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ANTIBACTERIAL ACTIVITIES AND PHENOLIC PROFILES OF SIX MONOFLORAL HONEY TYPES FROM DIFFERENT REGIONS OF MOROCCO

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ABSTRACT

Honey is renowned for its health benefits due to a variety of bioactive compounds that contribute to its antioxidant and antibacterial properties. This study aimed to evaluate the antibacterial activity of six Moroccan monofloral honeys against seven pathogenic bacterial strains (*Proteus mirabilis*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, all isolated from hospital patients. Thyme honey exhibited the highest phenolic content (188.71 ± 6.87 mg GAE/kg) and lowest IC50 in the DPPH assay (15.70 ± 5.15 mg/mL), indicating strong antioxidant activity. Antibacterial tests revealed *Enterobacter aerogenes and Klebsiella pneumoniae* as the most resistant strains, with minimum inhibitory concentration (MIC) values above 50% (w/v) across all honeys. Conversely, *Staphylococcus aureus* was the most sensitive, especially to honeys 2, 4, 5, and 6, with MIC values below 25% (w/v). HPLC analysis identified phenolic compounds such as gallic acid, vanillin, epicatechin, naringin, and rutin in honeys with high antibacterial activity, suggesting these compounds contribute to honey's bioactive properties.

Keywords: Moroccan honey, antibacterial activity, pathogenic bacteria, antioxidant activity, phenolic profile

INTRODUCTION

The prevalence of infectious diseases is increasing globally, driven largely by the rise of antibiotic-resistant bacteria. In fact, Multidrug-resistant (MDR) bacteria pose a critical global health threat, as they undermine the effectiveness of standard antibiotic treatments and lead to persistent, hard-to-treat infections. MDR pathogens have developed resistance mechanisms that render multiple classes of antibiotics ineffective, leaving limited treatment options and increasing healthcare costs, morbidity, and mortality rates worldwide. The rapid spread of MDR bacteria is exacerbated by factors such as antibiotic misuse, overuse, and lack of new antibiotic development **(Aslam *et al*., 2018)**. This phenomenon has intensified the search for alternative therapeutic options, particularly those derived from natural products that generally present minimal side effects. Honey has emerged as one of the most promising candidates for these applications due to its wide range of bioactive properties **(Meo et al., 2017).**

Natural compounds derived from plants have gained increasing recognition as potent therapeutic agents due to their broad spectrum of biological activities. These include antimicrobial properties that target a range of pathogens, anti-inflammatory effects that can modulate immune responses, and antioxidant properties that counteract oxidative stress linked to cellular damage. Among these, phenolic compounds and flavonoids are particularly valuable, as they can disrupt microbial cell membranes, inhibit bacterial growth, and neutralize free radicals. Consequently, plant-derived products, including honey, which is rich in such bioactive compounds, have become promising candidates for treating infections and mitigating the impact of antimicrobial resistance **(Pyrzynska & Biesaga, 2009).** Honey contains over 200 natural compounds, with its composition influenced by factors such as plant species, harvest conditions, and the geo-climatic characteristics of the production regions **(da Silva *et al*., 2016).** This variability contributes to honey's broad therapeutic potential, particularly its antioxidant and anti-inflammatory effects. Many of these effects are attributed to phenolic compounds and flavonoids, which play roles in free radical scavenging and cellular protection. Though the exact mechanisms remain to be fully elucidated, bioactive compounds such as polyphenols, flavonoids, and phenolic acids are believed to be central to honey health benefits, including its ability to mitigate oxidative stress and inflammation in the body **(Yaghoobi et al., 2013).**

Among honey most well-documented properties is its antibacterial activity, which is facilitated by a combination of physical and chemical factors. Six key factors have been identified as contributing to the antibacterial property of honey ; its osmolarity resulting from high sugar content, acidic pH (3.5–4) **(Almasaudi, 2021)**, hydrogen peroxide production **(Brudzynski, 2006)**, phytochemicals such as thymol and pinocembrin, defensin-1 which directly disrupts bacterial membranes and methylglyoxal involved in modifying the behavior and viability of bacteria (**Kwakman *et al*., 2010 ; Rabie *et al*., 2016)**. These combined properties allow honey not only to inhibit bacterial growth but also to support wound healing by creating a moist, antibacterial environment conducive to tissue regeneration **(Scepankova *et al*., 2021).** Research on the antibacterial activity of honey has gained significant attention globally, particularly in the Mediterranean region, where diverse plant species contribute to the unique properties of honey. Studies have demonstrated that honey exhibits a broad spectrum of antimicrobial effects against various pathogens, including multidrug-resistant bacteria. For instance, a study conducted on 21 types of honey from Mount Olympus in Greece revealed that all tested honeys showed antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*, attributed to their hydrogen peroxide and polyphenolic content **(Stagos *et al*., 2018)**. Similarly, research from Algeria highlighted the potent antibacterial properties of local honeys against several Gram-positive and Gram-negative bacteria, emphasizing the role of their physicochemical characteristics, such as low pH and high sugar concentration **(CHETTOUM *et al*., 2023)**. Furthermore, global studies have shown that honeys like Manuka, known for their high levels of methylglyoxal, possess superior antibacterial effects compared to other varieties, underscoring the importance of floral origin in determining honey's efficacy **(Mandal & Mandal, 2011)**. Moroccan honeys are of particular interest for their rich and diverse phenolic profiles influenced by Morocco's unique floral diversity and regional variations. For example, the study carried out by **Lakhmili et al. (2024)** indicates the presence of various phenolic compounds namely caffeic acid, (+)-catechin, vanillic acid, ferulic acid, quercetin, gallic acid and epicatechin in several Moroccan honey samples, such as fennel, eucalyptus, thistle, spurge and thyme honeys. Therefore, the aim of the current research is to analyze the phenolic profiles of six Moroccan monofloral honey samples and assess their antimicrobial efficacy against pathogenic bacteria, to identify specific bioactive compounds contributing to antibacterial activity

MATERIAL AND METHODS

Honey samples

Honey samples were collected directly from apicultures from different regions of Morocco and were freshly analyzed. The authenticity of the different honey samples was verified by melissopalynological analysis in the previous study of **Lakhmili et al. (2024)**. The main botanical origin and the geographical location of each sample are shown in the table 1. The six Moroccan honey samples were stored in sealed containers at a controlled temperature of 25 ± 2 °C until analysis.

Table 1 The different geographical and botanical sources of each honey sample

Botanical origin	Common name	Arabic name	Geographical origin
Euphorbia resinifera	Spurge	الدغموس	Mirleft (South-Morocco)
Thymus vulgaris	Thyme	زعتر	Rich (East- Morocco)
Foeniculum vulgare	Fennel	شمر البسباس	Rural municipality of Jaidat (Marrakesh region-Morocco)
Eryngium ilicifolium	Thistle	شوك	Rural municipality of Bourrous (Marrakesh region-Morocco)
Ceratonia siliqua	Carob	الخروب	Demnate (Beni Mellal region-Morocco)
Eucalyptus sp.	Eucalyptus	شجرة الكينا	Essaouira

Extraction of bioactive compounds

For extraction, 50 g of each tested honey sample was collected and added to 125 ml of methanol in a conical flask. The resulting mixture was vortexed for good homogenization of the components and then centrifuged at 1532 × g for 10 minutes at 25°C. The obtained extracts were filtered using Whatman N°1 filter paper in Stoppard test tubes. The final step involved drying the extracts under reduced pressure at 40 °C. The extracts were stored at a temperature of 4°C until use (**Dzomba, 2012**). Compared to other solvents, methanol offers a favorable polarity that enhances the extraction of both polar and moderately nonpolar compounds, which include the antioxidants and antibacterial agents relevant to this study.

Assessment of the antioxidant capacity (DPPH assay)

A volume of 2 ml of a methanolic solution of DPPH (100 µM) was mixed with 500 µl of honey sample. The resulting mixture was then kept away from light at room temperature for 30 minutes, and the absorbance was measured at 517 nm. A control, containing the methanolic solution of DPPH mixed with distilled water in place of the honey sample was performed to calculate the inhibition percentage (**Kumarasamy *et al.*, 2007**). Sample and control preparations were carried out under identical conditions. The decrease in absorbance was measured using a spectrophotometer, and the capacity of the tested samples to scavenge the DPPH radical was calculated as a percentage of DPPH inhibition (discoloration) using the following equation:

$$DPPH\,\% = \frac{\left(A_{control} - A_{sample}\right)}{A_{control}} \times 100 \qquad \text{Equation (1)}$$

Where $A_{control}$ was measured as the absorbance of DPPH without sample. IC50 values were calculated by linear regression, in which the abscissa is represented by the concentration of the test compounds and the ordinate by the percentage of inhibition (**El-Abbassi *et al.*, 2012**).

Determination of the total phenolic content

The total phenolic content of the honeys studied was determined using the spectrophotometric method of (**Singleton *et al.*, 1999**) with the Folin-Ciocalteu reagent. A volume of 250 µl of the honey extract was introduced into a test tube, to which 750 µL of Folin-Ciocalteu reagent and 750 µL of sodium carbonate (60 g/mL) were added. The mixture was vortexed and kept protected from light for 1 hour and a half at room temperature. Absorbance was measured at 725 nm against a blank containing water instead of a honey sample using a spectrophotometer. A standard curve was performed in parallel under the same operating conditions using gallic acid. The results were expressed in milligrams of gallic acid equivalent per 100 grams of honey (GAE mg/100 g).

Study of antibacterial activity

Strain collection

The bacterial strains used in this study were isolated and identified at the bacteriology laboratory of Ibn Tufail University Hospital Center (CHU) of Marrakech. The strains were obtained from hospitalized patients in different departments of the hospital. There were six strains studied, which are as follows: *Proteus mirabilis, Enterobacter aerogenes, Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Table 2).

Diffusion method or disc method

In this study, the disc-diffusion test was conducted using the method described by **Chandrasekaran & Venkatesalu, (2004**). Honey extracts (1 g) were dissolved in 5 ml of sterile distilled water to a 100% concentration. Sterilized filter paper discs were soaked with 10µl of the honey extract and allowed to dry.
The inoculum was prepared from a 18-hour pure culture in the exponential growth phase. Colonies were suspended in 5 to 10 mL of sterile physiological water with an opacity adjusted to 0.5 McFarland standard, corresponding to approximately 10^5 bacteria per milliliter. Mueller-Hinton agar plates were inoculated using a swabbing method. After applying the honey-treated discs, the plates were incubated at 37°C for 24 hours. Inhibitions zones were measured using a ruler.

Dilution method in liquid medium (MIC determination)

To determine the minimum inhibitory concentration (MIC) of the honey, three concentrations were prepared: 25%, 50%, and 75% (w/v). A bacterial suspension was diluted 1:50 from an 18-hour pure culture grown in Tryptic Soy Broth (TSA). A volume of 100 µL of each bacterial suspension was added to 4900 µL of sterile liquid culture medium. Due to the dark color of the honey, turbidity was difficult to assess, so the contents of each tube were also plated on agar media to observe bacterial growth. For this reason, each tube containing the honey sample and bacterial suspension was plated on agar media. The plates were incubated at 37 °C for 24 h, and the minimum inhibitory concentration (MIC) was determined as the lowest concentration of honey that completely inhibits the visible growth of bacteria.

Table 2 Sensitivity and resistance of the different strains tested against different antibiotics

Bacterial strain	Resistance	Sensitivity
Proteus mirabilis	**CTX**	**AMP, TIC, PIP, AMC, KF, CEFOR, CRO, CTX, CAZ, CFP, FOX, ATM, IMP, ERT, TOB, AK, CIP, NOR, SXT.**
Enterobacter aerogenes	**AMP, AMl, TIC, PIP, KF, CEFOR, CRO, AMC, CTX, CAZ, CFP, FOX, ATM, ERT, TOP, SXT**	**IMI, AK, CT, TOB, CIP, NORF**
Klebsiella pneumoniae	**AMP, TIC, PIP, AMC, KF, CEFOR, CIP, CRO, CTX, CAZ, CFP, FOX, ATM, ERT, TOP, SXT, NOR, TOB**	**AK, CT**
Escherichia coli	**AMP, TIC, CIP, SXT, NOR**	**AMC, KF, CRO, CTX, CAZ, CFP, FOX, G1, CEFOR, TOB, AK, CT**
Pseudomonas aeruginosa	**AMX**	**AMP, TIC, PIP, AMC, CÉFRO, CRO, CTX, CAZ, CFP, FOX, ATM, IMP, ERT, G, TOB, AK, CIP, NOR, SXT**
Staphylococcus aureus	**PG, AMP, AMX, TIC, PIP**	**OXA, AMC, CTX**

AMP: Ampicilline, **TIC:** Ticarcillin, **PIP:** Piperacilline, **AMC:** Amoxicillin/Clavulanic Acid, **CEFOR:** Cefuroxime, **CRO:** Ceftriaxone, **CTX:** Cefotaxime, **CAZ:** Ceftazidime, **CFP:** Cefoperazone, **FOX:** Cefoxitin, **ATM:** Aztreonam, **ERT:** Ertapenem, **TOB:** Tobramycin, **AK:** Amikacin, **CIP:** Ciprofloxacin, **NOR:** Norfloxacin, **SXT:** Sulfamethoxazole, **OXA:** Oxacillin.

HPLC analysis

The analysis was carried out using a Shimadzu LC20 HPLC system equipped with a C18 column (4.6 × 250 mm, 5.0 µm) and a diode array detector for UV–visible absorption spectra. The optimal performance was achieved using a linear gradient elution profile with a binary solvent system: methanol (A) and formic acid diluted in water (1:19) (B), at a flow rate of 1 mL/min. The elution process began with 5% A, maintained for 1 minute, followed by a linear increase to 100 % over 55 minutes, which was then held constant for 4 minutes. The injection volume was 20 µL, and

detection was performed at 280 nm. Phenolic compounds were identified by comparison with reference standards.

Statistical Analysis

Statistical analysis including one-way analysis of variance (ANOVA), and Tukey's post-hoc test was performed using SPSS (Statistical Package for the Social Sciences) version 18.0.0. All the assays were performed at least in triplicate and the results are expressed as the means ± standard deviation (SD). Differences between means with a confidence level of 95% ($p < 0.05$) were considered as statistically significant.
The results were also subjected to a multivariate analysis (principal component analysis).

RESULTS AND DISCUSSION

Anti-radical activity with DPPH and total polyphenol content

The results of the DPPH assay and the polyphenol content (Figure 1 and 2) show that carob honey had the highest IC50 value of 57.55 ± 11.44 mg/mL, while it also had the lowest total polyphenol content among the honey samples, with a value of 127.60 ± 1.25 mg EGA/kg. In contrast, thyme honey exhibited the lowest IC50 value of 15.70 ± 5.15 mg/mL, indicating the highest antioxidant potential, and had the highest polyphenol content at 188.71 ± 6.87 mg EAG/kg. These results confirm the possibility that thyme honey contains the largest amount of free radical scavenging compounds and has the greatest antioxidant potential. A lower IC50 value indicates a higher scavenging capacity of free radicals, as demonstrated by the determination of the polyphenol content of thyme honey.
The analysis revealed notable differences in the antioxidant potential of Moroccan honeys. Eucalyptus honey, which has one of the highest TPC values, demonstrated antioxidant power comparable to that of thyme honey, with no significant difference between these two types. Similarly, fennel and thistle honeys displayed a similar capacity to neutralize free radicals, showing nearly identical TPC levels ($p<0.001$). In contrast, spurge honey exhibited a lower radical-scavenging potential, comparable to that of carob honey, with an IC50 value of 47.2 ± 7.3 mg/mL.
The results indicate a strong correlation between the total polyphenol content and the antioxidant activity of the honey samples, as expected given the well-established antioxidant properties of polyphenols. This relationship is supported by a Pearson correlation coefficient of 0.8 ($p < 0.001$), providing quantitative evidence for this association. Notably, the honey sample with the highest total polyphenol content (thyme honey) also exhibited the lowest IC50 value, indicating the strongest antioxidant activity. Conversely, the honey sample with the lowest total polyphenol content (carob honey) showed the highest IC50 value, indicating weaker antioxidant activity. These findings suggest that the polyphenol content is an important factor to consider when evaluating the antioxidant potential of honey. These findings are consistent with those of **Mouhoubi-Tafinine *et al.* (2016)**, who reported levels of phenolic compounds in Algerian honey extract ranging from 15.84 to 61.63 EAG/100g. According to **Sagdic *et al.* (2013),** the phenolic content and the anti-radical activity in honey are strongly influenced by its floral source, which may explain the variation in the antioxidant activity among the honey samples studied. Furthermore, **Żak & Wilczyńska, (2023)** noted that darker, untreated honeys tend to have higher phenolic content and stronger antioxidant properties compared to lighter and processed samples. These results are consistent with those of **Aazza *et al.* (2014),** who reported that thyme honey from Errachidia region is particularly rich in phenolic compounds and exhibits the highest anti-radical activity among the honeys tested.
The strong antioxidant activity observed in thyme honey suggests potential applications in both food preservation and medical fields. Due to its ability to neutralize free radicals effectively, thyme honey could serve as a natural preservative to extend the shelf life of foods by reducing oxidation. In medical applications, its antioxidant properties might support wound healing and reduce oxidative stress in various therapeutic contexts. Highlighting these potential applications underscores the broader relevance of our findings and the value of thyme honey as a natural source of antioxidants.

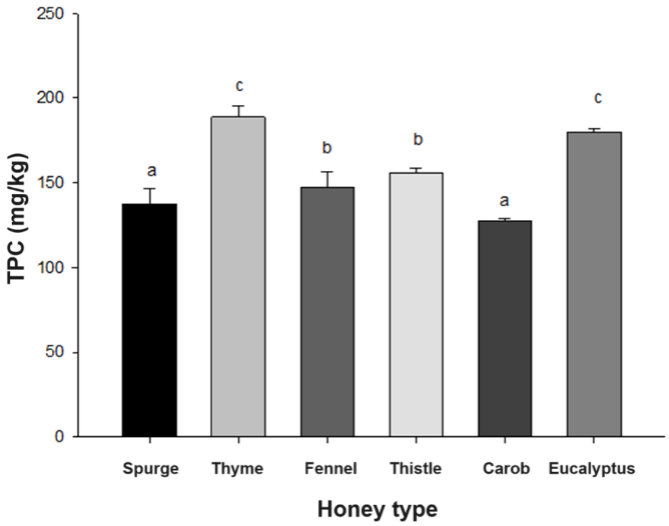


Figure 1. Total polyphenol content of the different honey samples expressed in mg EAG / kg of honey. Each value represents the mean ± standard deviation. Values with different letters are significantly different ($p < 0.05$).

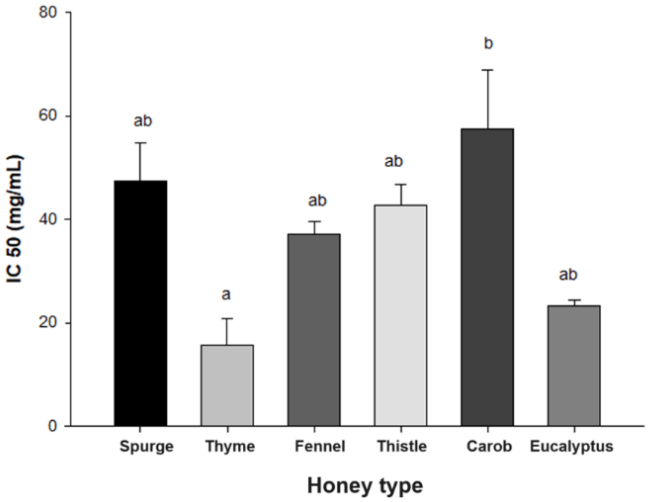


Figure 2 Anti-free radical activity in the DPPH expressed in half maximal (50%) inhibitory concentration of honey samples (IC50 mg/mL). Each value represents the mean ± standard deviation. Values with different letters are significantly different ($p < 0.05$).

Antibacterial activity of honey

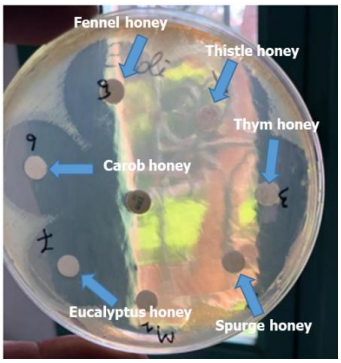


Figure 3 Antibacterial activity of different honeys on *Escherichia coli*.

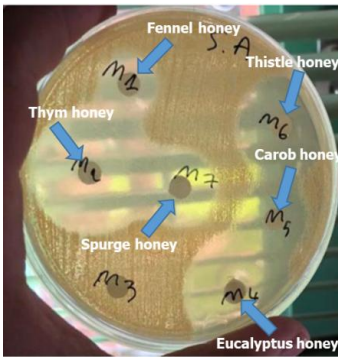


Figure 4 Antibacterial activity of different honeys on *Staphylococcus. aureus*

The study aimed to investigate the antibacterial activity of different types of honey using both the agar diffusion and minimum inhibitory concentration (MIC) methods. The honey concentrations tested ranged from 25% to 100% (w/v), with 100% as the highest concentration. Results demonstrated inhibition of bacterial growth across various strains, with notable sensitivity differences among them. *Staphylococcus aureus* was identified as the most sensitive strain, particularly for thyme, thistle, carob, and eucalyptus honeys (Table 3). As the only Gram-positive strain in the study, *Staphylococcus aureus* sensitivity may be due to its cell

structure, which is often more vulnerable to the phenolic compounds, hydrogen peroxide, and acidic properties of honey **(Bogdanov, 1997)**. Among the gram-negative bacteria, *Escherichia coli* exhibited sensitivity to honey, though to a lesser extent than *Staphylococcus aureus*. *Pseudomonas aeruginosa* and *Enterobacter aerogenes* emerged as the most resistant strains, showing limited inhibition across the honey samples. This resistance can be attributed to mechanisms such as efflux pumps and biofilm formation, particularly in *Pseudomonas aeruginosa*, which are known to inhibit the action of antimicrobial agents.

Table 3 Antibacterial activity of honey samples assessed by the diffusion method on agar medium.

Honey	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*	*Staphylococcus aureus*
Spurge	7.66 ± 0.57^{b}	5.33 ± 0.57^{ab}	2 ± 1^{a}	13.33 ± 0.50	8 ± 1	13.8 ± 0.28^{b}
Thyme	7.33 ± 1.15^{b}	3 ± 1^{a}	5.8 ± 0.28^{ab}	18 ± 0.57	3.63 ± 0.63	22.5 ± 0.50^{d}
Fennel	5 ± 0.28^{ab}	3.66 ± 1.52^{a}	4 ± 1^{ab}	15.33 ± 1.52	5.66 ± 1.15	3.33 ± 0.57^{a}
Thistle	11.33 ± 0.57^{c}	4 ± 0.57^{ab}	2.66 ± 0.57^{a}	16 ± 1	11.33 ± 1.50	14.33 ± 0.50^{b}
Carob	4 ± 1^{a}	3.66 ± 1.52^{a}	3.5 ± 0.86^{a}	10.66 ± 1.80	10.33 ± 0.50	13.83 ± 0.28^{b}
Eucalyptus	11 ± 0.43^{c}	8 ± 1.15^{b}	8 ± 1^{b}	11.33 ± 0.50	5.33 ± 1.15	20 ± 1^{c}
P value	*< 0.001*	*< 0.01*	*< 0.05*	*> 0.05*	*> 0.05*	*< 0.001*

The results are expressed in mean ± standard deviation. Results are statistically significant at ($p < 0.05$). Values in the same column with different letters are significantly different by Tukey's multiple range. Significantly different from baseline, P <0.05; significantly different from baseline, P < 0.01; significantly different from baseline, P <0.001

The MIC revealed that *Enterobacter aerogenes and Klebsiella pneumonia* were the most resistant organisms, with MIC values generally above 50% (w/v) for all honey samples studied. In contrast, *Staphylococcus aureus* demonstrated greater sensitivity, with MIC values often below 25% and not exceeding 50%. The comparison between the honey samples revealed that thyme honey exhibited the strongest antibacterial activity, with the lowest MIC values recorded. This was anticipated, as thyme is known for its high content of bioactive compounds with antibacterial and antifungal properties (**Lee *et al.*, 2005**).

Table 4 The minimum inhibitory concentration of different honey type against pathogenic bacteria**.**

Honey		*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*	*Staphylococcus aureus*
Spurge	**75%**	-	-	-	-	+	-
	50%	+	-	+	+	+	+
	25%	+	<25%	+	+	+	+
Thyme	**75%**	-	> 75%	-	-	+	-
	50%	-	+	+	-	+	-
	25%	<25%	+	+	<25%	+	<25%
Fennel	**75%**	+	+	> 75%	-	-	+
	50%	+	+	+	-	+	+
	25%	+	+	+	<25%	+	+
Thistle	**75%**	-	-	+	-	-	-
	50%	-	+	-	-	+	+
	25%	<25%	+	-	<25%	+	+
Carob	**75%**	-	-	-	+	+	-
	50%	+	-	+	+	+	+
	25%	+	<25%	+	+	+	+
Eucalyptus	**75%**	-	-	> 75%	+	> 75%	-
	50%	-	+	+	+	+	-
	25%	<25%	+	+	+	+	<25%

-: No bacterial development; +: Bacterial development; > 75%: The minimum inhibitory concentration is above the 75% (w/v) concentration; <25%. The minimum inhibitory concentration is lower than 25% (w/v).

These findings are consistent with previous research on thyme honey. For instance, the study by **Melliou & Chinou (2011)** reported strong antimicrobial effects of thyme honey and its isolated compounds against several Gram-positive and Gram-negative bacteria, as well as pathogenic fungi. Similarly, **Voidarou *et al.* (2011)** found that coniferous and thyme honeys displayed the highest antibacterial activity, with MICs of 17.4% and 19.2% (w/v), respectively, compared to citrus and multifloral honeys, which exhibited MICs of 20.8% and 23.8% (w/v). **Kuś *et al.* (2016)** also highlighted the antimicrobial potential of thyme, cornflower, and buckwheat honeys, particularly against *Staphylococcus aureus*, with MICs ranging from 3.12 to 25%. The antibacterial potency of thyme honey can be attributed to its specific bioactive compounds, particularly phenolic compounds and flavonoids known for their antimicrobial properties. Key compounds such as thymol and carvacrol are abundant in thyme honey and are recognized for their ability to disrupt bacterial cell membranes, thereby enhancing its efficacy against various pathogens. Additionally, intrinsic properties of honey itself, including its high sugar concentration, low water activity, and acidic pH (typically between 3.5 and 4), create an inhospitable environment for bacterial growth. The presence of hydrogen peroxide, naturally produced by the enzyme glucose oxidase in honey, further contributes to its antibacterial effect by causing oxidative damage to bacterial cells **(Cebrero *et al.*, 2020; Du *et al.*, 2015)**.

The variations in bacterial sensitivity to different honey types align with findings from **Merah *et al.* (2010)** suggesting that honey's antibacterial effect depends on its composition and characteristics, which can vary by origin, storage conditions, and floral source. These results highlight the significance of understanding honey's diverse properties in developing its potential applications for food preservation and medical use.

HPLC analysis of phenolic compounds

The polyphenolic profiles of the six Moroccan honey samples were determined using HPLC-DAD. Teen polyphenolic compounds were identified, including three phenolic acids, (gallic acid, chlorogenic acid and caffeic acid,), five flavonoids (rutin, quercetin, kaempferol, naringin and epicatechin), one phenolic aldehyde (vanillin), and one benzenetriol (phloroglucinol).

In general, the HPLC analysis (Table 5, Figure 5) revealed that the polyphenolic profiles of the studied Moroccan honey samples were diverse and dependent on the floral source of the honey. Thyme honey was found to have the richest phenolic profile among the studied samples, containing high levels of phloroglucinol (0.31 ± 0.01 mg/100g) , vanillin (2.47 ± 0.02 mg/100g), naringin (2.35 ± 0.03 mg/100g) and rutin (5.00 ± 0.2 mg/100g). These findings suggest that the composition of honey's polyphenolic compounds could contribute to the honey's antimicrobial activity, and that thyme honey may have a higher potential for therapeutic applications due to its rich phenolic profile.

Similarities and differences have been reported in terms of phenolic composition between the honey samples. In fact, a comparison of the phenolic compound profiles of honey samples with high antibacterial activity provided evidence that gallic acid, vanillin, epicatechin, naringin, and rutin, may be mainly responsible for the bioactivity of honey. The study carried out by **Borges *et al.* (2013)** investigated, using several physiological indices, the mechanisms involved in the antimicrobial activity of gallic acid against four human pathogenic bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Listeria monocytogenes*). The results of this work demonstrated that gallic acid led to irreversible changes in membrane properties (charge, intra and extracellular permeability, and physicochemical properties) through hydrophobicity changes,

decrease of negative surface charge, and occurrence of local rupture or pore formation in the cell membranes with consequent leakage of essential intracellular constituents. The phenolic structure of gallic acid, characterized by multiple hydroxyl groups, enhances its ability to interact with lipid bilayers. These hydroxyl groups can form hydrogen bonds with the polar head groups of phospholipids, while the hydrophobic aromatic ring can insert itself into the lipid bilayer. This dual interaction disrupts the integrity of the membrane, leading to increased permeability and potential cell lysis. Studies have shown that gallic acid's amphiphilic nature allows it to effectively penetrate and destabilize bacterial membranes, contributing to its antimicrobial activity against various pathogens **(Mwangi *et al*., 2024; Sang *et al*., 2024)**.

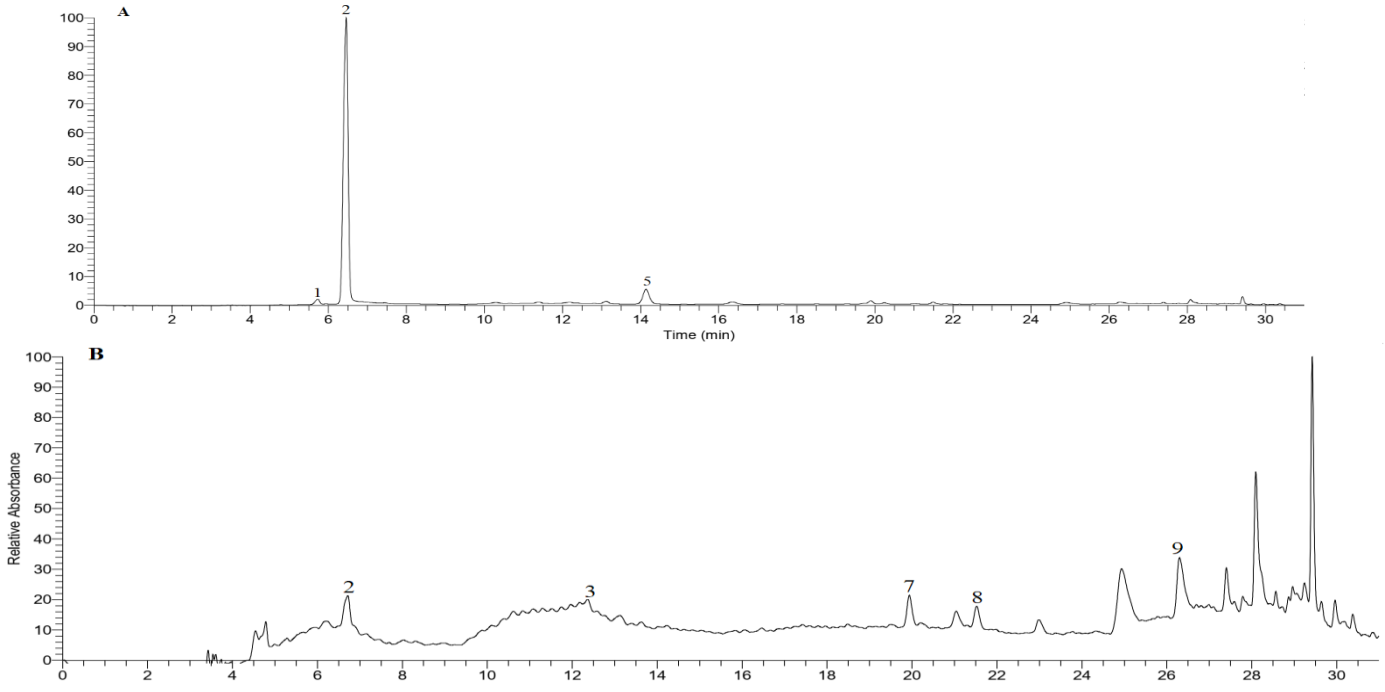
The antibacterial activity of naringin and its derivatives has been documented against pathogens like Listeria monocytogenes, *Escherichia coli* O157, and *Staphylococcus aureus* (**Céliz *et al.*, 2011**). **Dey *et al.* (2020**) demonstrated that combining naringin with antibiotics (ciprofloxacin and tetracycline) significantly reduced *Pseudomonas aeruginosa* biofilm formation, motility, and altered colony morphology. These findings suggest that naringin enhances the efficacy of these antibiotics against *Pseudomonas aeruginosa* biofilms, potentially serving as an effective adjuvant in combating biofilm-related antibiotic resistance. The ability of naringin to disrupt biofilm formation and increase antibiotic efficacy could be an important strategy to combat bacterial infections, especially those caused by antibiotic-resistant strains.

Rutin is one of the flavonoids identified in honeys with a high antibacterial activity. This finding is consistent with **Pimentel *et al.* (2013),** who reported that rutin was identified in the honey sample with the highest antimicrobial activity. In fact, rutin has been previously identified as the compound responsible for antibacterial activity in several natural sources, for example, a study by **Rym *et al.* (1996**) indicate that the antimicrobial activity of rutin isolated from Sophora japonica was the most potent against Mycobacterium smegmatis. Additionally**, Singh *et al.*, (2008**) showed that rutin from *Pteris vittata* exhibited potent activity against *B. cereus*, *P. aeruginosa* and *K. pneumoniae* with the MIC values of 0.03 mg/ml. One of the key results of this study is the identification of vanillin only in thyme honey. This compound and its derivative compounds have been reported to have important antibacterial activity (**Rym *et al.*, 1996**). **Fitzgerald *et al.* (2004**) investigated the mode of action of vanillin with regard to its antimicrobial activity against *Escherichia coli*, *Lactobacillus plantarum* and *Listeria innocua*. The authors reported that the inhibitory action of vanillin consists primarily of impairing cytoplasmic membrane integrity, resulting in the loss of ionic gradients, pH homeostasis, and inhibition of respiratory activity. They also indicated that the degree of membrane damage appears to be sub-lethal in the majority of cells in an inhibited microbial population, resulting in a bacteriostatic action of inhibition at MIC. Given these insights, it is hypothesized that the phenolic profile of thyme honey, specifically the presence of vanillin and other potent compounds, contributes to its efficacy against pathogens. This unique profile not only targets bacterial cell membranes but may also inhibit biofilm formation, positioning thyme honey as a promising natural antimicrobial agent with potential applications in both medical and food industries.

The key results of this study suggest that the antibacterial activity of Moroccan honeys is likely attributed to the combined effects of these polyphenols. The variability in bacterial sensitivity observed across the honey samples can be influenced by the diversity of phenolic compounds present, as well as differences in honey composition due to floral source and environmental factors. In general, studies allow us to state that the antibacterial mechanisms of phenolic compounds mainly include inhibition of nucleic acid synthesis, inhibition of cytoplasmic membrane function by influence biofilm formation, permeability, and interaction with some crucial enzymes (**Barbieri et al., 2017; Khameneh et al., 2019).** According to the literature, the level of sensitivity of the bacterial species to natural substances is highly diverse and strongly depends not only on the type of active compound but also on the selected strains, as shown by comparative analyses (***Özçelik et al., 2011***). Furthermore, it appears that modern clinical isolates are often far less susceptible to natural plant metabolites than normal strains. Although numerous standard strains have been discovered many years ago, with the currently developing resistance of bacteria, their applicability for the screening microbiological tests is restricted.

Table 5 Identified phenolic compounds content in the different type of Moroccan honeys (mg/100g).

Peak number	Compound	Retention Time	Fennel	Thistle	Eucalyptus	Spurge	Thyme	Carob
1	Phloroglucinol	5.85	0.34 ± 0.02	0.1 ± 0.001	0.12 ± 0.02	0	0.31 ± 0.01	0.08 ± 0.01
2	Gallic acid	6.86	2.58 ± 0.03	0	1.73 ± 0.02	0.12 ± 0.01	1.16 ± 0.04	1.48 ± 0.02
3	Chlorogenic acid	11.93	0	0.35 ±0.01	0	0	0	0
4	Epicatechin	13.48	0	0	0.02	0	0	0.56 ± 0.01
5	Caffeic acid	13.99	1.8 ± 0.02	0	0.1 ± 0.005	0	0	0.05 ± 0.01
6	Vanillin	15.10	0	0	0	0	2.47 ± 0.02	0
7	Naringin	20.35	0	0.02 ± 0.01	0.14 ± 0.01	0	2,35 ± 0.03	0
8	Rutin	21.50	0	0.23 ± 0.01	0.25 ± 0.012	0	5.00 ± 0.2	0.53 ± 0.1
9	Quercetin	26.49	0	1.10 ± 0.04	0.69 ± 0.05	1.02 ± 0.08	0.97 ± 0.01	0.08 ± 0.001
10	Kaempferol	27.45	0	0	0	0.013 ±0.002	0	0



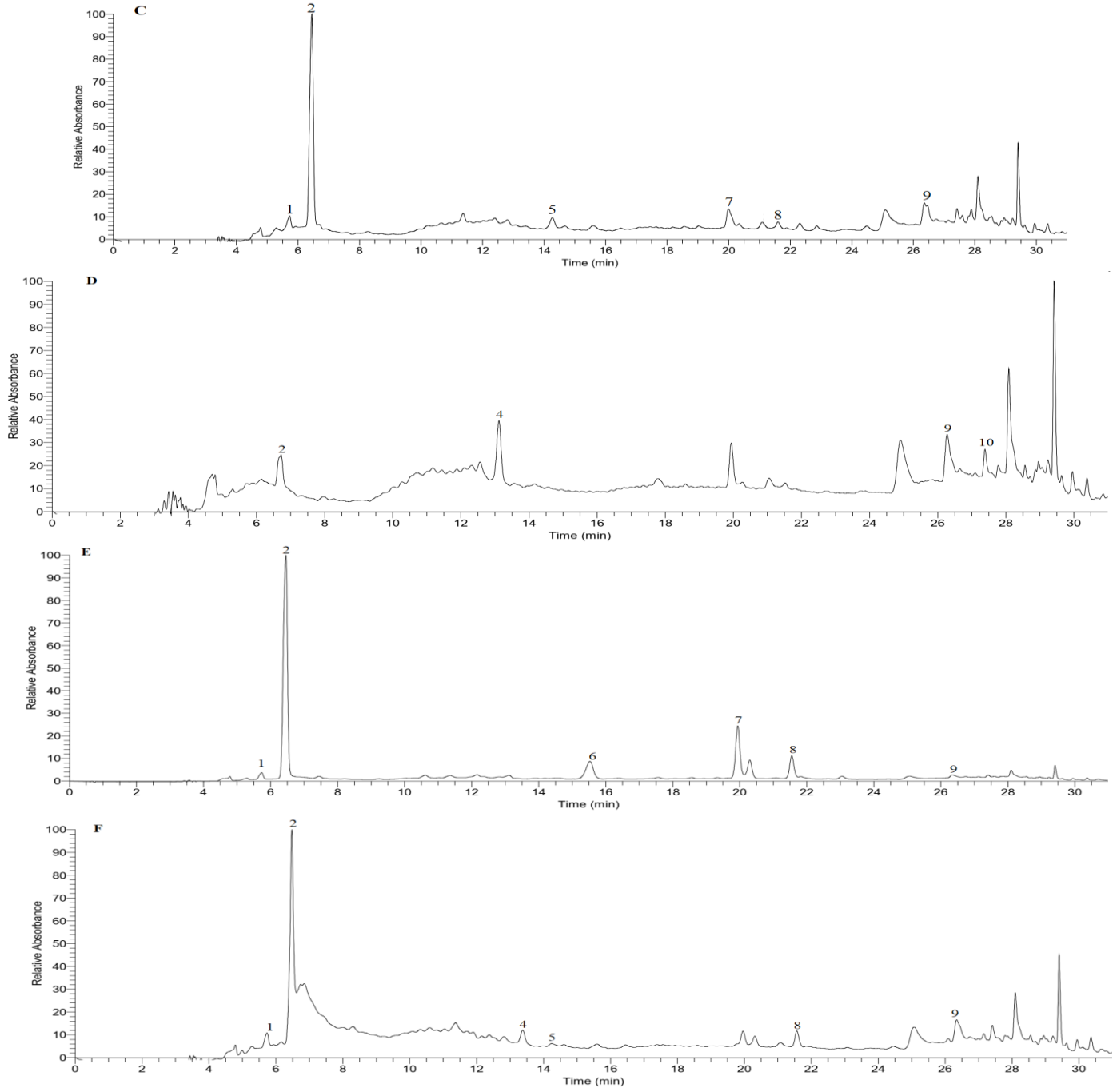


Figure 5. HPLC Chromatograms of different honeys extracts detected at 280nm, **A**, chromatogram of Fennel honey sample; **B**, chromatogram of Thistle honey extract; **C**, chromatogram of Eucalyptus honey sample; **D**, chromatogram of Spurge honey sample; **E**, chromatogram of Thyme honey sample; **F**, chromatogram of Carob honey sample. Peaks: 1, Phloroglucinol; 2, gallic acid; 3, chlorogenic acid, 4, Epicatechin; 5, Caffeic acids; 6, Vanillin; 7, Naringin; 8, Rutin; 9, Quercetin; 10, Kaempferol.

Principal Component Analysis

The biplot (Figure 6) illustrates the results of a PCA (Principal Component Analysis), highlighting the relationship between phenolic compounds in Moroccan honeys and their antibacterial activity against various bacterial strains. The axes F1 (33.38%) and F2 (29.17%) together explain 62.55% of the total variance. The phenolic compounds such as phloroglucinol, vanillin, naringin, rutin, and quercetin exhibit strong contributions, as indicated by their long vectors, correlating with higher antibacterial activities against strains such as Staphylococcus aureus, Escherichia coli, and Enterobacter aerogenes. Conversely, compounds like caffeic acid, gallic acid, and chlorogenic acid contribute moderately, correlating with specific bacterial inhibition patterns. The clustering of bacterial strains with certain compounds suggests differential antibacterial effectiveness based on phenolic profiles. For instance, thyme and eucalyptus honeys, associated with vanillin, naringin and rutin, appear particularly effective against Escherichia coli and Staphylococcus aureus. This analysis underscores the significant role of phenolic diversity in the antibacterial potential of Moroccan honeys.

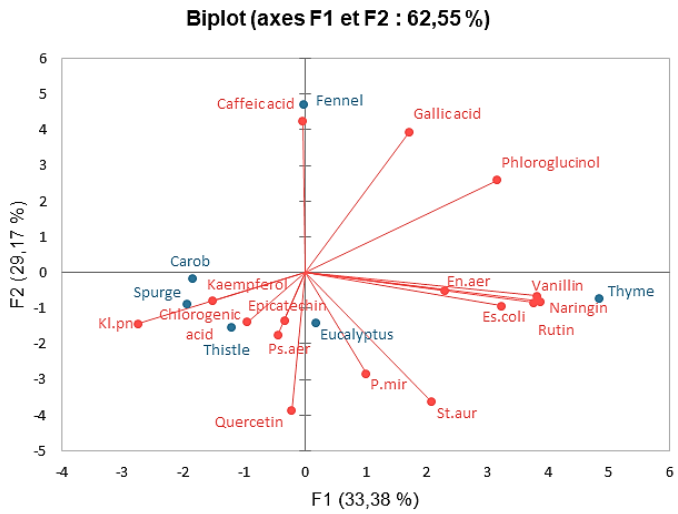


Figure 6 PCA biplot for the Moroccan honey samples with the identified phenolic compounds and their antibacterial activity.

CONCLUSION

Honey is a bee product with multiple therapeutic properties that has established an important role in natural medicine. This study demonstrates that thyme honey is particularly rich in phenolic compounds, which correlate with its significant antioxidant and antibacterial activities. Specifically, compounds such as rutin, and naringin in thyme honey may contribute to its strong bioactive effects. Future research should expand on these findings by evaluating the antibacterial efficacy of thyme honey against a broader range of pathogens, including biofilm-forming bacteria, and investigating its potential synergistic effects with conventional antibiotics. Furthermore, clinical trials are recommended to assess the therapeutic efficacy of thyme honey, particularly in applications like wound healing, under medical supervision. These results highlight thyme honey's potential as a natural antimicrobial agent, with promising applications in both medicine and food preservation.

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