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Bioactive molecules from Algerian propolis with therapeutic potential against the oral pathogen *Enterococcus faecalis* and *Chromobacterium violaceum CV026*

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Abstract

This research aims to analyse the richness of propolis ethanolic extract from Kherrata (EEPKh) in phenolic and flavonoid compounds and its antioxidant effects using different methods. Further investigations were conducted to evaluate the antibacterial and antibiofilm potential of propolis against *Enterococcus faecalis* strains originated from oral diseases. The antiquorum-sensing ability against *Chromobacterium violaceum CV 026* was also investigated. The results revealed that ethanolic extract contains a high content of phenolic and flavonoid compounds, with an amount of 734.3 9± 11.54µg GAE.mg^{-1} of Extract and 224.30 ± 0µg QE.mg^{-1}of extract respectively. Caffeic acid (23.79 mg.g^{-1}), hesperetin (15.42 mg.g^{-1}), cynarin (7.59 mg.g^{-1}), apigenin (5.91 mg.g^{-1}), naringenin (4.90 mg.g^{-1}), and kaempferol (3.43 mg.g^{-1}) were identified as the major compounds by the HPLC-DAD analysis. The antioxidant activity showed good scavenging and reducing abilities. Furthermore, EEPKh demonstrated high antibacterial potency against *E. faecalis* strain 2 at concentration 20 mg.mL^{-1} with an inhibition diameter of 20.33 ± 0.57 mm. The MIC and MBC values were found to range between 0.625 and 10 mg.mL^{-1}. Biofilm formation by *E. faecalis* strains was inhibited at MIC with a percentage ranging from 65.93 ± 1.11 to 51.54 ± 0.81%. Quorum sensing mechanisms in *CV 026* was inhibited by EEPKh, with diameter zone of 11.16 ± 0.29 mm at MIC. This study indicated that propolis extract is considered as a new source of natural medication with therapeutic potential against oral pathology caused by free radicals, *E. faecalis*, biofilm formation and quorum-sensing.

Introduction

Apitherapy is a branch of traditional and alternative medicine that uses products harvested and transformed by bees for therapeutic purposes (Kolayli and Keskin 2020). Bee glue is one of the most important products of the hive collected by honey bees from a diversity of botanical sources

(Zulhendri *et al*. 2021). It has been widely recognized for a long time for its numerous medicinal benefits including antimicrobial, antioxidants, and anti-inflammatory properties (Boulechfar *et al*. 2022; dos Santos *et al*. 2022). The medicinal efficacy of propolis is linked to its various chemical compounds, including over 300 biologically active components such as polyphenols, essential oils, amino acids, and waxes (Castaldo and Capasso 2002; Piccinelli *et al*. 2011). Extraction is a critical step that requires a proper protocol to obtain the desired propolis components. Maceration is the most common and traditional method to extract the active constituents of propolis (Bankova *et al*. 2021). Polyphenolic compounds in propolis increased with increasing concentration of ethanol in the solvent. This is probably because the polyphenolic compounds in propolis could be dissolved in ethanol better than in water. The optimum concentration of ethanol in water was found to be 70 – 95 % alcohol, most often 70 – 80 % (Dent *et al*. 2013; Bankova *et al*. 2021).

Oral infections are among the most common diseases that affect humans. The majority of these infectious diseases are induced by microorganisms living in biofilms (Colombo *et al*. 2015).Oral biofilm plays a crucial role in the development of several oral diseases, including periodontal disease and dental caries. Bacteria present in the biofilm can spread to other organs or tissues of the human body through bacteremia and cause systemic diseases (Larsen and Fiehn 2017; Mosaddad *et al*. 2019). Biofilms are among the well-known virulence factors adopted by bacteria. This phenomenon confers to bacteria a great resistance capacity toward multiple antibiotics, thus increasing mortality rates; biofilm eradication has become an important research topic in recent years (Ramachandran *et al*. 2023).

E. faecalis is an antibiotic-resistant microorganism, Gram-positive cocci, a facultative anaerobe, and an important opportunistic bacterium linked to a number of human diseases such as urinary tract infection, endocarditis, and oral infections (Kouidhi *et al*. 2011; Najafi *et al*. 2020). The pathogenicity of *E. faecalis*, as well as its resistance to the host immune system and antimicrobial medications, has been related to virulence factors such as extracellular surface protein, gelatinase, aggregation substance, collagen adhesion, and their ability to form biofilm, which are controlled by a quorum-sensing (QS) phenomenon (Ali *et al*. 2017; Bhardwaj *et al*. 2017; Najafi *et al*. 2020).

The QS system is a chemical communication process between bacteria that involves gene regulation in response to cell density, which influences a variety of functions such as virulence, acid tolerance, and biofilm formation (Basavaraju *et al*. 2016).The mechanism of QS differs between Gram-negative and Gram-positive bacteria. In Gram-negative bacteria, quorum sensing is effected by LuxI/LuxR, which uses acyl-homoserine lactones (AHLs) as signalling molecules, whereas in Gram-positive bacteria, quorum sensing is effected by oligopeptides, which use small peptides as signalling molecules (Yada *et al*. 2015). For instance, *Chromobacterium violaceum*is a pathogenic, Gram-negative mutant bacterium widely used as a model for quorum-sensing studies. This bacterium communicates by quorum sensing via the C6-homoserine lactone signal (C6-HSL) (de Oca-Mejía *et al*. 2015).

Antioxidant mechanisms are related to the homeostasis of the organism, an imbalance between both leads to oxidative stress, which is considered a significant factor in the pathogenesis of oral diseases (dental caries, lichen planus, oral cancer, chronic periodontitis) (Casas-Grajales and Muriel 2017; Kumar *et al*. 2017). The goal of this research was to investigate the phenolic and flavonoid contents in propolis extract as well as its chemical constitution, antibacterial, antibiofilm, and anti-QS properties.

Experimental

Harvesting and extraction of propolis

Propolis was collected in October 2018 in Kherrata district, situated in the Bejaia region (Algeria). The sample was extracted with 80 % ethanol in water for 24 h before being filtered, evaporated, and concentrated at 45 °C.

Phytochemical analysis

The content of total phenols (TPC) in the extract was evaluated according to Boulechfar *et al*.

(2022). Briefly, 20 µL of the prepared extract was combined with 100 µL of Folin Ciocalteu reagent, then 75 µL of Na_2CO_3 (7.5 %) was added. After incubation for 2 h in the dark, the absorbance was read at 765 nm. The result was expressed in terms µg of gallic acid equivalents mg^{-1} of extract (µg GAE.mg^{-1} E).

The total flavonoid content (TFC) was estimated according to Bensouici *et al.* (2020). 50 µL of diluted extract was added to 130 µL of methanol, 10 µL of potassium acetate (1M) and 10 µL of aluminium nitrate (10 %). The absorbance was read at 415 nm after incubation for 40 min. The result was expressed as µg quercetin equivalents per mg of extract (µg QE.mg^{-1} E).

The chemical components of propolis extract were identified by HPLC-DAD. The system is composed of Shimadzu reverse-phase high-performance liquid chromatography (Shimadzu Cooperation, Japan) including a Shimadzu model LC-20AT solvent delivery unit and the Shimadzu model SPD-M20A diode array detection system and controlled by LC-solution software (CBM-20A System Controller Shimadzu). The column temperature was adjusted to 35 °C and the injected volume was 20 µL. The separation was attained using an Inertsil ODS-3 column (4 µm, 4.0 mm × 150 mm) and an Inertsil ODS-3 guard column, with the mobile phase consisting of aqueous acetic acid 0.1% (A) and methanol (B). A sample stock solution was made in methanol at 8 $mg.mL^{-1}$ and filtered through an Agilent 0.45 µm filter. The diode array detector (DAD) was used for detection at a wavelength of 254 nm. The identification of EEPKh compounds was revealed by comparing the retention time of each detected compound with the retention time of different employed standards (fumaric acid, gallic acid, p-benzoquinone, protocatechuic acid, theobromine, theophylline, catechin, 4-hydroxybenzoic acid, 6,7-dihydroxycoumarin, methyl-1,4 benzoquinone, vanillic acid, caffeic acid, vanillin, chlorogenic acid, p-coumaric acid, ferulic acid, cynarin, coumarin, prophylgallate, rutin, trans-cinnamic acid, ellagic acid, myricetin, fisetin, quercetin, trans-cinnamic acid, luteolin, rosmarinic acid, kaempferol, apigenin, chrysin, 4-hydroxylresorcinol, 1,4-dichlorobenzene, pyrocatechol, 4-hydroxy benzaldehyde, epicatechin, 2,4-dihydroxybenzaldehide, hesperidin, oleuropein, naringenin, hesperetin, genistein, curcumin). The results were presented as $mg.g^{-1}$ of crude propolis (Boutellaa *et al.* 2019).

Antioxidant activity

The 2,2-diphenyl-1-picrylhydrazyl method (DPPH) was determined as described by Mazouz *et al.* (2020), using butylated hydroxyanisole (BHA) as a standard antioxidant. Briefly, 40 µL of EEPKh at different concentrations was added to 160 µL of DPPH solution. The absorbance was measured at 517 nm after incubation for 30 min in the dark. The percentage of DPPH scavenging effect was estimated using the formula (Eq. 1):

$$\text{Inhibition (\%)} = \frac{\text{AC-AS}}{\text{AC}} \times 100 \quad (1)$$

where, AC – absorbance of control, AS – absorbance of sample.

The results were presented as a 50 % inhibition concentration (IC_{50}).

The ability to scavenge 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was performed using the method of Mebrek *et al.* (2018). The reaction was generated by reacting 7 mM of ABTS with 2.45 mM of potassium persulfate ($K_2S_2O_8$), then kipping in the dark for 12 h before diluting it with distilled water to an absorbance of 0.700 ± 0.020 at 734 nm. Then, 160 µL of ABTS solution were added to 40 µL of sample solution prepared in methanol at various concentrations. After 10 min the absorbance at 734 nm was calculated. The following formula (Eq. 2) was used to determine the inhibition percentage:

$$\text{Inhibition (\%)} = \frac{\text{AC-AS}}{\text{AC}} \times 100 \quad (2)$$

where: AC – absorbance of control, AS – absorbance of sample.

Butylated hydroxytoluene (BHT) and BHA were used as standard antioxidants.

CUPRAC assay (cupric reducing power) was estimated using the method described by Lekouaghet *et al.* (2020). Extract solution (40 µL)

was combined with copper (II) chloride solution (50 µL), neocuproine ethanolic solution (50 µL) and 60 µL of $CH3COONH_4$ (1 M). The absorbance was read at 450 nm after one hour of incubation. The reduction capacity of the extracts was compared with BHA and BHT. The results were given as $A_{0.5}$ value ($\mu g.mL^{-1}$) corresponding to the concentration, indicating 0.50 absorbance.

The galvinoxyl radical scavenging (GOR) assay was determined using the procedure of Barzegar and Moosavi-Movahedi (2011). Briefly, 40 µL of sample at different concentrations was mixed with 160 µL of a methanolic solution of galvinoxyl (0.1 mM); the mixture was then incubated for 120 min in the dark at room temperature. Absorbance was measured at 428 nm. BHT and BHA were used as standard antioxidants.

The reducing power assay (RP) of the extract at various concentrations was determined using the method of Elkolli *et al.* (2022). 10 µL sample + 40 µL phosphate buffer (pH 6.6) + 50 µL potassium ferricyanide (1 %) $K_3Fe(CN)_6$ ((1 g $K_3Fe(CN)_6$ in 100 mL H_2O)) were mixed and incubated at 50 °C for 20 min. The absorbance was measured at 700 nm after adding 50 µL of trichloroacetic acid (10 %) (1 g of TCA in 10 ml H_2O), 40 µL of distilled water, and 10 µL of ferric chloride solution (0.1 %) (0.1 g of $FeCl_3$ in 100 mL H_2O).

As antioxidant standards, ascorbic acid, tannic acid, and α-tocopherol were used.

Phenanthroline-reducing activity was determined using the protocol of Aissaoui *et al.* (2020). 10 µL of extract solution was mixed with 50 µL ferric chloride (0.2 %) +30 µL phenanthroline (0.5 %) + 110 µL MeOH. The mixture was incubated at 30 °C for 20 min before measuring the absorbance at 510 nm. The results were given as $A_{0.5}$ value, which corresponds to the concentration giving a 0.5 absorbance.

Bacterial strains and culture conditions

Four oral clinical isolates of *E. faecalis* were collected in the Department of Biology, University of Oum el Bouaghi (Algeria). Isolates *E. faecalis AüCC 29212* and *CV026* were obtained from the Department of Microbiology, Mugla University (Tukey). Strains were purified by growing them on bile esculin agar for 48 h at 37 °C.

Antibacterial activity

The disc diffusion test was used to detect the antibacterial ability of the extract (Parija 2012). A volume of 100 µL of suspensions (adjusted to 0.5 McFarland) was spread on Mueller-Hinton Agar. The sterile filter disks, 6mm in diameter, were placed on the surface of the inoculated medium and impregnated with 20µL of the extract in concentrations 20 – 0.625 $mg.mL^{-1}$. Plates were maintained at 4 °C for 1 h to allow the extract to diffuse into the agar. The inhibition zones around the discs were measured in mm after 24 h of incubation at 37 °C. As a negative control, dimethyl sulfoxide (DMSO) was used.

Microdilution method

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined as described by Snoussi *et al.*(2016). In the presence of propolis extract at different concentrations (20 – 0.625 $mg.mL^{-1}$), 10 µL of diluted bacterial suspension (0.5 McFarland) was inserted into each well of sterile 96 well plates containing 170 µL Mueller-Hinton broth .The MIC was determined as the lowest concentration that inhibited bacterial growth. MBC was determined by taking 10 µL of liquid culture from each well that showed no growth, sub-cultured on Mueller-Hinton Agar, and incubated at 37 °C for 24 h. MBC was the lowest concentration, with no visible growth on MHA.

Antibiofilm assay

Antibiofilm capacity of propolis extract (MIC to MIC/16) was conducted according to Ceylan and Ugur (2015) method. 10 µL of diluted bacteria (5×10^5 $CFU.mL^{-1}$) were introduced into wells in the presence of 170 µL of medium and 20 µL of extract. The negative control contained only Tryptose-Soy Broth and bacterial cells. After incubation at 37 °C for 48 h, water was used to remove planktonic bacteria from the walls, which were then stained for 10 min at ambient temperature with a 0.1 % crystal violet solution. After that, the wells were washed once more to remove the crystal violet solution. A 200 µL

volume of glacial acetic acid (33 %) was added to the walls to dissolve the biofilm stains. Finally, at 550 nm, the optical density (OD) was calculated, and the proportion of inhibition of the tested extract was estimated using the equation (Eq. 3):

$$Biofilm\ inhibition\ (\%) = \frac{ODc - ODs}{ODc} \times 100 \quad (3)$$

where: ODc – OD control; ODs – sample.

Anti-QS activity

Five millilitres of warm molten Soft Top Agar were seeded with 100 µL of an overnight *CV026* culture and 20 µL of 100 µg.mL^{-1} C6HSL was added as exogenous AHL source. This was mixed and immediately poured onto the surface of Luria-*Bertani* agar. Wells of 5 mm in diameter were made on each plate after the overlay had solidified. Each well was filled with 50 µL of different concentrations (MIC to MIC/16) of sample. A white or cream-colored halo around this well against a purple lawn of activated *CV026* bacteria was an indication of QSI. After three days of overnight incubation at 30 °C, the inhibition zone was measured in millimetres (Koh and Tham 2011).

Statistical analysis

All operations were realized in triplicate. IC_{50} and $A_{0.5}$ were determined by linear regression analysis. Data were analysed by one-way ANOVA and Student t-test (for DPPH) using Graph Pad Prism software (version 8.0.2). *P*-values >0.05 indicated no significant differences, whereas *P*-values <0.05 were considered as significant.

Results

Amounts of polyphenols

The results of the extraction yield of phenolic and flavonoid contents are presented in Table 1. The EEPKh shows a high phenolic and flavonoid contents estimated to 734.39 ± 11.54 µg GAE.mg^{-1} and 224.30 ± 0 µg QE.mg^{-1}, respectively. On the other hand, the extraction yield showed a remarkable result with a percentage of 58.91 %.

Table 1. Extraction yield, TPCs, and TFCs in propolis ethanolic extract.

Extract	Extraction yield [%]	TPC [µg GAE.mg^{-1}]	TFC [µg QE.mg^{-1}]
EEPKh	58.91	734.39 ± 11.54	224.30 ± 0

The findings are presented as means standard deviations of three parallel measurements.

HPLC-DAD analysis

The identified compounds in the ethanolic extract of propolis were determined as mg.g^{-1} extract (Table 2, Fig.1). Caffeic acid (23.79 mg.g^{-1}), hesperetin (15.42 mg.g^{-1}), cynarin (7.59 mg.g^{-1}), apigenin (5.91 mg.g^{-1}), naringenin (4.90 mg.g^{-1}), kaempferol (3.43 mg.g^{-1}) were detected as the main compounds. Luteolin, quercetin, p-coumaric acid, ferulic acid, hesperidin, protocatechuic acid, and oleuropein were identified in trace amounts. However, the presence of cynarin was recorded for the first time in Algerian propolis extract.

Table 2. HPLC-DAD analysis of propolis ethanolic extract.

Compound	Retention time	Amount of compound [mg.g^{-1}]
Protocatechuic acid	22.39	0.12
4-oh-benzoic acid	31.69	0.07
Caffeic acid	35.19	23.79
p-coumaric acid	40.81	1.89
Ferulic acid	42.92	0.57
Cynarin	43.85	7.59
Quercetin	55.42	1.15
Luteolin	57.87	2.25
Kaempferol	62.48	3.43
Apigenin	64.07	5.91
1.4-diclorobenzene	73.81	87.74
Hesperidin	47.38	0.49
Oleuropein	49.54	0.11
Naringenin	55.51	4.90
Hesperetin	57.47	15.42

Antioxidant activity

The antioxidant capacities are given in Table 3. The results revealed that propolis extract has high

scavenging activity for ABTS and DPPH but less than BHA and BHT. On the other hand, EEPKh demonstrated similar GOR activity to BHA and BHT (P >0.05). For CUPRAC, phenanthroline, and reducing power, EEPKh showed stronger antioxidant activity than the antioxidant standards.

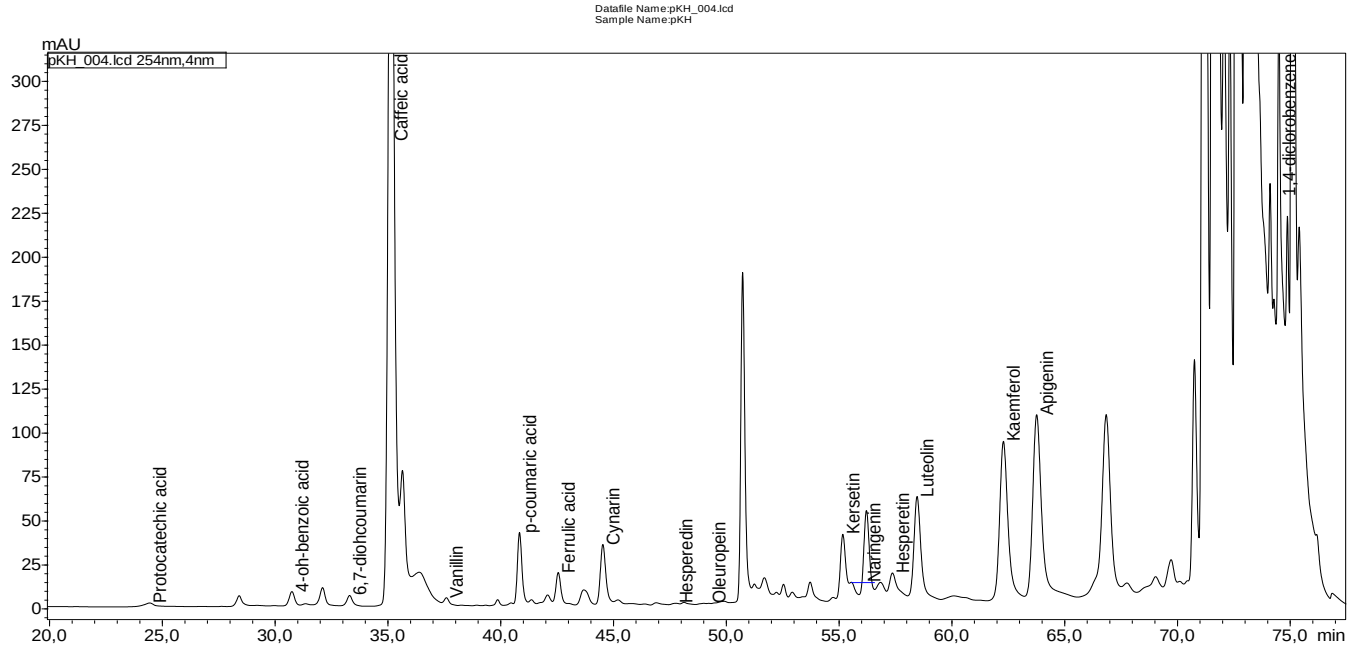

Fig.1. HPLC chromatogram of EEPKh.

Table 3. Antioxidant capacity of propolis ethanolic extract.

Samples	ABTS	DPPH	GOR	CUPRAC	Phenan-throline	Reducing power
		IC_{50} [µg.mL^{-1}]			$A_{0.5}$ [µg.mL^{-1}]	
EEPKh	5.37±0.07^{a}	10.33±0.04^{a}	4.82±1.07^{ab}	0.96±0.69^{a}	0 .51±0.19^{a}	1.19±0.66^{a}
BHT	1.59±0.03^{b}	ND	3.32±0.18^{b}	9.62±0.87^{b}	0.93±0.07^{ab}	NA
BHA	1.03±0.00^{c}	5.73±0.41^{b}	5.38 ±0,06^{a}	3.64±0.19^{c}	2.24±0.17^{b}	NA
Ascorbic acid	NA	NA	NA	NA	NA	6.77±1.15^{b}
Tannic acid	NA	NA	NA	NA	NA	5.39±0.91^{b}
α-Tocopherol	NA	NA	NA	NA	NA	34.93±2.38^{c}

Results are shown as IC_{50} and $A_{0.5}$ mean standard errors (n = 3). Values with different superscripts (a, b, or c) in the same columns are significantly different (P <0.05). NA – not applicable.

Antibacterial activity

The diameter of inhibition zones, MICs, MBCs, and MBC/MIC ratios of the ethanolic extract of propolis are summarized in Table 4 and Table 5. This is the first study to show that Algerian propolis has an antibacterial action against *E. faecalis* in oral diseases. EEPKh exerted high antibacterial activity against all *E. faecalis* strains at 20 mg.mL^{-1} compared to the negative control. The highest activity was observed versus strain 2 with an inhibition diameter of 20.33±0.57 mm. MIC and MBC values were found between 0.625 and 10 mg.mL^{-1}.

Table 4. Anti-bacterial activity of propolis ethanolic extracts.

E. faecalis strains	Zone of inhibition [mm] Concentrations [mg.mL^{-1}]						DMSO
	20	**10**	**5**	**2.5**	**1.25**	**0.625**	**/**
1	16±0^{b}	14.67±2.5^{ba}	13±1.73^{bc}	12.33±1.52^{b}	11±0^{b}	10±0^{b}	-
2	20.33±0.57^{a}	18.67±1.52^{a}	17.67±2.30^{a}	16±1.73^{a}	15.33±0.57^{a}	14±1^{a}	-
3	17±1^{ba}	16.33±0,57^{ba}	15.67±0.57^{ba}	15±0^{a}	14±1^{a}	10±0^{b}	-
4	16±1.73^{b}	14.33±0.57^{b}	12.33±1.15^{c}	-	-	-	-
ATCC 29212	16.67±2.82^{ba}	14.67±1.52^{ba}	13±2^{bc}	12±1^{b}	11±1^{b}	-	-

The results are given as the means and standard deviations of three parallel measurements. The values with different superscripts (a, b) in the same columns are significantly different ($P < 0.05$). (-) – No activity.

Table 5. MIC, MBC, and MBC/MIC ratios of propolis ethanolic extract.

E. faecalis strains	MIC	MBC	MBC/MIC
1	2.5	5	2
2	0.625	1.25	2
3	5	10	2
4	5	10	2
ATCC 29212	1.25	2.5	2

Antibiofilm activity

The biofilm inhibition potential was examined at different concentrations (MIC to MIC/16) as shown in Table 6. The ethanolic extract of propolis exhibited significant (P <0.05) inhibition effects on the biofilm formed by clinical strains. The greatest reduction in biofilm was observed at MIC concentrations against all strains, with a percentage of inhibition ranging from 65.93 ± 1.11 % to 51.54 ± 0.81 %. This inhibition decreased as concentration decrease.

Anti-QS activity

The MIC values of EEPKh against mutant strain *CV 026* were found to be 20 mg.mL^{-1} and the anti-QS properties of propolis extract are presented in (Fig. 2).

The inhibition zone at MIC was found to be 11.16 ± 0.29 mm with no inhibition detected at MIC/4, MIC/8, and MIC/16 for extract and DMSO 10 %.

Table 6. Effects of different concentrations of the propolis ethanolic extracton *E. faecalis* biofilmformation.

E. faecalis strains	Concentration [mg.mL^{-1}]	EEPKh [%] of biofilm inhibition
1	MIC	55.52±1.19^{c}
	MIC/2	48.57±1.28^{a}
	MIC/4	38.91±1.6^{b}
	MIC/8	21.73±2.01^{b}
	MIC/16	12.95±2.29^{b}
2	MIC	51.93±1.2^{d}
	MIC/2	38.21±1.60^{c}
	MIC/4	35.02±0.68^{c}
	MIC/8	-
	MIC/16	-
3	MIC	60.19±0.69^{b}
	MIC/2	52.03±1.89^{ab}
	MIC/4	43.23±2.28^{a}
	MIC/8	22.29±1.91^{b}
	MIC/16	18.27±1.89^{a}
4	MIC	65.93±1.11^{a}
	MIC/2	55.19±0.49^{b}
	MIC/4	35.77±0.49^{c}
	MIC/8	32.09±0.60^{a}
	MIC/16	-
ATCC 29212	MIC	51.54±0.81^{d}
	MIC/2	38.20±1.19^{c}
	MIC/4	-
	MIC/8	-
	MIC/16	-

Results are expressed as means ± SD of three parallel measurements. The values with different superscripts (a, b, c or d) in the same columns are significantly different (P <0.05). (-) – No activity.

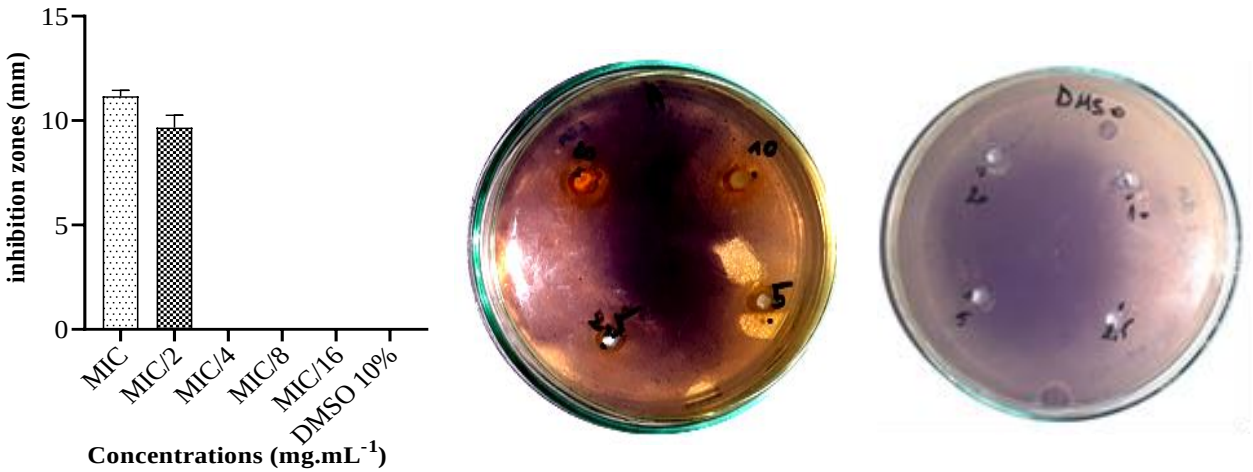

Fig. 2. Anti-quorum sensing activity of different concentration of propolis extract in mm on *CV026*. The data is shown as mean SD (n = 3).

Discussion

Propolis has a rich phenolic profile with numerous pharmacological effects. In this regard, much research seeks to investigate the main compounds responsible for these powerful activities (Aliyazıcıoglu *et al.* 2013). Extraction is a critical step in the isolation and purification of bio-compounds from plants (Jha and Sit 2021). In the present study, the percentage yield of EEPKH (58.91%) obtained by ethanolic maceration was higher than the yields of propolis extract obtained by ethanolic maceration for the cities of Skardu (31%), Islamabad (32%) situated in Pakistan, and Minas Gerais (36.8%) localized in Brazil (Shabbir *et al.* 2016; Saito *et al.* 2021). EEPKh contains a higher amount of polyphenols (TPC: 734.39 ± 11.54 µg GAE.mg^{-1}E, TFC: 224.30 ± 0 µg QE.mg^{-1} E) compared to the amount of polyphenols in propolis from other localities in Algeria (Oum El Bouaghi (TPC: 270.62 ± 1.91 µg GAE.mg^{-1} E, TFC: 54.53 ± 0.20 µg QE.mg^{-1} E), Skikda (TPC: 524.95 ± 2.54 µg GAE.mg^{-1} E, TFC: 47.31 ± 2.54 µg QE.mg^{-1} E)) (Boulechfar *et al.* 2022). Furthermore, Segueni *et al.* (2020) discovered a lower content of total phenolic and flavonoid compounds (TPC: 219.66 ± 1.23 mg GAE.g^{-1}, 61.04 ± 0.45mg GAE.g^{-1}, 148.73 ± 0.93 mg GAE.g^{-1}, 56.98 ± 0.22 mg GAE.g^{-1}, TFC: 41.80 ± 0.84 mg QE.g^{-1}, 17.00 ± 0.97 mg QE.g^{-1}, 21.03 ± 0.04 mg QE.g^{-1}, 10.21 ± 0.0mg QE.g^{-1}) in propolis extracts from four locations in Turkey (Ankara, Bursa, Bilecik, Istanbul).

The chemical profiles of EEPKh after HPLC analysis revealed that the most abundant phenolic and flavonoid compounds identified in propolis extract were caffeic acid, hesperetin, cynarin, apigenin, naringenin, and kaempferol, with quantitative differences between the compounds. Another study discovered that the main compounds in ethanolic extracts of propolis from Oumtboul, Ouadsabt, and Ferdjiwa in Algeria were caffeic and ferulic acids (Daikh *et al.* 2020). The most common compounds found in Turkish propolis samples were caffeic acid phenyl ester, caffeic acid and cinnamic acid, chrysin, and pinocembrin (Guler *et al.* 2021).These quantitative and qualitative differences in phenolic and flavonoid compounds are influenced by various factors such as geographical origin, botanical origin, honeybee's genetics, and season (Mountford-McAuley *et al.* 2021).

Polyphenolic compounds are among the most abundant secondary metabolites in the plant kingdom and have attracted much attention, mainly because of their broad-spectrum applicability in the prevention of human diseases (Bié *et al.* 2023). Propolis was identified as an important source of natural antioxidants such as caffeic acid, hesperidin, and cynarin (Wilmsen *et al.* 2005; Gülçin 2006; Topal *et al.* 2016). The results of the current study showed that EEPKh has strong antiradical activity against free radicals DPPH,

ABTS, GOR, CUPRAC, reducing power, and phenanthroline, which can be attributed to its high concentration of phenolic and flavonoid compounds. According to Boulechfar *et al.* (2022) ethanolic extracts of propolis from different areas of Algeria had higher free radical-scavenging activity for DPPH and ABTS radicals. Moreover, propolis from the region of Souk-Ahras, Algeria demonstrated a powerful antioxidant inhibitory activity against DPPH, ABTS, GOR, and CUPRAC radicals (Ouahab *et al.* 2023). Previous research by Miguel *et al.* (2014) demonstrates that propolis from different locations in Morocco exhibited high antiradical activity regarding both ABTS and reducing power assays. On the other hand, Ozdal *et al.* (2018) reported that Turkish propolis demonstrated high scavenging capacity against the CUPRAC radical. The phenolic and flavonoid compounds in propolis were found to correlate with its free radical scavenging activity (Kumazawa *et al.* 2010; Segueni *et al.* 2020).The emergence and progression of oral pathologies are linked to an increase in bacterial biofilm resistance to antibiotics; Due to the increasing bacterial resistance to currently used antibiotics, great importance is given to natural compounds for the prevention of oral bacterial growth, adhesion, and colonization (Kouidhi *et al.* 2015). Among them, propolis is well known for its powerful antimicrobial and antibiofilm effects against oral anaerobic bacteria, which can be attributed to its various chemical constituents (Uzel *et al*. 2005; de Sá Assis *et al.* 2022). In this context, EEPKh exerted antibacterial and antibiofilm action towards all *E. faecalis* strains, which can be attributed to their high concentration of phenolic and flavonoid compounds. It has been reported that apigenin, rutin, luteolin, naringin, morin, caffeic acid, and its esters effectively inhibit bacterial growth (Kartal *et al.* 2003; Gutiérrez-Venegas *et al.* 2019). The results of this study confirm the findings of other researchers who found that propolis extract had the highest efficacy against E. faecalis planktonic and biofilm forms (Wahjuningrum and Subijanto 2014; Carbajal Mejía 2014). According to Krishnan *et al.* (2010), the MBC/MIC ratios of propolis extract were equal to 2, suggesting that the extract has a bactericide effect against all *E. faecalis* strains. Propolis acts directly on the bacteria by damaging the cell wall, inhibiting bacterial DNA-dependant RNA polymerase and cell division (Bhandari *et al.* 2014). It also affects biofilm formation by damaging the biofilm membrane, decreasing the polysaccharide content in the biofilm, which then *release*s its cellular content (Wahjuningrum and Subijanto 2014). Bacteria regulate their biofilm formation and virulence factors through the QS system, which is controlled by chemical signalling molecules known as autoinductors (Paluch *et al.* 2020). QS can be blocked by stopping the production of the signal molecule, destroying the signal molecule, and preventing the signal molecule from binding to its receptor (Yada et al. 2015). The use of QS inhibitory agents to reduce, or even completely repress, biofilm formation by pathogenic bacteria appears to be a promising approach for the control of bacterial infections (Zhou et al. 2020). This study revealed that EEPKh has antiquorum sensing ability against *CV026*, which appeared as a cream-colored halo around the well against a purple lawn of activated *CV026* bacteria. A previous study by Savka *et al.* (2015) reported that propolis samples from different regions of the United States disrupt QS autoinducer signalling in *CV026*. Ceylan and Alıç (2020) showed that propolis ethanolic extracts originated from possess antiquorum-sensing activity toward *CV026*. The ability of propolis to inhibit and Turkey disrupt QS, biofilm formation in pathogenic bacteria may be attributed to chemical components such as caffeic acid (Kasote *et al.* 2015).

Conclusion

The present study describes the chemical profile of polyphenol-rich propolis collected in the Algerian region of Kherrata, as well as its effect on free radicals, the pathogen *E. faecalis* in both forms (planktonic and biofilm), as well as the QS activity against *CV026*.The extract was found to be rich in phenolic compounds, in which a new flavonoid compound, cynarin, was identified for the first time in Algerian propolis. The results also show that the extract showed significant inhibitory potential against free radicals, *E. faecalis*, and *CV026*, suggesting the potential health benefits of propolis as a natural drug for future therapy of oral infections. Further research is needed to isolate and

identify active chemical compounds that can be used to study their mechanisms of action.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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