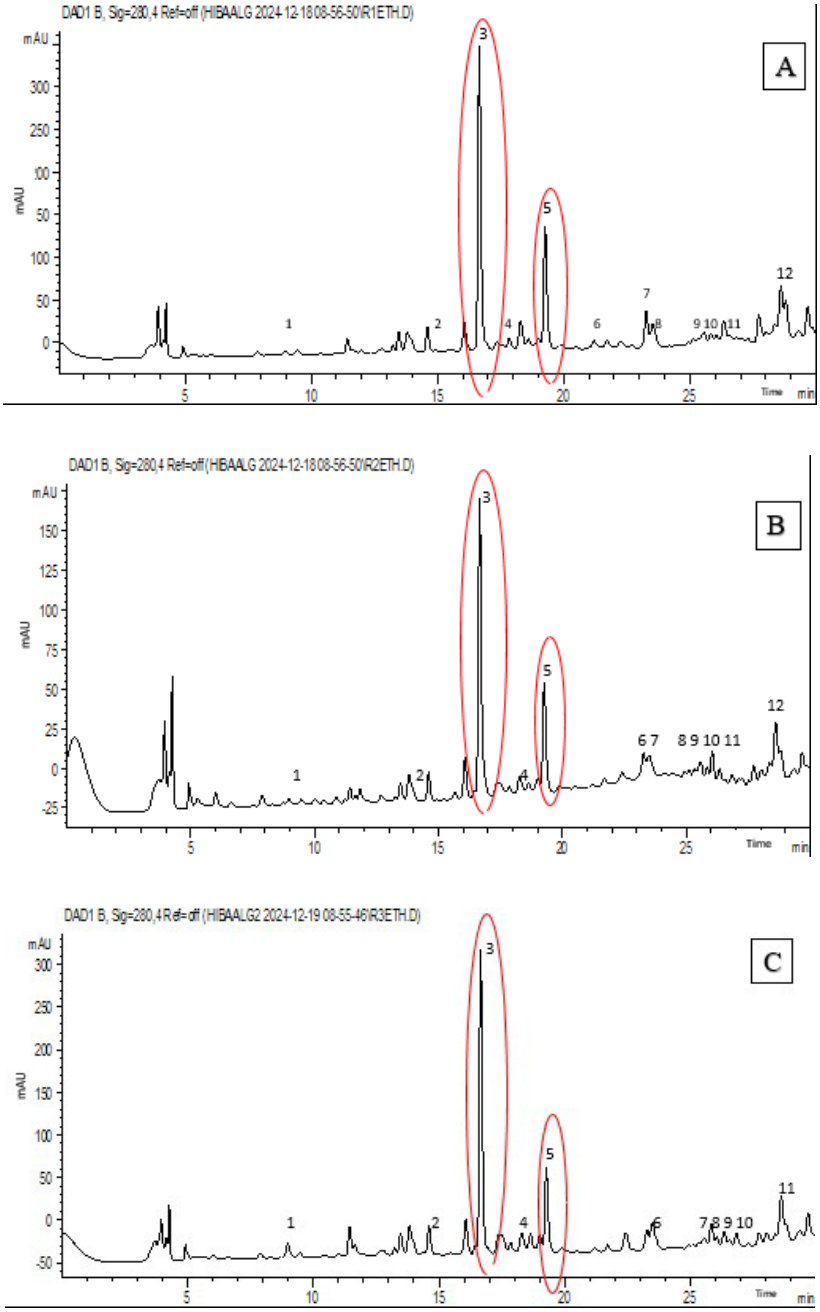

Figure 3. HPLC chromatographic profiles of polyphenols compounds at 280 nm; A) HPLC chromatogram of EEP1; B) HPLC chromatogram of EEP2; C) HPLC chromatogram of EEP3. (3 Epicatechin; 5 *P*-coumaric acid).

As indicated in Figure 3, epicatechin peak was detected in all samples at a retention time of 16.65 min. The corresponding concentrations were 8.5 µg/g, 4.63 µg/g and 8.41 µg/g in EEP1, EEP2 and EEP3, respectively. Although galangin was identified in both extracts EEP2 and EEP3, it was not present in EEP1. Additionally, rutin and laricitine were not detected in the EEP3 extracts. The differences are mainly attributed to variations in plant sources and the ecological environments from which Algerian propolis was harvested (Teggar *et al.*, 2023).

3.2. Scanning Electron Microscope (SEM) analysis

The surface of the propolis and the materials of the envelope have been studied by transmission electron microscopy using a high energy electron beam, allowing their structure to be recorded. The micromorphology of the propolis shows that it is spherical and crystallised, with a smooth surface. Figure 4 shows different morphologies of the samples.

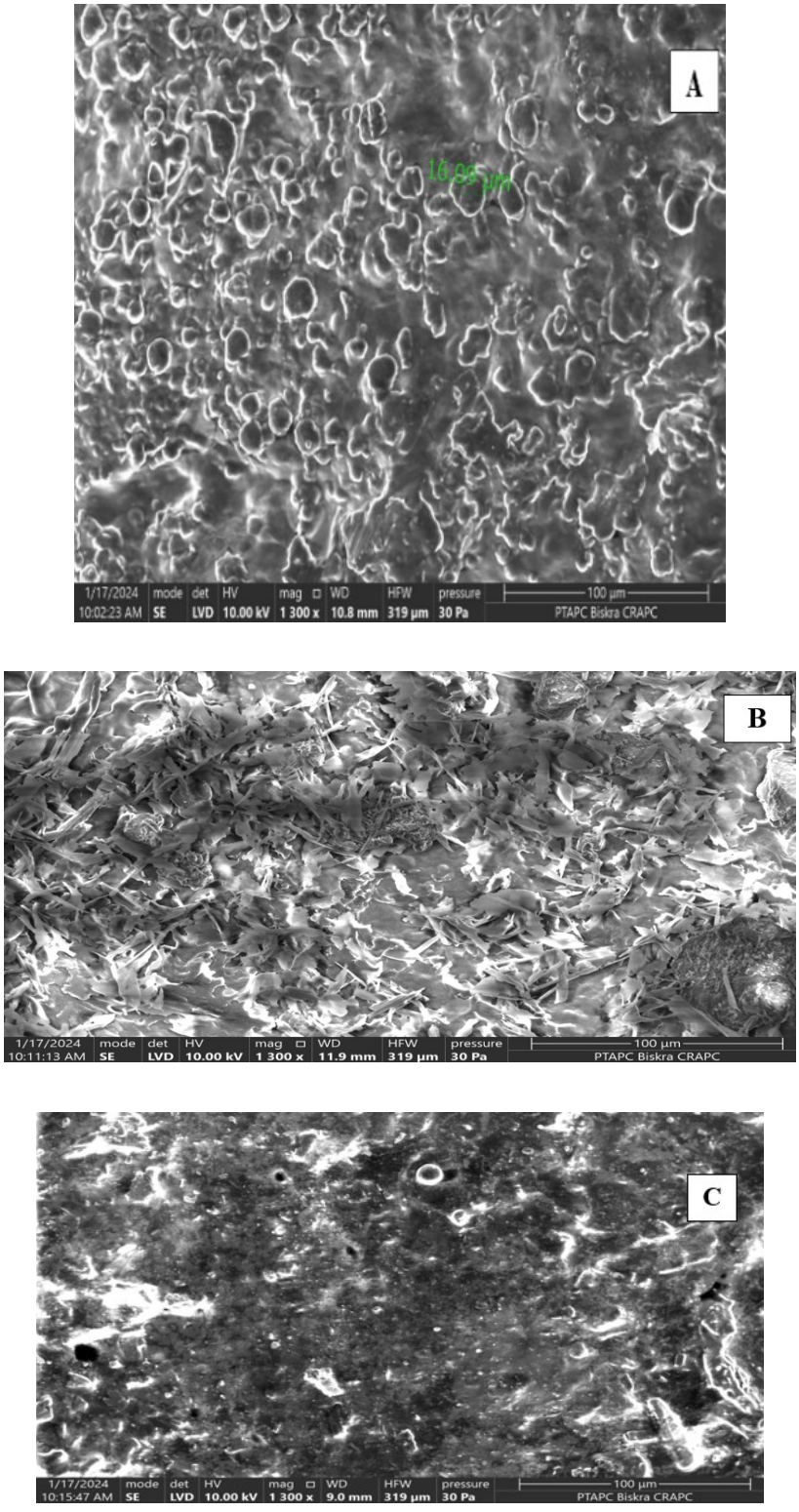

Figure 4. Observation of the micro morphological structure of propolis by scanning electron microscopy (SEM). A: EEP from Ouled Madhi (EEP1); B: EEP from Maadid (EEP2); and C: EEP from Boussada (EEP3).

3.3. Antibacterial activity against two *Paenibacillus larvae* isolates

The various samples analyzed allowed the isolation of two strains of *P. larvae* on the selective MYPGP medium the PP strain isolated from pollen and the PM strain isolated from honey. These two strains show the phenotypic characteristics of the *P. larvae* species indicated in the bibliography (small, regular, flat, or slightly raised shape, etc.). Moreover, they have a whitish colour tending slightly towards beige, and the following biochemical and physiological characteristics: catalase, nitrate reduction, oxidase test (Hamdi *et al.*, 2013). Thus, the antibacterial potential of ethanolic extracts of propolis harvested from Ouled Madhi, Maadid and Bousaada (EEP1, EEP2 and EEP3 respectively) against the strains PP and PM, was determined by the disc diffusion method. The results showed the high sensitivity to the different concentrations of propolis tested with inhibition zones (IZ) from 11.33 ± 1.52 to 41.66 ± 2.88 mm for the strain PP and higher for the PM from 29.66 ± 0.28 mm to 54.66 ± 0.57 mm. On the other hand, the antibiotic oxytetracycline (OT) gave low IZ ranging from 9.33±0.57 to 13.66±1.52 mm. According to the values recorded, the propolis extracts tested at different concentrations exerted a considerable and significant effect against the PP and PM strains compared with the antibiotic oxytetracycline. We also noted that propolis (EEP3) has better antimicrobial properties than propolis (EEP1) and (EEP2) (Figure 5).

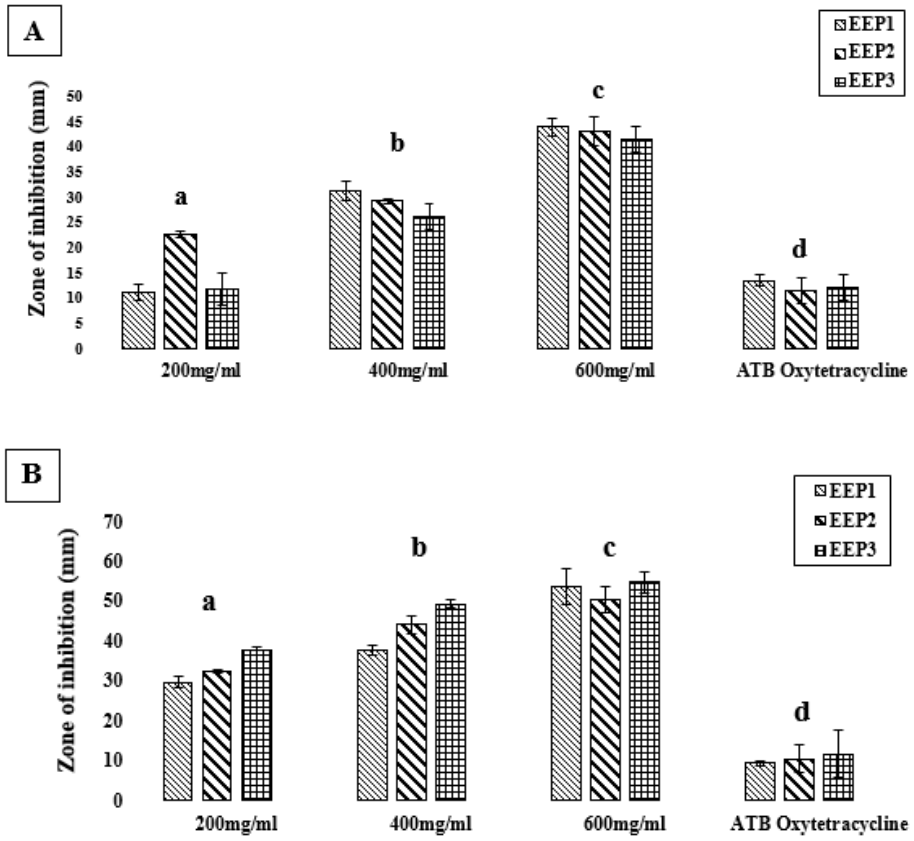

Figure 5. Antibacterial activity of the three propolis extracts (EEP1. EEP2 and EEP3) on the PP isolate (A) and the PM isolate (B) of *P. larvae* (ANOVA indicates a significant difference between the different concentrations tested (p <0.0001) and Tukey classifies each in a separate group a, b, c and d, n =3).

The results of the sensitivity/resistance test of the two bacterial isolates PP and PM with the antibiotic OT are shown in Figure 6.

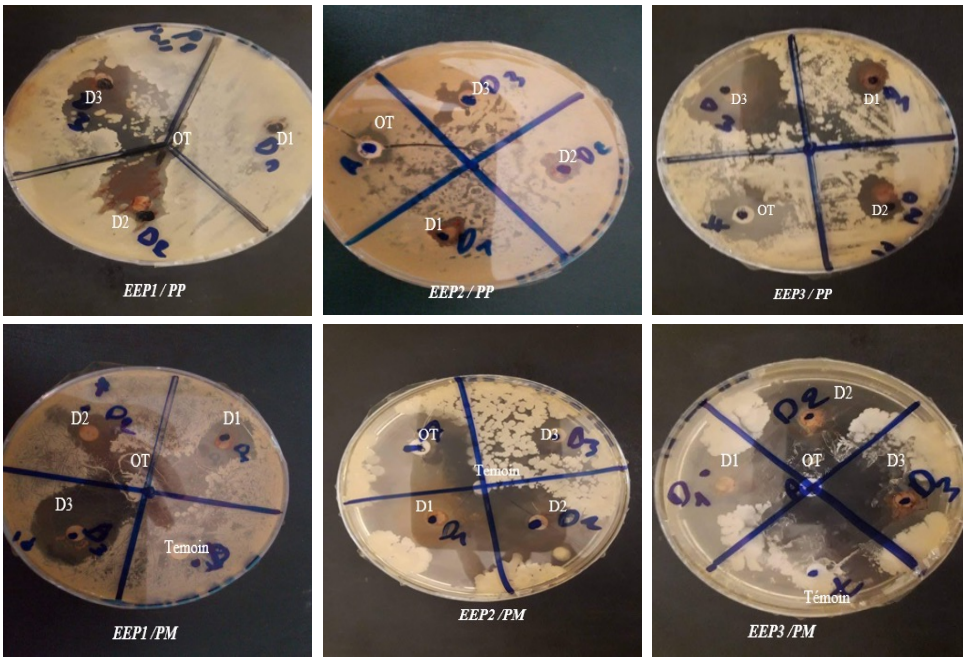

Figure 6. Antibacterial activity of three doses of the ethanolic propolis extracts against different isolates of *P. larvae* (PP and PM strains). (Dose: D1: 200 mg/ml, D2: 400 mg/ml, D3: 600 mg/ml and temoin: control).

We have considered bacteria with a diameter of less than 12 mm as resistant (non-sensitive), those with a diameter greater than 16 mm sensitive, and those with a diameter of greater than 46 mm highly susceptible (Kochansky *et al.*, 2001) (Table 2).

Table 2. Activity comparison of ethanolic propolis extracts (EEP1, from Ouled Madhi; EEP2, from Maadid; EEP3, from Bousaada) against bacterial strains isolated from *Paenibacillus larvae* (PP isolated from pollen; PM isolated from honey).

Bacterial strains	Ethanol Extracts	Dose (mg/ml)	Zone of inhibition (mm)	Categories
PP	EEP1	200	11.33±1.52	resistant
		400	31.33±1.15	susceptible
		600	44±1.73	susceptible
	EEP2	200	22.66±0.57	susceptible
		400	29.33±0.57	susceptible
		600	43.16±2.75	susceptible
	EEP3	200	11.83±1.75	resistant
		400	26.33±1.15	susceptible
		600	41.66±2.88	susceptible
PM	EEP1	200	29.66±0.28	susceptible
		400	37.67±1.15	susceptible
		600	53.6±2.42	very sensitive
	EEP2	200	23.33±0.57	susceptible
		400	44.16±1.04	susceptible
		600	50.33±4.16	very sensitive
	EEP3	200	37.5±0.86	susceptible
		400	49.33±1.15	very sensitive
		600	54.66±0.57	very sensitive

3.4. *Nosema* spp. antagonism

3.4.1. Detection and screening of *Nosema* spp

In the course of our research, we assessed the infection rate of microsporidian *Nosema* spp. in the ten colonies of the apiary surveyed in the region of Blida. To count the number of spores per bee, individuals taken at random were divided into 10 crates at a rate of 20 bees per crate (Figure 7) a total of 200 bees were studied. These individuals were subsequently used in biological treatments with propolis.

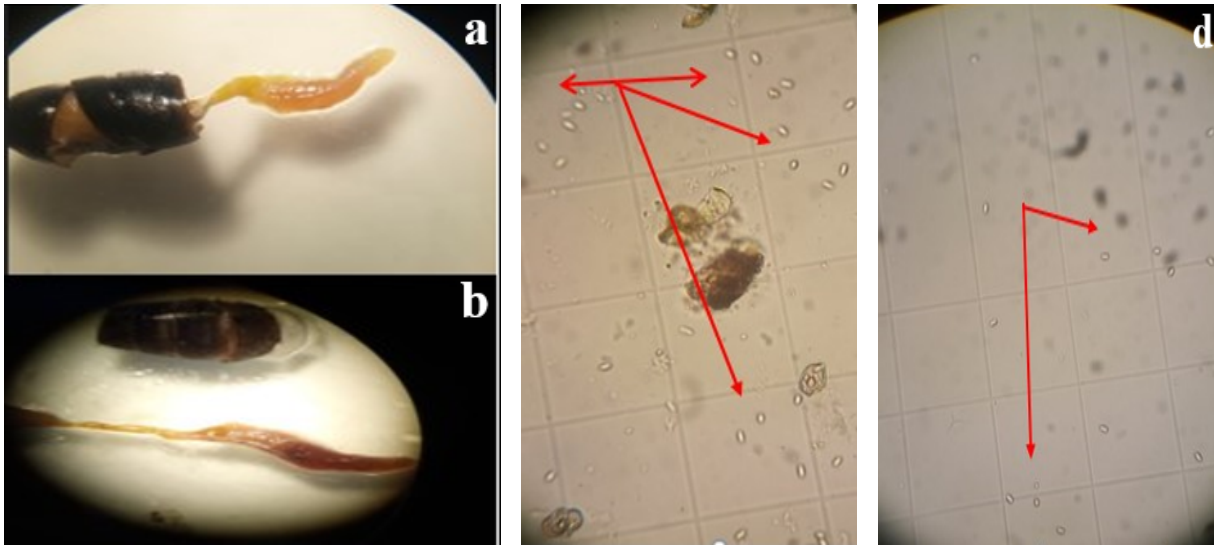

Figure 7. Detection of *Nosema* spp. in honeybees (a: healthy ventricle; b: ventricle with *Nosema*; c: quantification of *Nosema* spores under the light microscope (Gr x 40) before treatment; d: quantification of *Nosema* spp. spores after treatment).

3.4.2. Antiparasitic activity

The antiparasitic activity of the ethanolic extracts of propolis in Figure 8 was evaluated by calculating the number of spores per Malassez slide for 10 days after treatment. The results obtained enabled us to assess the resistance/sensitivity of *Nosema* sp. to propolis by comparing the number of spores detected after treatment with that obtained before treatment (see previous section). We found that the antiparasitic power varied from one extract to another and from one concentration to another. The efficacy of EPs is very marked, with a significant decrease for EEP1, EEP2, and EEP3 in all the concentrations tested (60 mg/ml, 40 mg/ml, and 20 mg/ml). The number of spores present in the intestines of treated bees was very low in the high-concentration EEP3 extract. On the other hand, the spore load in bees treated with diprim (TMP-SMX) remained high.

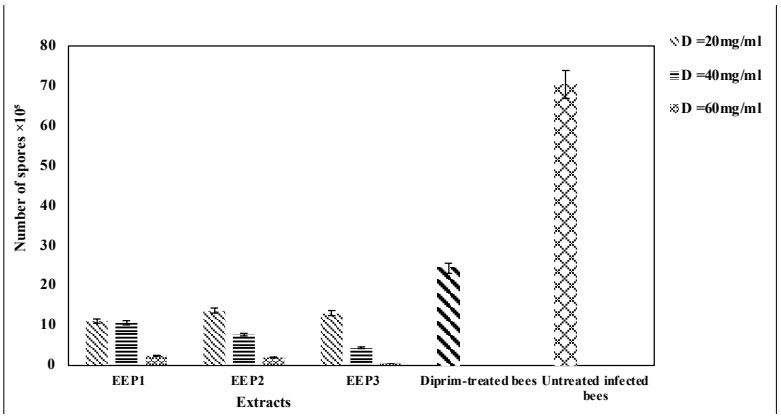

Figure 8**.** Antiparasitic effect of different propolis extracts applied at three doses (D) on bees infected with *Nosema* spp. (ANOVA shows a highly significant difference at a 5% threshold for EEP1 and EEP3 and $p<0.006$ for EEP2).

In addition, for the individuals treated with EEP1, EEP2, and EEP3, the consumption of syrup and pollen was recorded over the 10 days of the experiment (Figure 9).

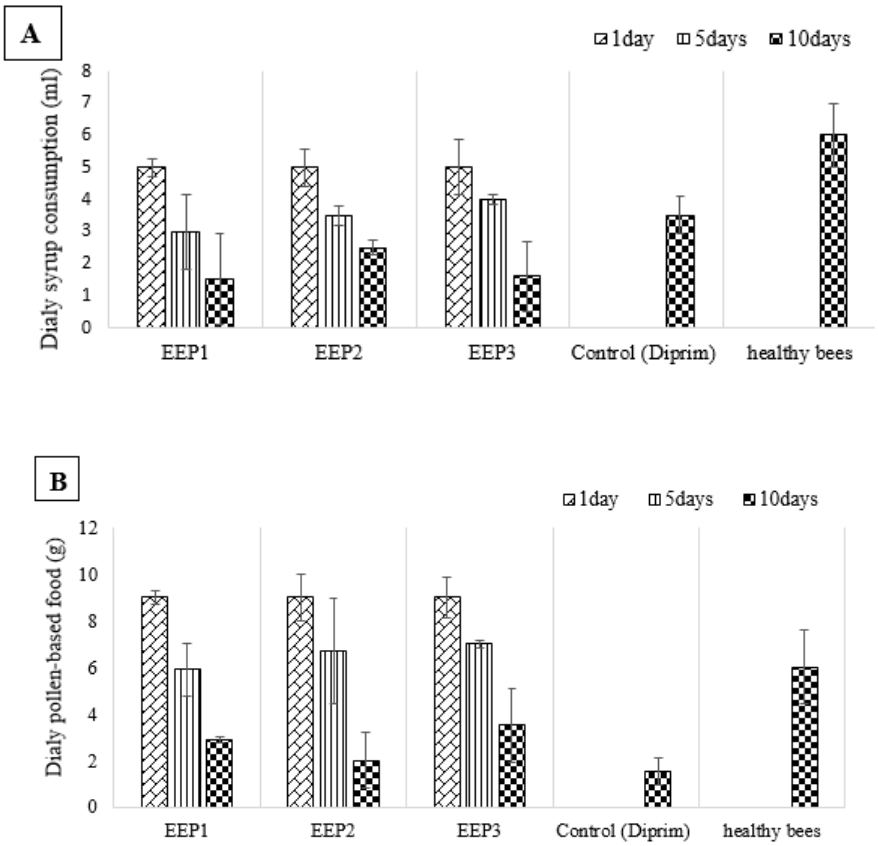

Figure 9. (A): Daily consumption of syrup (ml) and (B): pollen-based food (g) by infected individuals of *Apis mellifera* honeybees and treated with three doses (D) of ethanolic extracts of propolis compared to healthy individuals for 10 days (D1: 20 mg/ml, D2: 40 mg/ml, D3: 60 mg/ml).

In the case of syrup, we observed a reduction in consumption in EP-treated colonies compared to healthy colonies. This variability in food consumption appears to be a characteristic of *Nosema,* which develops rapidly and produces millions of spores. As mentioned in the literature, particularly in studies on *Nosemosis* infection (Martín-Hernández *et al.*, 2011; Mura *et al.*, 2020; Gómez-Moracho *et al.*, 2021). To this end, we carried out tests and statistical analyses using generalized linear regression on the effect of diet at different doses of propolis different types. We compared bee parasitic charge-using univariate generalized linear models (GLM), as well as negative binomial generalized linear analysis (GLM negative binomial) and Poisson test generalized linear analysis (GLM test Poisson) (Figure 10).

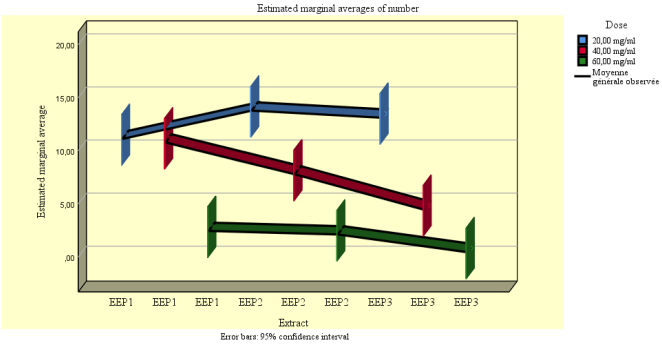

Figure 10**.** GLM test results: the antiparasitic effect of propolis was tested at different concentrations. The difference between treatments was significant (GLM, $p < 0.05$) using univariate generalized linear models.

It seems that the antagonistic effect had a significant influence on the number of spores after exposure to the treatment. Interestingly, the number of spores decreased significantly more when the bees were fed a diet containing propolis extracts. We also observed that the univariate GLM test (GLM: F = 7.156, $p < 0.000$). As well as the Poisson GLM test, all three extracts have values between (GLM test Poisson: $p < 0.000$ and $p < 0.001$, and for the concentration 40 mg/ml: $p < 0.001$ and $p < 0.002$; GLM test Poisson: $p < 0.069$ and $p < 0.097$ for the 60 mg/ml). It should also be noted that all extracts tested positive for *Nosema* spp. It was also found that the statistical results of the GLM negative binomial test showed considerable variability in spore loads after propolis treatment. This information is also illustrated in Figure 8. As indicated in the previous report, the linear regression model (GLM negative binomial) applied to extracts EEP1, EEP2 and EEP3 revealed significant results. Indeed, the *p-values* are less than 0.007, 0.08 and 0.148 for EEP1 extracts respectively. In our statistical analysis, the results of the linear regression model (GLM negative binomial) reveal that the variables Dose 1, Dose 2 and Dose 3 are statistically significant at the confidence level of $p < 0.004$, $p < 0.16$ and $p < 0.186$ respectively of the EEP2. Furthermore, regression analysis of the EEP3 extract indicates that variables D1, D2 and D3 are statistically significant at the threshold of $p < 0.005$, $p < 0.052$ respectively. We observed a decrease in syrup consumption in infected groups compared to healthy bees (Figure 10). This variability in food consumption appears to be a distinctive characteristic of *Nosema* spp., which develops rapidly, producing several million spores.

4. DISCUSSION

Analysis of the chemical composition of propolis extracted from M'sila using HPLC chromatography has revealed the presence of several chemical compounds. The ethanolic extract rich in gallic, rutin, *p*-coumaric, caffeic acids corresponds to the results reported by Segueni *et al.* (2022) in their UFLC/MS-MS study on Algerian propolis extracts. In addition, phenolic compounds have also been identified in Chilean propolis and shown to contain key compounds such as pinocembrin, quercetin and CAPE by Alvear *et al.* (2021). Then, similar results were obtained for Turkish propolis with *p*-coumaric acid 124 mg/g EEP and epicatechin 6.40 mg/g EEP (Sevim *et al.*, 2021). Caffeic acid, *p*-coumaric acid, ferulic acid, naringenin, pinocembrin, chrysin, galangin, pinobanksin and quercetin were also detected in Moroccan propolis (Touzani *et al.*, 2019). In addition, *p*-coumaric acid levels between 0.6 and 2.73 mg/g EPA (aqueous propolis extract) were also observed in extracts of propolis samples collected from three different locations in the province of Buenos Aires, Argentina (Fangio *et al.*, 2019). The results of a study by Giménez-Martínez *et al.* (2024) showed that the main component was epicatechin, which accounted for 40% of the total analysed, with a concentration of 135.06 ppm. Woźniak *et al.* (2022) found that the propolis from Poland contained a high concentration of epicatechin. Some studies have revealed a relationship between biological activities and the phenolic compounds in propolis (Touzani *et al.*, 2019; Bouzahouane *et al.*, 2021; Ożarowski *et al.*, 2022; Widelski *et al.*, 2023). Furthermore, other investigations have revealed a correlation between anti-*Paenibacillus larvae* activity and phenolic compounds in propolis (Giménez-Martínez *et al.*, 2019; Fangio *et al.*, 2019). However, the chemical structure of propolis is complex. It varies considerably depending on environmental factors and the flora and vegetation of the region where it is produced (Ecem Bayram *et al.*, 2020). In the context of this research, similar results have been obtained by authors who have reported on the micromorphology of propolis analysed by means of SEM (Zhang *et al.*, 2023; Saleh *et al.*, 2023).

The Food and Agriculture Organization of the United Nations (FAO) has identified three factors that contribute to the emergence of diseases in bees. These elements include (i) the genetic origins and hygienic behaviour of queens; and (ii) pathogens, such as parasites, bacteria, viruses, fungi, and protozoa, which vary in terms of presence, virulence, and infectious load. Moreover (iii) environmental factors, such as humidity, temperature, and the presence of nectar plants, as well as incorrect beekeeping practices (El-Seedi *et al.*, 2022). Our study detected *Paenibacillus* in honey and pollen collected using a selective culture medium (MYPGP). Similar results have been reported in other studies (Bakonyi *et al.*, 2003; Grubbs *et al.*, 2021). A study has already been carried out by Ansari *et al.* (2017) in Saudi Arabia and revealed that all colonies of *P. larvae* isolates are distinguished by their small size (3 mm in

diameter), regular shape, roughness, elevation, and brightness in MYPGP medium. Bees, like all other living species, are sensitive to bacterial infections, particularly those caused by American foulbrood. To combat these bacterial diseases, numerous studies in the literature (Bastos *et al.*, 2008; Isidorov *et al.*, 2017) highlight the antibacterial capacity of propolis. Our study confirms this efficacy and the significant inhibitory effect of this substance against the bee pathogen *P. larvae* according to a study by Bilikova *et al.* (2013), the bioactive constituents of propolis, such as pinocembrin, may be responsible for this inhibitory activity.

The substances chrysin, rutin, CAPE, myricetin and pinocembrin substances present in samples of chestnut propolis could be responsible for antibacterial activity against Gram+, Gram- bacteria and against yeasts and fungi (Sönmez *et al.*, 2023) and Altuntaş *et al.* (2023) revealed the presence of caffeic, *p*-coumariac and ferulic acids, as well as pinobanksin and apigenin in twenty-four samples of propolis from Turkey, these compounds were identified as being responsible for antimicrobial activities against the microorganisms *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. Other bioactive compounds identified and studied in propolis in fifteen regions of Poland are apigenin, catechin, chrysin, epicatechin, galangin, kaemperole, myricetin, naringenin, pinobanksin, pinocembrin, pinostrobin, quercetin, caffeic acid, cinnamic acid, coumaric acid, 3-hydroxycinnamic acid, ferulic acid, syringic acid, sinapic acid, vanillic acid and CAPE (caffeic acid phenethyl ester), which have remarkable antimicrobial properties (Woźniak *et al.*, 2022). Iranian propolis has remarkably effective antimicrobial properties against the pathogens *Candida albicans, Escherichia coli* and *Staphylococcus aureus*. This remarkable antimicrobial activity could be attributed to the presence of active components such as flavonoids, phenolic acids and terpenes in the composition of Iranian propolis (Afrouzan *et al.*, 2018).

The study of the association between the chemical composition of propolis from different geographical regions, such as Brazil (Salomao *et al.*, 2008) and Romania (Nichitoi *et al.*, 2021; Vică *et al.*, 2024), and its biological activities, such as antimicrobial properties, makes it possible to identify the active principles and is a fundamental tool for determining the substance responsible for this beekeeping product.

Furthermore, worldwide, and especially in the United States, several studies have reported the use of antibiotics, including oxytetracycline hydrochloride (OTC) to treat honey bee colonies infected with *Paenibacillus larvae* (Özkırım *et al.*, 2014), this justifies our choice for this substance to compare the propolis results obtained. Nevertheless, Özkırım *et al.* (2014) in a comparative study of the activities of Greek and Turkish propolis against *Paenibacillus larvae* noted that the intensive use of antibiotics (OTC) could cause the formation of residues in hive products, particularly honey, which diminishes their quality and makes them more difficult to market.

This is another reason why it is important to replace current methods of combating these diseases with natural, healthy methods. We observed that the three types of propolis taken from different locations in the M'sila region (Maadid, Ouled Madhi, and Boussada) were effective against *P. larvae* but they did not have the same antimicrobial activity. We noted that propolis (EEP3) had a significant antimicrobial power compared with propolis EEP1 and EEP2; this variation in results may be due to the phytochemical composition of each propolis as well as the botanical origin of each. Algerian propolis is of exceptional quality and is also made up of various phytochemicals such as phenolic compounds and flavonoids (Segueni, 2011; Soltani, 2017).

For *Nosema* spp., the results of our treatments are very positive, but their effectiveness differs from one concentration to another. During screening, we noticed that the colonies of the bees studied showed particular symptoms such as weakening, and diarrhoea, and the bees clustered together to die. To combat *Nosema*, in recent years many scientists have experimented with natural methods, in particular made from plants and their derived compounds. As a possible anti-*Nosema* treatment, including essential oils, ethanolic and methanolic extracts (Dumitru *et al.*, 2017; Yilmaz *et al.*, 2020). In addition, research is currently underway to develop and evaluate potential alternative treatments (Porrini *et al.*, 2011, 2017, 2020). The propolis extracts tested in our study were generally beneficial for bee survival and reducing the *Nosema* spp. load of infected bees, while being non-toxic to healthy bees. Recent research by Arismendi *et al.* (2018) in Chile has shown that adding propolis to the diet over

some time can cause physiological alterations in treated bees, making it important to rationalize the concentrations applied. The authors added that the methanolic extract of propolis decreased the load of *N. ceranae* in bees that underwent treatments compared to untreated bees. Our results are also in line with those of Suwannapong *et al.* (2018) who showed in an interesting study that bees treated with propolis extract after infection with *N. ceranae* spores had a significantly longer lifespan and reduced parasite load. In addition, they confirmed that the use of bee propolis extract can be an effective treatment for not only improving the health of bees by reducing the parasite load of *N. ceranae* but also counteracting one of the main pathological effects, known as energy stress, which is manifested by a decrease in trehalose levels in the haemolymph. Recently, research carried out in Italy by Mura *et al.* (2020) showed that the propolis used prevented the proliferation of *N. ceranae* spores in the bees' intestines. In addition, the bees did not show any clinical signs or other adverse effects. Another *in vitro* study was conducted to assess the efficacy of a natural product based on herbal essential oils for treating *Nosema* in bee colonies. Certain compounds of laurel (*Laurus nobilis*), eucalyptus (*Eucalyptus globulus*), thyme (*Thymus vulgaris*), and garlic (*Allium sativum*) have an inhibitory effect on *Nosema* (Yılmaz *et al.*, 2020; Formato *et al.*, 2022; ÖzüiÇli *et al.*, 2023). Finally, with regard to phytochemical analysis, the data obtained confirmed that phenolic molecules are one of the main components of propolis; they are responsible for its antagonistic activity.

Therefore, the use of propolis as an insecticide can help us to minimize the problem of environmental pollution caused by the use of synthetic insecticides. It can also reduce the ever-growing problem of insecticide resistance. Propolis is a complex natural product, and it is unlikely or very slow to have various components with different actions (Ararso & Legesse, 2016, Brahimi *et al.*, 2025).

5. CONCLUSION

Our study showed that the three propolis extracts had antibacterial activity against *Paenibacillus larvae* and antiparasitic activity against *Nosema* spp. It is a promising alternative for biological control of biotic stressors in *Apis mellifera*. These effects are in addition to the other beneficial effects of propolis in beehives. All three extracts were practically equally effective. Therefore, this confirms that Algerian propolis is rich in sources of natural compounds with a biological impact on bee diseases. This activity is strongly linked to the composition and concentration of the active compounds in propolis. It was important to identify them, purify them and study their biological composition.

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Supplementary material

Not applicable.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Authorship contribution statement

Hibat-Allah Brahimi: investigation, formal analysis, writing – original draft, Software, Conceptualization, Visualization, Writing – review & editing.

Hakima Oulebsir-Mohandkaci: investigation, formal analysis, Validation, Visualization, Writing – review & editing.

Abdelhamid Guelil: methodology, formal analysis, Validation, Writing – review & editing.

Moufida Saidani-Tounsi: Formal analysis, Resources, Validation.

All authors read and approved the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Statement on the use of Artificial Intelligence

Not applicable.

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