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Dedication/ إهداء

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Abstract

Bee Products as Natural Strategy for the Control of Bovine Mastitis in Dairy Production: Insights from Algerian Honey and Propolis

Bovine mastitis remains one of the most serious health challenges affecting dairy herds worldwide, leading to substantial economic losses and contributing significantly to the global escalation of antimicrobial resistance (AMR). In Algeria, these constraints further exacerbate milk production shortages, posing a threat to national food and nutritional security. In response to the urgent need for natural and sustainable alternatives to conventional antibiotics, this study investigated Algerian propolis and honey as potential therapeutic agents for mastitis control. A multi analytical strategy was employed, integrating advanced metabolomic profiling, *in vitro* biological assays, and *in silico* modeling. Phytochemical analyses using GC MS, and LC-MS identified more than 60 bioactive compounds in propolis and 17 phenolic constituents in honey, including two molecules reported for the first time in Algerian samples from previously unexplored regions, such as 1,3-*O*-caffeoyl dihydrocaffeoylglycerol. *In vitro* investigations demonstrated marked biological activity, with propolis showing strong antibacterial effects against clinical mastitis pathogens (*S. aureus*, *S. uberis*, *S. dysgalactiae*, *Escherichia coli*, and *Klebsiella* spp.). Minimum inhibitory and bactericidal concentrations ranged from 20.08 to 300 µg/mL for propolis and from 0.44 to 28% for honey. Notably, both propolis and honey remained effective against multidrug resistant *E. coli* and linezolid resistant *S. aureus*, indicating an absence of cross resistance with conventional antibiotics. Combination testing revealed additive, indifferent, and antagonistic interactions, with synergy (FICI ≤ 0.5) observed only for specific propolis–honey pairings against certain streptococcal isolates. Propolis also exhibited high antioxidant capacity, with IC_{50} values of 0.27 mg/mL (DPPH) and 0.52 mg/mL (FRAP), comparable or superior to standard antioxidants such as quercetin and ascorbic acid. Molecular docking analyses revealed strong binding affinities for key bioactive compounds against bacterial targets (DNA gyrase and PBP2a: −7.45 to −10.05 kcal/mol) and the Keap1–Nrf2 antioxidant pathway (−7.2 to −11.02 kcal/mol), providing mechanistic insights into the observed biological effects. Furthermore, ADME-Tox predictions suggested favorable pharmacokinetic profiles for several major compounds. Collectively, these findings identify Algerian propolis and honey as rich reservoirs of bioactive molecules with significant antibacterial and antioxidant potential, supporting their development as safe, natural alternatives for addressing bovine mastitis and mitigating AMR.

Keywords: Phytochemical Profiling; Antibacterial activity; Antioxidant capacity; Antimicrobial resistance (AMR); Molecular docking; ADME-Tox; Synergy.

Produits de la ruche comme stratégie naturelle pour le contrôle de la mammite bovine en production laitière : enseignements tirés du miel et de la propolis algériens

Résumé : La mammite bovine demeure l'un des problèmes de santé les plus graves affectant les troupeaux laitiers à l'échelle mondiale, entraînant des pertes économiques considérables et contribuant significativement à l'augmentation globale de la résistance aux antimicrobiens (RAM). En Algérie, ces contraintes aggravent davantage les pénuries de production laitière, constituant une menace pour la sécurité alimentaire et nutritionnelle nationale. Face au besoin urgente d'alternatives naturelles et durables aux antibiotiques conventionnels, cette étude a évalué la propolis et le miel algériens en tant qu'agents thérapeutiques potentiels pour le contrôle de la mammite. Une approche multi-analytique a été adoptée, intégrant le profilage métabolomique avancé, des essais biologiques *in vitro* et la modélisation *in silico*. Les analyses phytochimiques (GC-MS, et LC-MS) ont permis d'identifier plus de 60 composés bioactifs dans la propolis et 17 constituants phénoliques dans le miel, dont deux molécules rapportées pour la première fois dans des échantillons algériens issus de régions jusque-là inexplorées, telles que le 1,3-*O*-caffeoyl-dihydrocaffeoylglycérol. Les investigations *in vitro* ont mis en évidence une activité biologique marquée, la propolis présentant de forts effets antibactériens contre les principaux agents pathogènes de la mammite (*S.aureus, S. uberis, S. dysgalactiae, E. coli* et *Klebsiella* spp.). Les concentrations minimales inhibitrices et bactéricides variaient de 20,08 à 300 µg/mL pour la propolis et de 0,44 à 28 % pour le miel. Notamment, la propolis et le miel sont restés efficaces contre des souches multirésistantes d'*E. coli* et des souches de *S. aureus* résistantes à la linézolide, indiquant l'absence de résistance croisée avec les antibiotiques conventionnels. Les tests de combinaison ont révélé des interactions additives, indifférentes et antagonistes, avec une synergie (FICI ≤ 0,5) observée uniquement pour certaines associations propolis–miel contre des isolats streptococciques spécifiques. La propolis a également montré une forte capacitée antioxydante, avec des valeurs d'IC_{50} de 0,27 mg/mL (DPPH) et 0,52 mg/mL (FRAP), comparables ou supérieures à celles d'antioxydants standards tels que la quercétine et l'acide ascorbique. L'amarrage moléculaire a révélé de fortes affinités de liaison des principaux composés bioactifs avec des cibles bactériennes (ADN gyrase et PBP2a : −7,45 à −10,05 kcal/mol) ainsi qu'avec la voie antioxydante Keap1–Nrf2 (−7,2 à −11,02 kcal/mol), apportant des éléments mécanistiques expliquant les effets biologiques observés. Par ailleurs, les prédictions ADME-Tox ont suggéré des profils pharmacocinétiques favorables pour plusieurs composés majeurs. Dans l'ensemble, ces résultats mettent en évidence la propolis et le miel algériens comme des sources riches en molécules bioactives dotées d'un fort potentiel antibactérien et antioxydant, soutenant leur développement en tant qu'alternatives naturelles et sûres pour la gestion de la mammite bovine et la lutte contre la aux antimicrobiens.

Mots-clés : Profilage phytochimique ; Activité antibactérienne ; Capacité antioxydante ; Résistance aux antimicrobiens (RAM) ; Docking moléculaire ; ADME-Tox ; Synergie.

منتجات النحل كاستراتيجية طبيعية للحد من التهاب الضرع البقري في مزارع إنتاج الحليب: إسهامات العسل والعكبر (البروبوليس) من أصل جزائري

ملخص :يُعدّ التهاب الضرع البقري من أبرز المشكلات الصحية التي تؤثر على الأبقار الحلوب على المستوى العالمي، لما يسببه من خسائر اقتصادية معتبرة، فضلًا عن مساهمته في تفاقم ظاهرة مقاومة المضادات الحيوية. وفي الجزائر، تزداد أهمية هذه الإشكالية في ظل العجز المسجل في إنتاج الحليب، وما يترتب عنه من انعكاسات على الأمن الغذائي والتغذوي الوطني. وفي هذا السياق، ومع تزايد الحاجة إلى بدائل علاجية طبيعية ومستدامة للمضادات الحيوية التقليدية، هدفت هذه الدراسة إلى تقييم العسل والعكبر من أصل جزائري باعتبارهما مصدرين محتملين لمركبات حيوية قادرة على المساهمة في مكافحة التهاب الضرع البقري .اعتمدت الدراسة على مقاربة متعددة التخصصات، جمعت بين التحاليل الكيميائية المتقدمة، والتجارب البيولوجية المخبرية (*in vitro*) ، إضافة إلى دراسات النمذجة الحاسوبية (*in silico*). وقد كشفت التحاليل الكيميائية النباتية، باستخدام تقنيات (GC-MS، وLC-MS)، عن وجود أكثر من 60 مركبًا حيويًا في العكبر، و17 مركبًا فينوليًا في العسل، من بينها جزيئات تم الإبلاغ عنها لأول مرة في عينات جزائرية من مناطق لم تُدرس سابقًا، مثل مركب -1,3-*O*-caffeoyl-dihydrocaffeoyl-glycerol.وأظهرت الاختبارات البيولوجية نشاطًا مضادًا للبكتيريا ذا دلالة مهمة، حيث أبدى العكبر فعالية ملحوظة ضد أبرز البكتيريا المسببة لالتهاب الضرع، بما في ذلك *S. aureus, E. coli, S. dysgalactiae, S. uberis*:و*Klebsiella* spp.وقد تراوحت القيم الدنيا المثبطة والقاتلة للبكتيريا بين 20.08 و300 ميكروغرام/مل بالنسبة للعكبر، وبين 0.44 و28٪ بالنسبة للعسل. كما حافظ كل من العكبر والعسل على فعاليتهما تجاه سلالات *E. coli* متعددة المقاومة، وسلالات *S. aureus* المقاومة للينيزوليد، مما يشير إلى غياب مقاومة متصالبة مع المضادات الحيوية التقليدية .أما اختبارات الدمج، فقد أظهرت تأثيرات مضافة أو محايدة أو متضادة، في حين سُجّل تأثير تآزري ($FICI \leq 0.5$) فقط في بعض التركيبات التي جمعت بين العكبر والعسل ضد بعض السلالات العقدية. ومن جهة أخرى، أظهر العكبر قدرة عالية مضادة للأكسدة، حيث بلغت قيم IC_{50} حوالي 0.27 ملغ/مل في اختبار (DPPH) و0.52 ملغ/مل في اختبار(FRAP) ، وهي نتائج مماثلة أو متفوقة مقارنةً بمضادات الأكسدة المرجعية مثل الكيرسيتين وحمض الأسكوربيك .كما كشفت دراسات الارتباط الجزيئي عن وجود ألفة ارتباط مرتفعة بين المركبات الحيوية الرئيسية وبعض الأهداف البكتيرية، مثل (DNA gyrase وPBP2a)، بقيم تراوحت بين −7.45 و−10.05 كيلوكالوري/مول، إضافة إلى ارتباطها بمسار (Keap1–Nrf2) المرتبط بالنشاط المضاد للأكسدة، وهو ما يساهم في تفسير الآليات المحتملة لهذه الأنشطة البيولوجية. كذلك، أظهرت تنبؤات (ADME-Tox)خصائص دوائية واعدة ومستويات سمية منخفضة متوقعة لبعض المركبات المدروسة. وبصفة عامة، تبرز نتائج هذه الدراسة العسل والعكبر من أصل جزائري كمصدرين غنيين بالمركبات الحيوية ذات الخصائص المضادة للبكتيريا والمضادة للأكسدة، مما يدعم إمكانية توظيفهما كبدائل طبيعية وآمنة للمضادات الحيوية التقليدية في مجال مكافحة التهاب الضرع البقري.

الكلمات المفتاحية:

التحليل الكيميائي النباتي؛ النشاط المضاد للبكتيريا؛ القدرة المضادة للأكسدة؛ مقاومة المضادات الحيوية؛ الإرساء الجزيئي؛ ADME-Tox؛ التآزر.

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List of Abbreviations

ABTS : 2:2′ -azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)

ADME-Tox : absorption, distribution, metabolism, excretion, and toxicology

CCE : caffeic acid cinnamyl ester

CDG : 1,3-O-caffeoyl-dihydrocaffeoylglycerol

CIE : caffeic acid isoprenyl ester

CPE : caffeic acid phenylethyl ester

CUR : curcumin

DPPH : 2,2-diphenyl-1-picrylhydrazyl

FAG : ferulic acid 4-O-glucoside

FRAP : ferric reducing antioxidant power

GAG : gallic acid 4-O-glucoside

GC-MS : gas chromatography–mass spectrometry

HES : hesperetin

ISO : isorhamnetin

KAE : kaempferol

KFD : kaempferide

KOJ : kojic acid

LUT : luteolin

MBA : 2-methoxybenzoic acid

MCE : *p*-methoxycinnamic acid cinnamyl ester

NAR : naringenin

NAL : nalidixic acid

NHX : naringenin hexoside

OLA : oleanolic acid

PCA : principal component analysis

PCH : *p*-coumaric acid hexose

PCI : *p*-coumaric acid isoprenyl ester

PDB : Protein Data Bank

PE1 : propolis extract 1

PE2 : propolis extract 2

PEN : penicillin

Phen : 1,10-phenanthroline

PIN : pinobanksin

PME : pinobanksin-5-methyl ether

PNA : pinobanksin-3-O-acetate

PNP : pinobanksin-3-O-pentanoate

PNR : pinobanksin-3-O-propionate

QDE : quercetin-dimethyl ether

QME : quercetin-3-methyl-ether

QOR : quercetin-3-O-rhamnoside

QUE : quercetin

RBF : riboflavin

RES : resveratrol

RMSD : root mean square deviation

RUT : rutin

TFC : total flavonoid content

TPC : total phenolic content

UHPLC-DAD-ESI/MS : ultra-high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry

USD : United States Dollar (or US Dollar)

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Introduction

Bovine mastitis remains one of the most significant challenges to the sustainability of the global dairy industry, affecting animal welfare, economic performance, and public health. The management of this disease is further complicated by the increasing emergence of antimicrobial resistance (AMR), highlighting the need to explore alternative therapeutic approaches. In this context, natural products derived from beehives, particularly honey and propolis, have attracted growing scientific interest due to their multifaceted chemical composition and well-documented biological properties, along with their relatively low propensity to induce bacterial resistance. This chapter provides a comprehensive overview of these aspects in relation to mastitis control.

1. Current Status and Challenges of Dairy Farming in Algeria

The dairy sector is a cornerstone of global food security, providing essential nutrients while supporting the livelihoods of millions worldwide. In Algeria, domestic production faces significant structural challenges that hinder long-term growth. Annual fluid milk production is currently estimated at 2.5 million metric tons, 73% of which is cow's milk (Hales, 2024).

Cattle represent only 6% of Algeria's livestock population, which is heavily dominated by sheep (79%) (Eulmi, 2024). The national cattle herd comprises approximately 2 million heads, including more than 900,000 dairy cows, with dairy production concentrated primarily in the northern regions (MADR, 2022). Production is predominantly small-scale, averaging approximately 6–10 dairy cows per farm, and relies mainly on imported Holstein and Montbéliarde breeds, predominantly kept in free-stall housing. Daily milk yields average approximately 15 L/cow/day, ranging from 10.7 L/cow/day in arid zones to 16.6 L/cow/day in semi-arid areas (Eulmi, 2024).

However, this level of domestic production falls far short of the estimated domestic demand of 4.5 million metric tons, resulting in a substantial deficit. Consequently, Algeria depends heavily on imported milk powder for reconstitution (Hales, 2024). While national policies aim to expand milk collection networks, persistent challenges in infrastructure, farm management, and animal health continue to constrain productivity and national self-sufficiency (Kacimi El Hassani and Belmahdi, 2025).

Within this context, animal health issues represent an important constraint on dairy productivity, among which bovine mastitis emerges as one of the most critical challenges requiring particular attention. This disease, defined as an inflammatory reaction of the

mammary gland, is considered one of the most complex and economically impactful conditions affecting dairy farming worldwide (Sharun *et al.*, 2021). Although mastitis can be caused by physical, chemical, or thermal injuries to the udder, it most commonly results from an immune response to the invasion of pathogenic microorganisms, primarily bacteria, through the teat canal (Wieland, 2024; Sharun *et al.*, 2021).

In this regard, the disease is commonly classified according to the severity of its clinical manifestations into two main forms. Clinical mastitis is characterized by visible abnormalities in the milk, such as flakes, clots, or a watery consistency (mild clinical mastitis), along with clear signs of inflammation in the mammary tissue, including swelling, redness, and pain (moderate clinical mastitis). In more severe acute cases, the condition may extend beyond the udder, leading to systemic symptoms such as fever and loss of appetite (Ruegg, 2017).

Subclinical mastitis, in contrast, is characterized by the absence of visible changes in both the milk and the udder, making it far more difficult to detect without the use of diagnostic tools (Ruegg, 2017). It is typically identified through measurements such as the somatic cell count (SCC), where an elevated level reflects the migration of inflammatory cells, mainly neutrophils, into the mammary gland (Ruegg, 2017). This form of mastitis is particularly insidious, as it often persists unnoticed within a herd and acts as a silent reservoir of infection. As a result, it is responsible for the majority of economic losses in the dairy industry, primarily through chronic reductions in milk yield and deterioration in milk quality (Halasa *et al.*, 2007). Given its widespread and often hidden nature, mastitis has significant implications for both productivity and economic performance at a global scale. Early studies demonstrated that subclinical mastitis alone can reduce milk yield by 5 to 20%, making it a major contributor to production losses, while clinical mastitis leads to substantial direct costs associated with treatment and discarded milk (Halasa *et al.*, 2007). More recent economic analyses estimate the annual global economic losses associated with clinical mastitis and subclinical mastitis at approximately US$13 billion and US$9 billion, respectively (Rasmussen *et al.*, 2024). These findings consistently underline the heavy financial burden that mastitis imposes on dairy farm profitability (Ruegg, 2017).

In Algeria, the situation reflects this global trend, as mastitis remains a major obstacle to the development and economic sustainability of the national dairy sector (Akkou *et al.*, 2024). Epidemiological studies conducted across various regions of the country have highlighted the high prevalence of both clinical, and subclinical mastitis reaching up to

68.5% at the cow level (Seddar-Yagoub *et al.,* 2023). Notably, subclinical mastitis accounted for approximately 65.4%, of the reported mastitis cases, highlighting its silent yet pervasive impact on dairy production systems (Seddar-Yagoub *et al.,* 2023).

In eastern Algeria, another epidemiological study involving 600 lactating cows from 30 dairy farms reported a subclinical mastitis prevalence of 37.7% at the cow level and 27.2% at the quarter level (Zaatout *et al.,* 2020). Similarly, a cross-sectional study conducted across 32 dairy herds in northeastern Algeria found that 52.3% of the examined cows (116/222) were affected by mastitis, including 6.3% with clinical cases and 45.9% with subclinical cases. This study also investigated the molecular characteristics of *Staphylococcus aureus* (*S. aureus*) isolates infecting the udders and highlighted concerning patterns of antibiotic resistance (Akkou *et al.,* 2024). Taken together, these findings highlight the high burden of mastitis in Algerian dairy herds and underscore its importance as a major animal health issue affecting dairy production in the country. The high prevalence of mastitis may also contribute to economic losses in the dairy sector and further increase the need to rely on imported dairy products, particularly milk powder, to meet domestic demand (Kebbal *et al.,* 2024; Seddar-Yagoub *et al.,* 2023).

The pathogens responsible for bovine mastitis are diverse and broadly classified into contagious and environmental microorganisms. Contagious pathogens, such as *S. aureus*, *Streptococcus agalactiae*, *Mycoplasma* spp., and coagulase-negative staphylococci, primarily spread during milking and persist within infected mammary glands (Wieland, 2024; Sharun *et al.,* 2021). Environmental pathogens, including *Escherichia coli* (*E. coli*), *Streptococcus uberis* (*S. uberis*), *Klebsiella spp.,* and *Streptococcus dysgalactiae* (*S. dysgalactiae*) (frequently linked to teat lesions), originate from the cow's surroundings, and are heavily driven by poor hygiene and organic bedding. Occasionally, yeasts are also involved in environmental infections (Wieland, 2024; Sharun *et al.,* 2021).

Antimicrobial resistance (AMR) has become a critical global health concern, with an estimated 4.95 million deaths attributed to it in 2019, positioning it as one of the leading causes of mortality worldwide (Okeke *et al.,* 2024). This issue, part of the "One Health" challenge, is fueled by a 46% increase in antimicrobial usage across human, animal, and environmental domains (Okeke *et al.,* 2024). In the dairy sector, the unselective use of antibiotics for mastitis control, often the primary reason for antimicrobial use in adult cows, has led to the development of resistance to many previously effective drugs (Roope *et al.,* 2019). The situation is worsened in regions with inadequate diagnostic infrastructure, where

broad-spectrum antibiotics are frequently misused empirically (Eloit, 2021). Algeria illustrates this issue, with studies showing *S. aureus* isolates exhibiting 100% resistance to penicillin and 20% to oxacillin, the defining phenotype of Methicillin-Resistant *S. aureus* (MRSA), which is classified by the WHO as a "High Priority" pathogen (World Health Organization, 2024a; Saidi *et al.,* 2019). Additionally, a significant 76.9% of *E. coli* strains isolated from dairy herds were multidrug-resistant and produced extended-spectrum beta-lactamases (ESBLs) (Tahar *et al.,* 2020), aligning them directly with the WHO's "Critical Priority" category for 3rd-generation cephalosporin-resistant *Enterobacterales* (World Health Organization, 2024b). This rise in antimicrobial resistance poses a significant challenge to both animal health and public safety, as well as the long-term sustainability of the dairy sector. The growing prevalence of resistant pathogens, along with the environmental and health risks posed by antibiotic residues, underscores the need for a shift toward more sustainable alternatives. As a result, the search for natural bioactive compounds has gained considerable attention, with beehive products emerging as a promising alternative. These products are increasingly recognized for their scientifically validated biological potential, offering a viable solution for managing resistant infections in the dairy sector, a therapeutic potential that warrants closer examination.

2. Beehive Products: From Ecological Significance to Therapeutic Potential

Beekeeping plays a crucial role in global agriculture, supporting both food security and biodiversity through the vital process of pollination (Etxegarai-Legarreta and Sanchez-Famoso, 2022). Beyond their ecological contributions, honey bees (*Apis mellifera*) also produce a range of natural substances, including honey and propolis, which are valued for their rich array of biologically active compounds (Durazzo *et al.,* 2021). These substances result from intricate biochemical transformations where bees process plant-derived materials into pharmacologically active matrices (Bava *et al.,* 2024).

In Algeria, the beekeeping sector benefits from considerable genetic diversity, including endemic subspecies such as *Apis mellifera intermissa* and *Apis mellifera sahariensis* (Ayad *et al.,* 2024). The country's vast botanical diversity further suggests that local bee products may exhibit distinct phytochemical fingerprints, offering significant potential for investigation against multidrug-resistant veterinary pathogens (Loucif-Ayad *et al.,* 2015). Honey and propolis are of particular interest due to their rich bioactive composition. While previous studies suggest promising therapeutic properties, their efficacy remains to be fully

validated for Algerian hive products, particularly against antibiotic-resistant strains.

Propolis is a resinous substance produced by *Apis mellifera* from plant exudates, which are mixed with beeswax and salivary enzymes (Asma *et al.,* 2024; Kasote *et al.,* 2022). Its composition is highly variable, influenced by local flora, geographical origin, and seasonal conditions, all of which shape its biochemical profile and bioactivity (Kasote *et al.,* 2022; Wali *et al.,* 2017). This variability is what drives its traditional uses and contemporary scientific interest. Propolis is now recognized for its wealth of bioactive compounds, particularly for its antimicrobial and anti-inflammatory properties (Boulechfar *et al.,* 2022; Cruz *et al.,* 2022). These therapeutic qualities are especially relevant in the context of antimicrobial resistance (AMR) in veterinary medicine, with particular promise for the treatment of bovine mastitis (Mikniene *et al.,* 2026; Abu-Seida, 2023; Klhar *et al.,* 2019; Wang *et al.,* 2016). Physically, propolis is a lipophilic and thermolabile substance, which remains hard and brittle in colder environments but becomes pliable and sticky when heated (Hausen *et al.,* 1987). Its organoleptic properties, ranging from dark yellow to deep brown, with a characteristic resinous aroma, are influenced by the specific plants from which it is derived (Sosa-López *et al.,* 2017; Huang *et al.,* 2014).

Typically, propolis is composed of about 50% resins, 30% waxes, and 20% pollen or other organic matter, further underlining the diversity of its chemical makeup and therapeutic potential (Kasote *et al.,* 2022). Botanical dependency forms the basis for the global classification of propolis into distinct chemotypes, each defined by a specific chemical fingerprint (El-Guendouz *et al.,* 2019; Bankova, 2005). These primary categories include the Poplar type, prevalent in temperate regions and rich in flavonoids and cinnamic acids, and the Mediterranean type, which characterizes North Africa and Southern Europe propolis through a high diterpene content, particularly labdane-type acids derived from the Cupressaceae family (Wali *et al.,* 2017), a chemotype relevant to the Algerian context. Other specialized classifications include the Brazilian Green and Red types, defined by prenylated phenylpropanoids and isoflavonoids, as well as the Birch, Clusia, and Pacific types, which are distinguished by unique markers such as prenylated benzophenones and specialized flavones (Rufatto *et al.,* 2018).

The pharmacologically active fraction of propolis is primarily composed of polyphenols and terpenoids, which are the main contributors to its antimicrobial efficacy. In addition to these compounds, propolis also contains volatile oils and inorganic elements that enhance its bioactivity (Teggar *et al.,* 2023; Stojanovic *et al.,* 2020). Owing to this complex

chemical composition, propolis exhibits a wide range of pharmacological properties, including antioxidant, antimicrobial, anti-inflammatory, and anticancer effects, supporting its importance in both traditional and modern medicine (Taleb *et al.*, 2020; Anjum *et al.*, 2019; Zabaiou *et al.*, 2017). Historically, propolis has been valued for its medicinal and preservative qualities. It was used by ancient Egyptians in mummification, while Greek and Roman civilizations applied it for wound healing and infection control (Teggar *et al.*, 2023; Kuropatnicki *et al.*, 2013). In contemporary research, propolis is increasingly investigated as a potential "natural antimicrobial" agent, particularly against multidrug-resistant (MDR) pathogens. Its biological activity is attributed to multiple mechanisms of action, which are still being actively explored. A summary of its major bioactivities, associated bioactive compounds, and representative targets is presented in Table 1.1.

Honey is a natural substance produced by honeybees from floral nectar. It has long been used served as a food source and for its medicinal properties, which are attributed to its complex chemical composition and diverse biological activities (Tafere, 2021; Eteraf-Oskouei & Najafi, 2013). Its physicochemical characteristics and therapeutic potential vary considerably depending on geographical and botanical origin, making honey a highly heterogeneous natural product (Tafere, 2021).

As a complex biological matrix containing more than 180 constituents, its composition is primarily influenced by the botanical origin of nectar, while environmental factors such as climate, hive conditions, and storage also contribute to its final quality (Tafere, 2021; Eteraf-Oskouei & Najafi, 2013). Sugars represent the major component, accounting for approximately 85–95% of its content, mainly as fructose (32–41%) and glucose (28–34%). Beyond their contribution to the physicochemical properties of honey, the high sugar concentration also contributes directly to its antibacterial activity through osmotic effects and reduced water availability. The minor constituents, including enzymes (e.g., glucose oxidase), phenolic compounds, organic acids, minerals, and vitamins, further contribute to its biological and functional properties. Physicochemical parameters such as color, clarity, moisture content (<20%), and acidic pH (3.4–6.1) contribute to its stability, while its high sugar concentration determines its viscosity and hygroscopic nature, influencing shelf life and consistency (Sharma *et al.*, 2024).

Throughout history, honey has been revered in various cultures for its therapeutic and preservative properties. In Islamic medicine, the Qur'an describes it as a healing "drink of varying colors," and Avicenna notably recommended honey for treating tuberculosis nearly a

thousand years ago (Eteraf-Oskouei & Najafi, 2013). Modern science has since uncovered the biochemical mechanisms behind these ancient uses, revealing several synergistic pathways that contribute to honey's clinical effectiveness.

The therapeutic properties of honey are grounded in well-defined physicochemical and biochemical mechanisms. Its broad-spectrum antimicrobial activity, often described as "natural antibiotic", stems from a combination of factors: low water activity and high osmolarity causing bacterial dehydration; an acidic pH (3.2–4.5); enzymatic production of hydrogen peroxide (H_2O_2); and the direct antimicrobial action of bioactive polyphenols (Hossain *et al.,* 2021; Tafere, 2021; Mandal & Mandal, 2011). These synergistic mechanisms account for honey's effectiveness against a wide range of pathogens, reinforcing its longstanding medicinal reputation.

Beyond its antimicrobial activity, honey exhibits strong antioxidant properties mainly attributed to phenolic compounds, flavonoids, and enzymes, which neutralize free radicals and reduce oxidative stress, thereby protecting cells and preventing chronic diseases (Ahmed *et al.,* 2018; Eteraf-Oskouei & Najafi, 2013). These effects, in turn, contribute to its anti-inflammatory activity through the modulation of pro-inflammatory cytokines and enzymes such as cyclooxygenase (COX) (Poulsen-Silva *et al.,* 2023; Silva *et al.,* 2021). Together, these complementary mechanisms support the therapeutic potential of honey, particularly in conditions associated with oxidative stress and inflammation.

The relationship between the chemical composition of bee products and their bioactivity relies on comprehensive chemical characterization. Such analysis is essential to understand how specific constituents translate into biological effects. Detailed physicochemical and biochemical profiling ensures product quality, reproducibility, and authenticity, as environmental factors and adulteration can significantly alter their properties (Svečnjak *et al.,* 2020). By elucidating structure–activity relationships, this approach links compound classes such as polyphenols and terpenoids to biological activities, including antioxidant and antimicrobial effects (Alvarez-Suarez *et al.,* 2014). Moreover, the influence of botanical and geographical origin is particularly relevant in regions such as Algeria, where local flora confers distinct chemical signatures to honey and propolis. These variations directly affect their therapeutic potential and highlight the importance of region-specific investigations (Kouadri *et al.,* 2021).

Table 1.1. Major Biological Activities of Propolis, Key Compounds, and Mechanisms.

Activity	Key Bioactive Compounds	Primary Mechanisms / Targets	Key Pathogens / Conditions	References
Antibacterial	Phenolic acids (e.g., caffeic acid), Flavonoids (e.g., pinocembrin, pinobanksin)	Membrane disruption; Inhibition of enzyme activity; protein synthesis, and cell division; bacteriostatic & bactericidal action.	High efficacy against Gram-positive (e.g., *S. aureus*); active against MDR[1] strains.	Kasote *et al.*, 2022; Anjum *et al.*, 2019; Silva-Carvalho *et al.*, 2015 ; Bankova *et al.*, 2000.
Antifungal	Pinocembrin, *p*-coumaric acid, caffeic acid.	Inhibition of growth & toxin production (e.g., aflatoxins); Cell integrity disruption.	*Aspergillus flavus, Candida albicans, C. krusei, and C. guilliermondii;* dermatophytes (e.g., *Trichophyton* spp.).	Bouchelaghem, 2022; Silva *et al.*, 2017; Silva-Carvalho *et al.*, 2015; da Silva *et al.*, 2006.
Anti-inflammatory	Flavonoids.	Enzyme Inhibition (NADPH oxidase; myeloperoxidase; hyaluronidase…); Suppression of prostaglandins & leukotriene synthesis.	Wound healing, skin conditions; efficacy depends on origin and concentration.	Anjum *et al.*, 2019; Sforcin, 2016; Banskota *et al.*, 2001.
Antioxidant	Phenolic compounds (flavonoids, phenolic acids).	Free radical scavenging; Protection of lipids, proteins, and DNA from oxidative damage.	Cellular aging, disease prevention; natural food preservative.	Alvarez-Suarez, 2017; Zabaiou et al., 2017; Socha *et al.*, 2015.
Anticancer	Flavonoids, phenolic compounds, baccharin, artepillin C.	Apoptosis induction, antiproliferative, anti-angiogenic and immunomodulatory effects.	Breast, colon, lung, prostate, cervical, pancreatic and other cancers.	Hossain *et al.*, 2022 ; Kasote *et al.*, 2022.

[1] Multidrug-Resistant.

Building upon the importance of chemical characterization, the therapeutic relevance of these compositional profiles becomes particularly evident when considering their activity against mastitis-associated pathogens. Bee products, particularly honey and propolis, have attracted increasing attention as potential alternatives for the management of bovine mastitis, demonstrating varying degrees of efficacy against both Gram-positive and Gram-negative pathogens. Propolis has consistently shown strong antibacterial activity, especially against Gram-positive bacteria such as *S. aureus*, while its effectiveness against Gram-negative species like *E. coli* may vary depending on its botanical and geographical origin (Fiordalisi *et al.,* 2016). This variability reflects differences in chemical composition associated with botanical and geographical origin, which can significantly influence antimicrobial and antioxidant potency. The antibacterial mechanisms of propolis are multifactorial and include disruption of bacterial cell membranes, inhibition of enzymatic systems, and interference with quorum sensing and biofilm formation, which may explain its higher efficacy against Gram-positive bacteria (Rana *et al.,* 2025; Bouchelaghem, 2022; Almuhayawi, 2020). In addition to its antimicrobial properties, propolis has been shown to modulate inflammatory responses in mammary epithelial tissues, supporting its potential as a dual-action therapeutic agent (Wang, K., *et al.,* 2016).

Honey also exhibits well-established antibacterial activity and is increasingly investigated as a complementary or alternative therapeutic agent in mastitis management. Its antimicrobial effects are attributed to multiple factors, including high osmotic pressure, acidity, hydrogen peroxide production, and the presence of bioactive compounds. Recent studies have highlighted the potential of intramammary honey formulations, particularly against biofilm-forming and ESBL-producing *Enterobacterales*, although their clinical application remains at an early stage (Özdikmenli Tepeli *et al.,* 2022; Benhanifia *et al.,* 2020). However, variability in composition and the frequent use of non-standardized samples limit reproducibility, emphasizing the need for medical- or veterinary-grade honey to ensure consistent therapeutic outcomes (Benhanifia *et al.,* 2020; Aamer *et al.,* 2014).

Despite the growing evidence supporting the antibacterial potential of bee products, the precise molecular mechanisms underlying their activity remain incompletely understood. This limitation, combined with the chemical complexity of honey and propolis, highlights the need for complementary approaches capable of linking bioactive compounds to their

biological targets.

In this context, *in silico* techniques have emerged as valuable tools in natural product research. Molecular docking, in particular, enables the prediction of interactions between bioactive compounds and specific protein targets at the molecular level, providing mechanistic insights into their potential biological activity (Dnyandev *et al.*, 2021; Chaudhary and Mishra, 2016). In addition, virtual screening facilitates the investigation of bioactive constituents from complex natural products by predicting their interactions with disease-related molecular targets, thereby contributing to the molecular interpretation of experimentally observed biological activities (Asiamah *et al.*, 2023). Complementary ADME-Tox (Absorption, Distribution, Metabolism, Excretion, and Toxicity) analysis further characterizes the predicted physicochemical, pharmacokinetic, and safety properties of these constituents. Although these computational approaches require subsequent experimental validation, they provide a valuable molecular framework for interpreting the biological activities of complex natural products and guiding future mechanistic investigations (Wu *et al.*, 2020)

By integrating phytochemical characterization, *in vitro* biological evaluation, and *in silico* modeling, this approach facilitates a deeper understanding of the molecular basis underlying the antibacterial activity of bee products and provides a scientific framework for the further investigation of their therapeutic potential against mastitis-associated pathogens.

3. Problem Statement and Justification

Bovine mastitis remains a major health challenge in the global dairy industry, leading to substantial economic losses due to reduced milk yield, increased treatment costs, and premature culling (Halasa *et al.,* 2007). In Algeria, this challenge is further compounded by the high prevalence of mastitis, contributing to the persistent gap between domestic milk production and national demand and, consequently, affecting food security (Seddar-Yagoub *et al.,* 2023). Antibiotic therapy remains a cornerstone of mastitis management; however, the widespread use of antibiotics has raised growing concerns regarding antimicrobial resistance, highlighting the need for alternative or complementary therapeutic strategies. This concern is particularly relevant in local dairy systems, where resistant strains, including methicillin-resistant *S. aureus* and ESBL-producing *E. coli*, have been reported (Bounoua and Souna, 2025; Akkou *et al.*, 2024).

This trend compromises treatment efficacy and raises serious concerns for both animal and public health. In this context, natural products such as propolis and honey have gained increasing attention as potential alternative therapeutic agents. However, significant knowledge gaps remain regarding their effectiveness against prevalent mastitis pathogens. In particular, limited data exist on the chemical characterization of Algerian bee products and the relationship between their phytochemical composition and antibacterial activity against key mastitis pathogens, including *S. aureus*, *E. coli*, *Klebsiella spp.*, *Streptococcus dysgalactiae*, and *Streptococcus uberis*. Furthermore, the potential synergistic effects of propolis–honey combinations remain insufficiently explored, particularly in the context of polymicrobial infections. Furthermore, mechanistic insights into their modes of action remain limited, particularly at the molecular level. Computational approaches such as molecular docking can provide insight into compound–target interactions, while ADME-Tox (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiling contributes complementary information on the predicted physicochemical, pharmacokinetic, and safety characteristics of the identified bioactive constituents.

To address these gaps, this study proposes an integrated approach combining chemical characterization, *in vitro* biological evaluation, synergy assessment, and *in silico* analysis to investigate the therapeutic potential of Algerian bee products against bovine mastitis pathogens.

4. Objectives & Hypothesis

The main objective of this study is to evaluate the bioactive potential of Algerian honey and propolis as alternative therapeutic agents against a clinically relevant panel of five major bovine mastitis pathogens, with each species represented by multiple distinct strains to account for microbial variability through an integrated physicochemical, biological, and computational approach.

The specific objectives are:

1. To characterize the physicochemical and phytochemical profiles of selected Algerian honey and propolis samples.
2. To assess their *in vitro* antibacterial activity, individually and in combination, against clinical isolates of bovine mastitis pathogens (*S. aureus*, *E. coli*, *Klebsiella spp.*, *S. dysgalactiae*, and *S. uberis*).
3. To investigate the molecular basis underlying the bioactivity through molecular docking of the identified bioactive constituents and complementary ADME-Tox analysis.

The study is based on the following hypotheses:

- Algerian honey and propolis exhibit distinct physicochemical and phytochemical profiles influenced by their botanical and geographical origin.
- Both products display dose-dependent antibacterial activity against mastitis-associated pathogens.
- Their combination may produce additive or synergistic effects.
- Specific bioactive compounds are hypothesized to exhibit favorable interactions with key bacterial targets and favorable predicted pharmacokinetic properties.

Chapter 1. Algerian Propolis and Honey: Multi-Analytical Profiling, Bioactivity, and Quality Assessment.

I. Introduction

Honey and propolis are complex apicultural matrices acknowledged for their intricate chemical compositions and rich bioactive compound content. These constituents form the basis of their pharmacological properties, rendering them promising natural alternatives for the treatment of various infections (Al-Kafaween *et al.*, 2023). Propolis reportedly contains over 800 identified constituents (Šturm and Ulrih, 2020), whereas honey comprises a complex matrix of over 180 compounds and nearly 600 volatile constituents in addition to its primary carbohydrate fraction (Tafere, 2021). These bioactive properties, including antimicrobial and antioxidant activities, are directly linked to the specific chemical constituents present in each product. However, the chemical fingerprints of both honey and propolis are highly plastic and vary significantly according to botanical sources, environmental conditions, and geographic origin (Al-Kafaween *et al.*, 2023).

Despite increasing global interest, the compositional and bioactive characterization of Algerian honey and propolis remains limited. This gap is particularly relevant given Algeria's ecological diversity, ranging from Mediterranean to Saharan environments, and its rich flora, with over 1000 medicinal plant species reported (Yabrir *et al.*, 2018). Such diversity suggests that Algerian bee products may possess unique phytochemical signatures and bioactivities, offering a substantial opportunity for investigation.

In this context, the present study aims to characterize the physicochemical and phytochemical profiles of selected Algerian honey and propolis samples using advanced multi-analytical approach. Complementary techniques were employed, including Gas Chromatography–Mass Spectrometry (GC–MS) for volatile compounds and liquid chromatography–mass spectrometry platforms (UHPLC-DAD-ESI/MS and UPLC-ESI-MS/MS) for detailed profiling of phenolic and non-volatile constituents.

This integrated analytical strategy provides a comprehensive basis for exploring the relationship between chemical composition and biological activity, particularly in the context of antibacterial potential against bovine mastitis pathogens.

II. Materials & Methods

1. Chemicals and Reagents

All chemicals and reagents employed in this study were of analytical or HPLC (High-Performance Liquid Chromatography) grade. Acetonitrile and formic acid were obtained from Panreac (Barcelona, Spain), and dichloromethane (DCM) and methanol were sourced from VWR International (Radnor, PA, USA). Pyridine (≥99%), trimethylchlorosilane (≥99%), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA, ≥99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), trichloroacetic acid (TCA), iron (III) chloride ($FeCl_3$), 1,10-phenanthroline, potassium ferricyanide ($K_3Fe(CN)_6$), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ascorbic acid, and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Folin–Ciocalteu reagent was supplied by Biochem Chemopharma (Cosne-Cours-sur-Loire, France). All phenolic standards used for identification and quantification were provided by Extrasynthèse (Genay, France). Ultrapure water was prepared with a Direct-Q® water purification system (Merck Life Science, Darmstadt, Germany).

2. Propolis Samples

2.1. Sample Origin, Collection and Preparation

Propolis specimens were procured from *Apis mellifera* colonies in two distinct regions: Batna (P1) and Biskra (P2) in Algeria. The sampling period spanned from late June through late July 2021. The samples were selected based on their availability and accessibility during the sampling period and originated from distinct geographical and climatic environments. Detailed site-specific metadata, including geographical coordinates, altitudinal data, and climatic profiles, are consolidated in Table 2.1 and illustrated in the geolocalization map in Figure 2.1. The raw material was harvested by scraping the resin from the flexible top-bar grids of multiple hives at each site, which was then homogenized to form representative composite samples. After manual purification to remove all visible exogenous matter, 100 g aliquots of each sample were transferred to hermetically sealed containers. These were preserved at 4 °C to maintain chemical stability prior to subsequent analysis.

Table 2.1. Geographic and Climatic Characteristics of the Algerian Propolis Collection Sites.

Sample	Site name	Latitude (N)	Longitude (W)	Altitude (m)	Climate	Temp (°C / °F)	Pluviometry (mm / inch)
P1	Ouled Si-Slimane (Batna)	35°36'39"	5°37'58"	1045	Cfa	13.5 / 56.4	496 / 19.5
P2	Sidi Masmodi (Biskra)	34°51'10.5"	6°18'51.5"	121	BWh	21.1 / 69.9	125 / 4.9

*-According to the Köppen climate classification, **Cfa** is a humid subtropical climate and **BWh** is a hot desert climate. The data were obtained during 2021.*

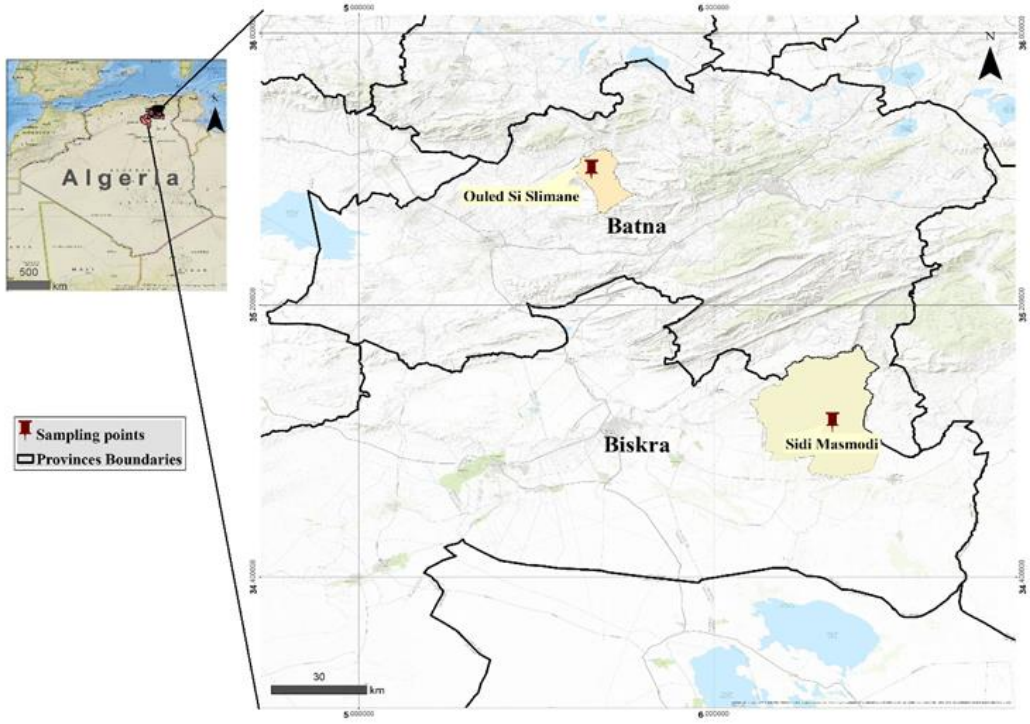


Figure 2.1. Geographic Locations of Algerian Propolis Samples.

2.2. Extraction Protocol and Yield Determination

Dry propolis extracts from P1 and P2 were prepared with minor modifications to the method described by Jug *et al.* (2014). Briefly, 100 g of each crude propolis sample underwent three successive macerations, each lasting 3 days, using 70% ethanol at a 1:10 (w/v) ratio. The extractions were carried out at room temperature in the dark with occasional shaking. The resulting ethanolic mixtures were then filtered and concentrated using a rotary evaporator. The crude extracts, referred to as propolis extract 1 (PE1) and propolis extract 2 (PE2), were subsequently lyophilized using a freeze-dryer (Martin Christ Alpha 2-4 LSCplus, Osterode am Harz, Germany) to ensure complete removal of the solvent and enable long-term storage.

2.3. Phytochemical Screening

2.3.1. Total Phenolic Content (TPC)

The total phenolic content (TPC) of propolis extracts was determined using a modified Folin-Ciocalteu method (Singleton *et al.,* 1999). Briefly, 20 µL of dry propolis extract was combined with 100 µL of Folin-Ciocalteu reagent (diluted 1:10) and 75 µL of 7.5% sodium carbonate (Na_2CO_3), and the mixture was incubated for 2 h. Absorbance was recorded at 765 nm using a microplate reader (Thermo Scientific™ Multiskan™ Sky, Waltham, MA, USA) with the SkanIt™ Upgrade software (version 5.0). TPC was quantified using a gallic acid calibration curve ($y = 0.005x + 0.09$, $R^2 = 0.995$), and the results were expressed as mg of gallic acid equivalents per g of dry extract (GAEs mg g^{-1}). All measurements were performed in triplicates.

2.3.2. Total Flavonoid Content (TFC)

The total flavonoid content (TFC) was determined following the procedure described by Moreno *et al.* (2000). Briefly, 50 µL of propolis extract was mixed with 50 µL of aluminum trichloride ($AlCl_3$) and 150 µL of sodium acetate (CH_3COONa), followed by incubation in the dark for 2.5 h. Absorbance was measured at 440 nm, and quantification was performed using a quercetin calibration curve ($y = 0.0048x + 0.0121$, $R^2 = 0.997$).

TFC values were expressed as mg of quercetin equivalents per gram of dry extract (QEs mg g^{-1}), and all assays were conducted in triplicates.

2.4. Chromatographic Profiling

2.4.1. Volatile Compound Analysis by GC-MS

Propolis extracts were derivatized for GC-MS analysis, following the method described by Oliveira *et al.* (2006). Briefly, 20 mg of each sample was dissolved in 450 µL of dichloromethane and combined with 500 µL docosane as an internal standard (IS), 250 µL pyridine, 250 µL N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), and 50 µL trimethylchlorosilane (TMSCl). The reaction mixture was incubated at 70 °C for 30 min, cooled to room temperature, and subsequently injected into the GC-MS in triplicate.

Analyses were performed using a Shimadzu GCMS-QP2010 Ultra system (Shimadzu Corporation, Kyoto, Japan) equipped with a ZB-5ms capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium served as the carrier gas at a flow rate of 1.17 mL/min, and the injector was maintained at 320 °C with a split ratio of 10:1. The oven temperature was programmed as follows: initially held at 90 °C for 4 min, then ramped at 16 °C/min to 180 °C, at 6 °C/min to 250 °C, and finally at 3 °C/min to 300 °C, where it was held for 5 min, yielding a total run time of 42.96 min. The transfer line temperature was set at 200 °C.

Mass spectra were recorded using electron ionization (EI) at 70 eV with a scan range of m/z 50–1000 at 1 scan/s. Compound identification was achieved by comparison with the NIST14 and WILEY mass spectral libraries.

2.4.2. Phenolic Compound Identification by UHPLC-DAD-*ESI/MS*

Propolis extracts were analyzed using an Ultimate 3000 UHPLC system (Dionex Co., San Jose, CA, USA) coupled to an LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA). Separation was achieved using a Hypersil Gold C18 column (100 mm × 2.1 mm, 1.9 µm particle size). The mobile phase consisted of 0.1% (v/v) formic acid in water (A) and acetonitrile (B) solutions. The flow rate was maintained at 0.2 mL/min, and the injection volume was 10 µL (1 mg extract/mL of ethanol).

Mass spectrometric detection was carried out using electrospray ionization (ESI) in a mass scan range of m/z 100–2000. Nitrogen (>99% purity, 520 kPa) served as the nebulizing gas, and helium was used as the collision gas. The collision-induced dissociation (CID) energy was set between 25 and 35 arbitrary units. Compound identification was based on matching retention times and MS/MS spectra with authentic standards and previously published data. Quantification was performed using an external standard approach with MS^2 calibration curves.

2.5. Antioxidant capacity evaluation

2.5.1. *2,2* -Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

The DPPH radical scavenging activity of the propolis extracts was assessed following the method of Kurek-Górecka *et al.* (2022). In a 96-well microplate, 40 µL of propolis extract or standard (final concentrations: 4-6.25 × 10^{-2} mg/mL for extracts and 4-4.88 × 10^{-4} mg/mL for standards, prepared via two-fold serial dilutions) was mixed with 160 µL of DPPH solution. The reaction mixtures were incubated in the dark for 2 h, after which absorbance was measured at 517 nm using a PerkinElmer EnSpire Multimode Plate Reader (Waltham, MA, USA).

2.5.2. *2,2′*-Azino-bis (3-Ethylbenzothiazoline-6-sulfonic Acid) (ABTS) Radical Scavenging Assay

The ABTS radical cation decolorization assay, which evaluates the ability of the extracts to scavenge free radicals and inhibit oxidation (Re *et al.*, 1999), was performed according to Xiao *et al.* (2020). Briefly, 40 µL of propolis extract or standard was added to 160 µL of ABTS solution in a 96-well plate. After incubating in the dark for 10 min, the reduction in ABTS radical cation was determined by measuring absorbance at 734 nm with a spectrophotometer. The antioxidant activity for both assays was expressed as the half-maximal inhibitory concentration (IC_{50}, µg/mL).

2.5.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay was carried out following Oyaizu (1986). Briefly, a reaction mixture containing 10 µL of propolis extract, 40 µL phosphate buffer (pH 6.6), and 50 µL of 1% potassium ferricyanide (K_3 [Fe $(CN)_6$]) was incubated at 50 °C for 20 min. After incubation, 50 µL of 10% trichloroacetic acid (TCA) and 40 µL of water were added, followed by 10 µL of 0.1% iron (III) chloride ($FeCl_3$). The absorbance of the resulting solution was measured at 700 nm.

2.5.4. Metal Chelating Activity (1,10-Phenanthroline) (Phen) Assay

The Phenanthroline (Phen) assay was conducted as described by Szydłowska-Czerniak *et al.* (2008). In brief, 10 µL of propolis extract was mixed with 50 µL of 0.2% $FeCl_3$ and 30 µL of 0.5% phenanthroline, followed by the addition of 110 µL of methanol. The mixture was incubated in the dark at 30 °C for 20 min, and absorbance was measured at 510 nm. Antioxidant activity was expressed as $A_{0.50}$, corresponding to the concentration that produced an absorbance of 0.500.

3. Honey Samples

3.1. Sample Origin, Collection and Preparation

Three honey specimens (H1, H2, and H3) were procured from *Apis mellifera* apiaries managed by local beekeepers across diverse regions of Algeria, with the collection period occurring between May and June 2021. The samples were selected based on their availability and accessibility, with consideration given to their botanical diversity, including monofloral and multifloral honeys, and their respective flowering and production periods. The specimens were sourced from distinct provinces to represent varied ecological and climatic zones: H1 from Skikda (Collo), H2 from Biskra (Doucen), and H3 from Laghouat (Brida). Detailed environmental metadata, including geographical coordinates, altitudes, and specific climatic parameters for each site, are consolidated in Table 2.2 and illustrated in the geolocalization map in Figure 2.2.

Approximately 500 g of honey was obtained from each apiary, representing a composite of multiple hives. Extraction methods followed local apicultural practices, utilizing either

traditional manual pressing or modern centrifugal techniques. Following collection, samples were transferred to sterile containers and stored under dark conditions at 4 °C until laboratory analysis.

Table 2.2. Geographic Characteristics of the Algerian Honey Sample Collection Sites.

Sample	Site name	Latitude (N)	Longitude (E)	Altitude (m)	Climate	Temp (°C / °F)	Pluviometry (mm / inch)
H1	Collo (Skikda)	37◦00′20″	6◦33′37″	24	Csa	17.3 / 63.1	821 / 32.3
H2	Doucen (Biskra)	34◦36′00″	5◦05′60″	191	BSh	22.4 / 72.3	196 / 7.7
H3	Brida (Laghouat)	33.9501°	1.7502°	1,378	BSk	16.5 / 61.7	161 / 6.3

-According to the Köppen climate classification, ***Csa****, is a Hot-summer Mediterranean,* ***BSh,*** *is a Hot Semi-arid and* ***BSk,*** *is a Cold Semi-arid / Steppe. The data were obtained during 2021.*

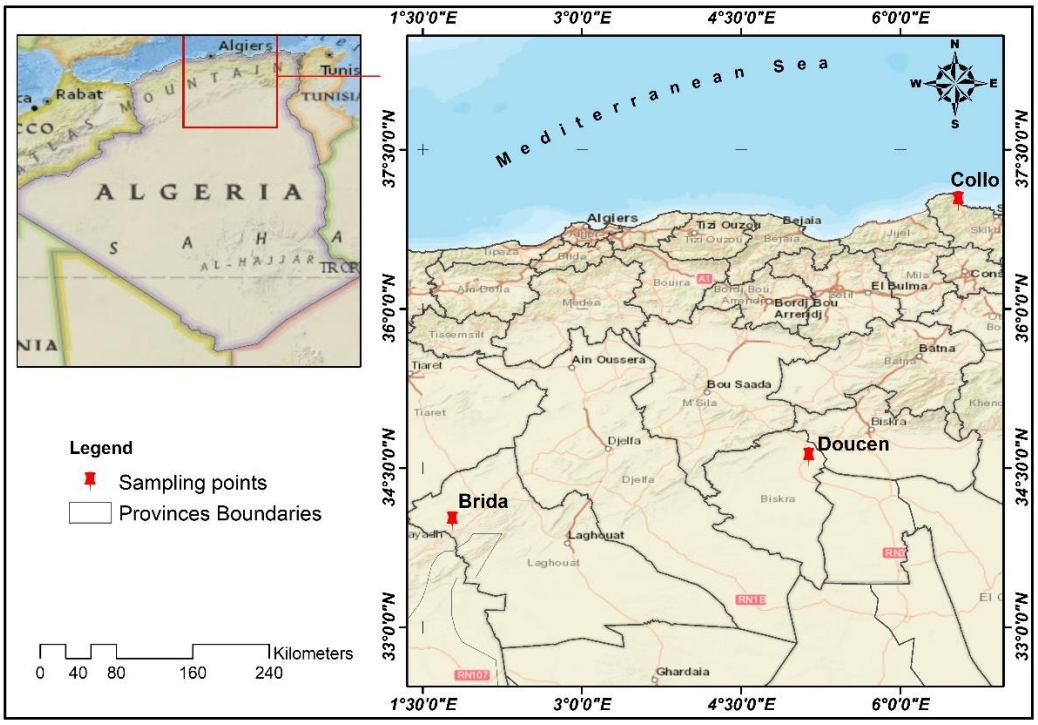


Figure 2.2. Cartographic Representation of Honey Sampling Sites Across Algeria.

3.2. Physicochemical Quality Parameters

The physicochemical properties of the honey samples were determined according to the official methods established by the Algerian Standard NA 15304 (2016), and the International Honey Commission (Bogdanov, 2002). All analytical determinations were performed in triplicate to ensure statistical reproducibility.

3.2.1. Moisture, pH, Free Acidity, and Electrical Conductivity

Determination of moisture content: Moisture was determined indirectly via the refractive index using a digital refractometer (HI 96800, Hanna Instruments, Italy). Samples were gently warmed in a water bath to achieve complete liquefaction. A droplet was placed on the prism plate, and the refractive index was recorded at a stabilized ambient temperature of 20°C. Moisture percentages were obtained using the Chataway conversion table (NA 15304, 2016).

Determination of pH and Free Acidity: These parameters were measured by potentiometric titration. A 10 g honey sample was dissolved in 75 mL carbon dioxide-free distilled water. The initial pH was recorded using a calibrated digital pH meter (Starter 3100-F, OHAUS, USA). The solution was then titrated with standardized 0.1 N sodium hydroxide (NaOH) to an endpoint of pH 8.30. Free acidity was calculated as: $Free\ Acidity\ (meq/kg) = V\ (mL) \times 10$, where V is the volume of 0.1 N NaOH consumed (mL).

Determination of Electrical Conductivity: Following the method of Vorwohl (1964), a solution was prepared containing 20.0 g of honey dry matter in 100 mL of distilled water. Conductivity was measured at 20°C (± 0.1°C) using a calibrated conductivity meter (Starter 300C, OHAUS, USA) equipped with an STCON3 electrode (cell constant K = 1.0 cm^{-1}). Results are expressed in milliSiemens per centimeter (mS/cm).

3.2.2. HMF Content, and Specific Optical Rotation

Hydroxymethylfurfural (HMF) Content: HMF was determined spectrophotometrically according to White (1979). A honey sample (5.00 ± 0.01 g) was dissolved in distilled water, clarified with 0.5 mL each of Carrez I and Carrez II solutions, and diluted to 50 mL.

After filtration, two 5 mL aliquots of the clear filtrate were prepared: one was mixed with 5 mL of distilled water (sample solution), and the other with 5 mL of 0.2% sodium bisulfite solution (reference solution). Absorbance was measured at 284 nm using a UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan). HMF concentration (mg/kg) was calculated as: $HMF = (A_sample - A_reference) \times 149.7 \times 5 / W$, where A represents absorbance and W is the exact honey weight (g).

Specific Optical Rotation: Determined according to the harmonized method of Bogdanov (2002). A 12 g honey sample was accurately weighed, dissolved in distilled water, and clarified by sequential addition of Carrez I and Carrez II solutions (1.0 mL each) in a 100 mL volumetric flask. The solution was diluted to volume, mixed thoroughly, and allowed to stand overnight at room temperature for complete clarification. The clarified solution was filtered, and the optical rotation (α) was measured at 20°C using a manual polarimeter (Polamat, Carl Zeiss, Jena, Germany) equipped with a 100 mm polarimeter tube and sodium D-line illumination (589 nm). The specific optical rotation $[\alpha]_{20D}$ was calculated using the formula: $[\alpha]_{20D} = (\alpha \times 100)/(l \times c)$, where α is the measured angular rotation (degrees), l is the tube length (dm), and c is the concentration of dry matter (g/100 mL), calculated as: $c = W \times (100 - M) / 100$, where W is the honey sample weight (12 g) and M is the moisture content (%).

3.3. Phytochemical Screening

3.3.1. Total Phenolic Content (TPC)

The TPC was determined according to the method of Meda *et al.* (2005) using the Folin-Ciocalteu assay. Briefly, 1 mL of a 5% (w/v) honey extract was mixed with 2.5 mL of 0.2 N Folin-Ciocalteu reagent. The mixture was vortexed and allowed to stand at room temperature for 5 minutes. Subsequently, 2 mL of a 6% (w/v) sodium carbonate

(Na_2CO_3) solution was added. The final mixture was incubated in the dark for 2 hours, after which the absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Jenway 6300, Bibby Scientific Ltd., UK). A standard curve was prepared using gallic acid at concentrations of 20, 40, 60, 80, and 100 µg/mL. The results were expressed as milligrams of gallic acid equivalents (GAE) per 100 grams of honey.

3.3.2. Total Flavonoid Content (TFC)

TFC was quantified using the aluminum chloride ($AlCl_3$) colorimetric method, as described by Marquele *et al.* (2005). A 5 mL aliquot of a 5% (w/v) honey solution was mixed with 5 mL of a 2% (w/v) aluminum trichloride ($AlCl_3$) solution in methanol. The solution was vortexed and incubated in the dark for 30 minutes. The absorbance of the reaction mixture was then measured at 415 nm. TFC was determined using a quercetin standard curve prepared at concentrations of 20, 40, 60, 80, and 100 µg/mL, and expressed as milligrams of quercetin equivalents (QE) per 100 g of honey.

All analyses were performed in triplicate, and the results are presented as mean values ± standard deviation.

3.4. Chromatographic Profiling

3.4.1. Solid Phase Extraction (SPE) and UPLC–ESI–MS/MS Analysis

To achieve a high-resolution characterization of individual phenolic compounds, samples were subjected to solid-phase extraction (SPE) followed by tandem mass spectrometry.

Sample Pre-treatment (SPE): Phenolic compounds were isolated according to the method described by İzol *et al.* (2025). Briefly, 5 g of honey was dissolved in 50 mL of ultrapure water, and the pH was adjusted to 2 using HCl to ensure that the polyphenols remained in their protonated, non-ionic form. The solution was purified using an Isolute C18 SPE cartridge, preconditioned with 10 mL of methanol: water (1:1, v/v). After loading, the cartridge was washed with 20 mL of acidified water (pH 2) to remove sugars and polar interference. The polyphenols were eluted with 2 mL of methanol and filtered through a 0.22 µm nylon syringe filter.

UPLC–ESI–MS/MS Conditions: Targeted profiling was performed on a Shimadzu 8040 system (Kyoto, Japan) equipped with Ultra-Fast Mass Spectrometry (UFMS) technology and a binary Nexera XR LC-20AD pump. Chromatographic separation was achieved on a Shim-pack C8 column (150 mm × 2.0 mm, 2.2 µm) maintained at 35 °C. The mobile phase consisted of (A) water and (B) methanol, both containing 2 mM ammonium formate and 0.002% formic acid. A linear gradient was applied, starting from 98% A and transitioning to 5% A over 7 minutes, followed by a re-equilibration period, for a total run time of 12 minutes. The injection volume was 10 µL.

Mass Spectrometry Parameters: The ESI source was operated in both positive and negative ionization modes using Multiple Reaction Monitoring (MRM). Parameters were set as follows: nebulizing gas flow, 3.0 L/min; drying gas flow, 10.0 L/min; desolvation line (DL) temperature, 250 °C; heat block temperature, 400 °C; and collision-induced dissociation (CID) gas pressure, 230 kPa. The conversion dynode voltage was set at −6.00 kV. Data acquisition was managed via LabSolutions software.

3.5. Botanical Origin Determination (Melissopalynology)

Pollen analysis was performed according to the standard melissopalynological methods described by Louveaux *et al.* (1970). Briefly, 10 g of each honey sample was dissolved in 25 mL of distilled water and centrifuged at 3000 rpm for 10 minutes to isolate the pollen fraction. The resulting sediment was resuspended and mounted on microscope slides with glycerin gelatin for examination under a light microscope (OPTIKA B-380 series, Italy) at 400x magnification. Pollen grains were identified to the lowest possible taxonomic level using reference pollen atlases and authenticated slide collections. A minimum of 800 pollen grains per sample was counted to ensure statistical reliability. Results were expressed as relative frequency percentages using the formula: P% = (n/N)*100, where ‘n’ represents the count for a specific pollen type and ‘N’ is the total pollen count. Following the International Commission for Bee Botany (ICBB) system, these frequencies were classified into: Dominant (>45%), Secondary (16–45%), Important Minor (3–<16%), and Minor Trace (<3%). This classification enables the objective determination of the botanical origin and floral authenticity of the investigated Algerian honey samples.

4. Statistical Analysis

4.1. Data Processing and Quality Control

All experiments were performed in triplicate, and the results are presented as the mean ± standard deviation (SD). Data normality and homogeneity of variance were verified using the Shapiro-Wilk and Levene's tests, respectively. For bioactive assays, IC_{50} values were determined through non-linear regression analysis using IBM SPSS Statistics 29.

4.2. Comparative and Metabolomic Analysis

Significant differences between honey and propolis samples based on geographic origin were evaluated using one-way analysis of variance (ANOVA), followed by Tukey's HSD post-hoc test ($p < 0.05$). To visualize the biochemical relationships between the regions (e.g., Batna vs. Biskra), Venn diagrams were used to identify shared and specific metabolites. Comparative distributions of major chemical classes and individual bioactive compounds detected by GC-MS and LC-MS/MS were illustrated using targeted bar charts and relative abundance plots, respectively.

4.3. Multivariate Data Analysis (PCA, HCA)

To explore the complex relationships between the physicochemical parameters and the chemical profiles of the samples, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were conducted using XLSTAT 2016 and R software (v4.0.0). PCA was performed on standardized data to identify the primary variables responsible for sample clustering. HCA was used to confirm the proximity between different Algerian terroirs based on Euclidean distance and Ward's linkage method.

III. Results & Discussion

1. Characterization of Algerian Propolis

1.2. Colorimetric Quantification of Bioactive Markers (TPC And TFC)

The quantitative assessment of total phenolic and flavonoid contents revealed significant chemical heterogeneity between the two Algerian propolis extracts (Table 2.3). Batna sample (PE1), harvested from a region characterized by a humid subtropical climate (Köppen: Cfa), exhibited markedly higher concentrations of both bioactive markers compared to the Biskra sample (PE2), obtained from a hot desert climate (Köppen: BWh). The latter showed significantly lower values for both total TPC and TFC ($p < 0.05$). These variations, confirmed via Tukey's HSD test, underscore the profound influence of regional bioclimatic conditions and local flora on the secondary metabolite profiles of the samples collected during the 2020 season.

The Cfa climate of Batna supports a diverse Mediterranean forest and mountain vegetation, providing a richer array of botanical precursors. In contrast, the environmental stress associated with the BWh desert climate in Biskra limits resin-producing vegetation to specific xerophytic species, resulting in a more specialized phenolic load. The TPC values obtained are consistent with ranges reported for other Algerian and Turkish samples, as well as Brazilian brown propolis, while remaining lower than those described for Brazilian green and red varieties (Kouadri *et al.*, 2021; Devequi-Nunes *et al.*, 2018). The TFC levels in this study exceeded several earlier reports for Algerian propolis (Teggar *et al.*, 2023; Kouadri *et al.*, 2021), though they remained lower than the levels documented by Ouahab *et al.* (2023) and those of Brazilian green and red propolis (Devequi-Nunes *et al.*, 2018). These discrepancies likely reflect the influence of geographical location and climatic conditions on resin availability and specific botanical sources.

The notable TPC and TFC values observed across the extracts highlight their significant bioactive potential, particularly given the well-established association between phenolic compounds and biological activities such as antioxidant capacity (Wang, X., *et al.*, 2016).

Table 2.3. Total Phenolic and Flavonoid Contents of Algerian Propolis Extracts.

Sample	TPC (mg GAE/g)	TFC (mg QE/g)
PE1	317.87 ±1.85[a]	50.68 ±4.09[a]
PE2	238.05 ±1.93[b]	43.47 ±0.96[b]

- Values are expressed as mean ± SD (n=3). Different superscript letters (a, b) within the same column indicate statistically significant differences ($p < 0.05$) according to Tukey's HSD test.

1.3. Volatile Compound Profiling by GC-MS

The volatile and semi-volatile chemical landscapes of the two Algerian propolis extracts were elucidated via GC-MS analysis, with the total ion current (TIC) chromatograms and identified constituents presented in Appendix A and (Table 2.4), respectively. A comparative analysis revealed that while both extracts share a fundamental chemical "core," they possess distinct metabolic signatures shaped by their respective environments.

Table 2.4. Volatile Compounds Identified in Algerian Propolis Extracts by Gas Chromatography–Mass Spectrometry (GC–MS).

Peak	Rt (min)	Profiled volatile	Quantification (µg compound/g extract)		*p*-value
			PE1	PE2	
1	6.74	Verbenyl ethyl ether	29.99 ± 0.01	-	-
5	8.22	α-Pinene	12.81 ± 0.03	-	-
7	8.44	α-Terpineol	55.75 ± 0.01	-	-
8	8.72	α-Terpinyl acetate	14.65 ± 0.01	-	-
11	10.38	δ-Cadinene	12.35 ± 0.01	-	-
12	10.74	β-Terpineol	35.01 ± 0.05	-	-
13	10.80	Cubebol	14.64 ± 0.01	-	-
14	11.51	Valerianol (4b*H*,5a-Eremophil-1(10)-ene)	28.88 ± 0.01	-	-
30	15.98	(13*R*)-8,13-Epoxylabd-14-ene	17.78 ± 0.04	-	
35	17.34	Pimaric acid	1631.19 ± 0.07	1505.00 ± 0.65	0.756
39	20.36	Isopimaric acid	404.72 ± 0.01	298.50 ± 0.16	0.319
45	21.00	Callitrisic acid	98.39 ± 0.02	-	-
42	20.71	4-epi-Abietic acid	22.13 ± 0.07	-	-
43	20.97	Dehydroabietic acid	-	95.27 ± 0.03	-
47	21.93	Methyl 7-β-hydroxydehydroabietate	116.48 ± 0.09	41.96 ± 0.01	0.001
48	22.38	Farnesol	-	9.08 ± 0.02	-
55	36.56	α-Amyrin	-	24.87 ± 0.01	-
56	38.15	Methyl commate C	-	12.82 ± 0.04	-
57	38.85	Lupeol	-	84.97 ± 0.04	-
Total terpenoids			***2494.77 µg/g***	***2072.47 µg/g***	*0.383*
4	8.04	Dihydrocinnamic acid	26.68 ± 0.01	-	-
10	9.93	Malic acid	-	148.60 ± 0.06	-
20	13.38	Citric acid	-	118.51 ± 0.01	-
22	13.85	Quininic acid	-	74.02 ± 0.03	-
26	15.01	10,12-Docosadiynedioic acid	344.62 ± 0.11	-	-
31	16.10	3,4-Dimethoxycinnamic acid	-	39.01 ± 0.01	-
33	16.79	Ferulic acid	-	55.10 ± 0.01	-
40	20.53	Caffeic acid	151.81 ± 0.03	297.22 ± 0.03	0.004
Total carboxylic acids			***523.11 µg/g***	***732.44 µg/g***	*0.004*
6	8.29	Butanedioic acid	55.75 ± 0.03	105.95 ± 0.05	0.205
19	13.50	Myristoleic acid	273.55 ± 0.07	-	-
32	16.37	Palmitic Acid	-	408.71 ± 0.14	-
36	18.596	Linoleic acid	-	436.92 ± 0.10	-
37	18.68	Oleic acid	-	994.56 ± 0.15	-
41	20.61	Oleamide	NQ	-	-
Total fatty acids			***329.30 µg/g***	***1946.14 µg/g***	*0.205*
17	12.85	D-Tagatose	-	62.95 ± 0.02	-
18	13.35	D-Fructose	90.64 ± 0.73	394.83 ± 0.15	0.032
21	13.63	D-Talose	-	43.39 ± 0.01	-
23	14.22	D-Psicose	50.26 ± 0.09	128.86 ± 0.04	0.031
24	14.28	D-Mannose	73.44 ± 0.02	102.69 ± 0.06	-
25	14.83	D-Galactose	43.75 ± 0.01	49.04 ± 0.01	0.505
28	15.38	D-Glucose	93.70 ± 0.02	147.76 ± 0.05	
52	24.36	Sucrose	-	96.33 ± 0.02	-
53	24.88	Methyl galactoside	-	101.45 ± 0.02	-

Peak	Rt (min)	Profiled volatile	Quantification (µg compound/g extract)		*p*-value
			PE1	**PE2**	
54	26.52	Mannobiose	-	54.98 ± 0.03	-
Total sugars			***351.79 µg/g***	***1095.64 µg/g***	*0.045*
38	20.24	Allocholic acid	38.99 ± 0.04	-	-
44	20.81	17-Methylandrosta-1,4-dien-3-one	26.59 ± 0.01	-	-
51	23.82	Androsta-3,5-diene-3,17-dione	43.16 ± 0.05	34.27 ± 0.07	0.151
Total steroids			***108.74 µg/g***	***34.27 µg/g***	*0.151*
3	7.81	Glycerol	15.26 ± 0.04	52.58 ± 0.03	0.140
27	15.09	*scyllo*-Inositol	7.43 ± 0.01	-	-
34	16.88	*myo*-Inositol	-	107.60 ± 0.04	-
Total alcohols			***22.69 µg/g***	***160.18 µg/g***	*0.140*
2	7.32	Epimethendiol	127.19 ± 0.08	-	-
9	9.61	1-Methyl 2-cyclohexene-1-methanol	5.47 ± 0.02	-	-
15	11.53	Gluconolactone	-	NQ	-
16	12.54	2-Furanacetaldehyde	-	NQ	-
29	15.66	Galactonic acid	-	86.65 ± 0.02	-
49	23.42	Retroretinol	15.78 ± 0.01	-	-
50	23.53	Naringenin	NQ	NQ	-
Other compounds					

-The results are presented as the mean ± standard deviation. ***(-),*** *not detected;* ***NQ,*** *not quantified;* ***PE1,*** *propolis extract 1;* ***PE2,*** *propolis extract 2;* ***Rt,*** *retention time.*

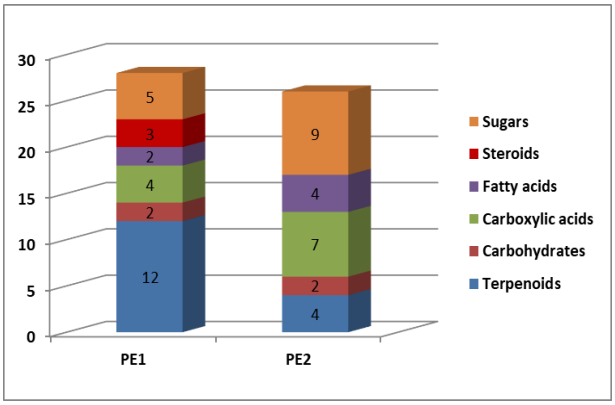
As illustrated in the comparative distribution of chemical families (Figure 2.3 (A)), both PE1 and PE2 are characterized by a marked predominance of terpenoids, a profile consistent with North African and Eastern Mediterranean propolis (Kasote *et al.*, 2022; Sarikahya *et al.*, 2021). In Batna sample (PE1), terpenoids clearly dominate the profile, followed by carboxylic acids, sugars, and fatty acids. Conversely, Biskra sample (PE2) is characterized by a higher relative proportion of fatty acids and sugars, suggesting a more polar chemical character compared to the resinous profile of PE1.

Among the 34 compounds identified in PE1 and 36 in PE2, pimaric acid, a tricyclic diterpene, was the most abundant constituent in both extracts. Other major diterpenes included isopimaric acid and 7-β-hydroxydehydroabietate methyl. The prevalence of these diterpenic structures is a defining feature of propolis from tropical and Mediterranean regions (Machado *et al.*, 2023; Kartal *et al.*, 2002) and is strongly associated with a broad spectrum of biological activities, including antibacterial, anti-inflammatory, and cytotoxic effects (Li *et al.*, 2022; Aminimoghadamfarouj and Nematollahi, 2017).

The qualitative relationship between the two samples was further explored using a Venn diagram (Figure 2.3 (B)). The two extracts share a biochemical "core" of 12 common compounds, representing approximately 26% of the total detected metabolites. This shared chemical signature likely constitutes the fundamental secondary metabolite framework of the studied propolis and may underlie the baseline biological properties of both samples. However, the high proportion of unique compounds in PE1 (34%) and PE2 (40%) highlights a significant degree of metabolic specificity, which may lead to distinct variations in their respective bioactive potencies.

These differences reflect the botanical richness and ecological diversity of Algeria (Boulechfar *et al.*, 2023). Specifically, the unique compounds in PE1 (Batna, Cfa climate) likely originate from the diverse forest resins of the Aurès mountains, whereas the specificities of PE2 (Biskra, BWh climate) point toward the specialized xerophytic flora of the desert steppe. In addition to the resinous terpenoids, the detection of caffeic acid, naringenin, and various fatty acids; which contribute to the biological and antioxidant potential of propolis (Naik *et al.*, 2021); further reinforces the value of these Algerian samples as high-quality bioactive sources.

(A) **(B)**



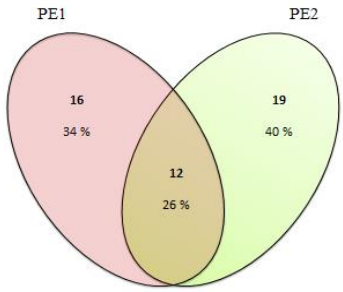


Figure 2.3. *Comparative chemometric analysis of Algerian propolis as determined by GC-MS profiling.* **(A)** Distribution of major chemical classes (by number of identified compounds) in propolis samples PE1 and PE2. **(B)** Venn diagram illustrating the distribution of shared and unique biochemical constituents identified in both extracts.

1.4. Targeted Phenolic Fingerprinting via UHPLC-DAD-ESI/MS

The high-resolution phenolic profiles of the two propolis extracts were elucidated using UHPLC-DAD-ESI/MS, with representative chromatograms and detailed chemical compositions presented in Appendix B and Table 2.5, respectively. This targeted analysis allowed for the identification of a sophisticated array of phenolic acids, flavonoids, and their derivatives, providing a deeper "fingerprint" than spectrophotometric methods.

As illustrated in the comparative analysis of chemical classes (Figure 2.4 (A)), both PE1 and PE2 are dominated by phenolic acids and flavonoids. However, PE2 exhibited a more diversified polyphenolic profile, identifying 11 phenolic acids and 12 flavonoids compared to 8 and 9 in PE1, respectively. The most abundant constituents in both extracts included caffeic acid isoprenyl esters (I, II, and III), pinobanksin, and pinobanksin-3-O-acetate. The detection of these compounds, along with kaempferol, chrysin, and quercetin, aligns with profiles previously reported for propolis from Algeria (Ayad *et al.,* 2024; Boulechfar *et al.,* 2022), Turkey (Ozdal *et al.,* 2019), and Brazil (Xu *et al.,* 2020). Notably, various phenolic and flavonoid glycosides were detected. These metabolites have been only sporadically documented in propolis literature prior to 2011, with occurrences mainly restricted to specific samples from Brazil, Portugal, and the United Kingdom (Kasote *et al.,* 2022). To the authors' knowledge, this is the first report on the detection of two specific phenylpropanoid compounds in Algerian propolis, both of which were identified in the Biskra sample (PE2). The first, *p*-methoxycinnamic acid cinnamyl ester, has been previously identified in propolis from Australia and Egypt (Kasote *et al.,* 2022). The second, 1,3-*O*-caffeoyl dihydrocaffeoylglycerol, is a phenylpropanoid glyceride described here for the first time in the propolis. While previously detected in sorghum grains (Kang *et al.,* 2016) and the Mediterranean shrub Myrtus communis (Okaiyeto *et al.,* 2023), its occurrence in PE2 suggests a botanical link to sorghum-derived sources, which are well-adapted to the arid BWh climate of Biskra (Kang *et al.,* 2016).

The qualitative relationship between the extracts was further explored via a Venn diagram (Figure 2.4 (B)). The 14 shared compounds (54%) constitute the core chemical signature of these regional samples. However, PE2 showed a significantly higher degree of specificity, with 9 unique metabolites (35%) compared to only 3 for PE1 (11%). This

greater chemical diversification in PE2 may correspond to a broader spectrum of bioactive potencies.

The superior phenolic diversity and concentration observed in PE2 may be attributed to a combination of altitudinal and climatic factors. The lower altitude of the Biskra site (121 m) compared to Batna (1045 m) may favor the accumulation of flavonoids, as lower elevations have been suggested to promote certain secondary metabolites (Sorucu & Oruç, 2019; Anđelković *et al.*, 2017). Furthermore, while humid periods (such as in Batna's Cfa climate) may favor primary metabolite accumulation, the drier, high-stress conditions of Biskra's BWh climate often enhance the synthesis of secondary metabolites as a plant defense mechanism (do Nascimento *et al.*, 2019; Regueira *et al.*, 2017).

Table 2.5. Phenolic Compounds Identified in Algerian Propolis Extracts Using UHPLC-DAD-ESI/MS.

Peak	Identified Compound	Rt (min)	λ (nm)	$[M-H]^-$	Quantification (µg/g Extract)		*p*-Value
					PE1	PE2	
1	Unknown	1.66	208, 258, 282, 319	377			-
2	Caffeic acid	2.38	208, 324	179	572.08 ± 0.04	-	0.003
		2.40	208, 290, 323		-	1666.71 ± 0.44	
3	*p*-Coumaric acid	3.44	208, 312	163	-	511.68 ± 0.27	-
4	Ferulic acid derivative	4.09	208,323	397	277.47 ± 0.17	-	
		7.45	208, 324		137.57 ± 0.07	-	0.023
		7.54	208, 322			456.98 ± 0.20	
5	Gallic acid 4-*O*-glucoside	17.37	208, 311	331 (377)	-	NQ	-
6	Caffeic acid isoprenyl ester I	21.44	209, 269, 322	247	3518.39 ± 0.14	-	<0.0001
		21.54	208, 268, 320		-	12,127.35 ± 0.02	
7	Caffeic acid isoprenyl ester II	22.27	207, 299, 325	247	2572.2 ± 1.63	-	0.349
		22.37			-	6820.26 ± 2.77	
8	Caffeic acid isoprenyl ester III	22.85	208, 290, 330	247	2743.55 ± 0.22	-	0.964
		22.93			-	8932.12 ± 1.39	
9	Caffeic acid phenylethyl ester	25.39	209, 326	283	449.38 ± 0.46	-	0.157
		25.50	208, 295, 326		-	2928.35 ± 1.62	
10	*p*-Coumaric acid isoprenyl ester	30.21	208, 314	231	-	728.37 ± 0.30	-
11	Caffeic acid cinnamyl ester	30.68	207, 294, 326	295	-	842.25 ± 0.39	-
12	*p*-Coumaric acid hexose	35.60	209, 290, 328	325	-	NQ	-
13	Caffeic acid derivative	36.85	209, 263, 318	315 (361)	314.24 ± 0.14	-	-
14	Ferulic acid 4-*O*-glucoside	39.21	208, 293, 335	355	-	NQ	-
15	*p*-Methoxycinnamic acid cinnamyl ester	41.55	206, 281	293 (339)	-	282.75 ± 0.35	-
16	Benzofuran derivative	45.83	229, 278	301	1273.47 ± 0.23	-	0.064
		45.86	231, 276		-	361.13 ± 0.07	
17	Ferulic acid derivative	47.35	252, 326	517	-	584.66 ± 0.12	-
Total phenolic acids					***11,858.35 µg/g***	***35,448.18 µg/g***	***0.550***
18	Querceti-3-*O*-rhamnoside	4.15	208, 297, 343	447	-	NQ	-
19	Pinobanksin-5-methyl ether	10.16	208, 287	285	-	1257.76 ± 1.28	-
20	Quercetin-3-methyl-ether	10.74	207, 265, 355	315	-	1398.68 ± 0.61	0.604
		36.17	207, 267, 353	315 (361)	1263.72 ± 0.63	-	

	Identified Compound	Rt (min)	λ (nm)	[M-H]⁻	Quantification (µg/g Extract)		*p*-Value
21	Pinobanksin	12.31	208, 292, 335	271	3140.4 ± 0.37	-	0.006
		12.41			-	7102.82 ± 0.82	
22	Kaempferol	12.80	207, 265, 368	285	1773.39 ± 1.53	-	0.598
		12.89	206, 266, 367		-	646.02 ± 0.05	
23	Kaempferide	14.27	207, 266, 351	299	292 ± 0.11	339.23 ± 0.24	0.132
24	Quercetin-dimethyl ether	19.41	207, 261, 356	329	-	470.40 ± 0.37	-
25	Apigenin	23.43	207, 266, 358	269	1894.54 ± 0.58	-	0.036
		23.53	202, 265, 358		-	4158.55 ± 1.62	
26	Pinobanksin-3-*O*-acetate	24.84	208, 293	313	4530.01 ± 1.47	-	0.038
		24.93			-	9730.09 ± 3.59	
27	Naringenin hexoside	26.13	208, 331	433	NQ	-	-
		26.24	203, 300, 331		-	NQ	-
28	Chrysin	29.35	208, 316	253	-	1151.80 ± 0.92	-
29	Pinobanksin-3-*O*-propionate	31.78	209, 292, 328	327	470.08 ± 0.09	1121.68 ± 0.13	0.026
30	Pinobanksin-3-*O*-pentanoate	35.21	288. 329	355	NQ	-	-
		35.26	208, 268, 329		-	NQ	-
31	Hesperetin	35.56	210, 289, 368	317 (363)	NQ	-	-
32	Isorhamnetin	35.85	210, 270, 372	315	907.19 ± 0.31	2767.44 ± 0.28	0.040
33	Quercetin	47.81	255, 367	301 (347)	129.67 ± 0.09	-	-
Total flavonoids					***14,401.00 µg/g***	***30,144.47 µg/g***	***0.009***
34	1,3-*O*-Caffeoyl-dihydrocaffeoylglycerol	35.88	208, 260, 294, 324	417	-	NQ	-
Other phenolic compounds							

-All results are expressed as mean ± standard deviation. Peak identification was performed either by comparing retention times, mass spectra, and diode array detector (DAD) spectra with those of authentic standards analyzed under identical conditions, or by referencing previously reported mass spectrometric and UV–visible data from the literature. Quantification of each compound was carried out using external calibration curves constructed with the most structurally similar pure standards. Abbreviations used in the tables are as follows: ***(−)****, not detected;* ***[M−H]⁻,*** *pseudomolecular ion;* ***NQ,*** *not quantified;* ***PE1,*** *Propolis Extract 1;* ***PE2,*** *Propolis Extract 2;* ***Rt,*** *retention time.*

(A) **(B)**

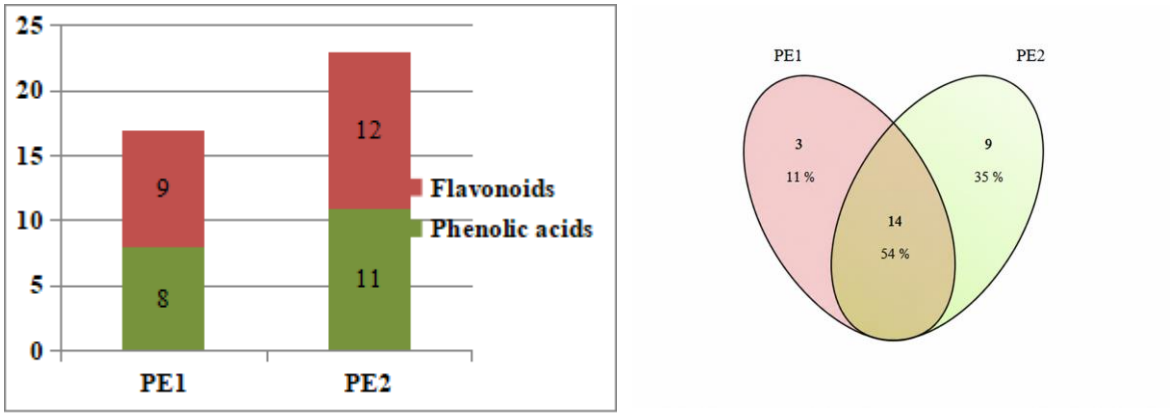


Figure 2.4. Comparative metabolomic analysis of Algerian propolis by UHPLC-DAD-ESI/MS. **(A)** Distribution of phenolic acids and flavonoids in propolis extracts PE1 (Batna) and PE2 (Biskra). **(B)** Venn diagram showing the number and percentage of shared and unique phenolic compounds detected in PE1 and PE2.

These complex environmental interactions should be carefully considered in future strategies aimed at standardizing propolis quality. Ultimately, these compositional differences; particularly the richer polyphenolic profile of PE2, suggest a potential for enhanced antioxidant and antimicrobial activities, which will be evaluated in the subsequent sections of this work.

1.5. *In Vitro* Antioxidant Capacity

The antioxidant potential of the Algerian propolis extracts was evaluated through a multi-mechanism approach, utilizing radical scavenging assays (DPPH and ABTS) alongside reducing power assays (FRAP and phenanthroline). Following the recommendations of Hossain *et al.* (2022) for robust antioxidant characterization, these complementary methods were employed to capture diverse single-electron transfer and hydrogen atom transfer dynamics. This integrative strategy ensures a comprehensive assessment of the extracts' ability to neutralize oxidative species through various chemical pathways. As summarized in Table 2.6 and visually depicted in Figure 2.5,

efficiency was expressed via IC_{50} and $A_{0.5}$ values, where lower concentrations signify superior antioxidant capacity.

Table 2.6. The antioxidant Capacity of the Algerian Propolis Extracts.

	IC_{50} (µg/mL)		$A_{0.5}$ (µg/mL)	
Sample	**DPPH**	**ABTS**	**FRAP**	**Phen**
PE1	150.68 ± 1.44 ^{e}	60.49 ± 0.22 ^{e}	110.09 ± 2.97 ^{d}	57.44 ± 1.35 ^{d}
PE2	27.74 ± 0.19 ^{d}	26.30 ± 0.12 ^{d}	5.16 ± 0.12 ^{a}	18.90 ± 1.13 ^{c}
Ascorbic acid *	4.70 ± 0.01 ^{b}	12.92 ± 0.18 ^{c}	5.47 ± 0.20 ^{a}	3.74 ± 0.17 ^{b}
BHT *	10.49 ± 0.07^{c}	2.68 ± 0.08 ^{b}	28.96 ± 1.87 ^{c}	1.84 ± 0.15 ^{a,b}
BHA *	5.41 ± 0.01 ^{b}	2.42 ± 0.11 ^{b}	9.33 ± 0.06 ^{b}	0.79 ± 0.09 ^{a}
Quercetin *	2.12 ± 0.04 ^{a}	1.52 ± 0.02 ^{a}	4.38 ± 0.09 ^{a}	0.75 ± 0.01 ^{a}

-All data are presented as mean ± standard deviation (SD) of three independent determinations. Samples indicated by an asterisk (*) correspond to reference standards. Lower values reflect stronger antioxidant activity. Values within the same column bearing different superscript letters (a–e) differ significantly ($p < 0.0001$), as determined by one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test. Abbreviations: $A_{0.5}$, concentration required to achieve an absorbance of 0.500; ***ABTS,*** *2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid);* ***BHA,*** *butylated hydroxyanisole;* ***BHT,*** *butylated hydroxytoluene;* ***DPPH,*** *2,2-diphenyl-1-picrylhydrazyl;* ***FRAP,*** *ferric reducing antioxidant power;* ***IC_{50}****, half-maximal inhibitory concentration;* ***PE1****, propolis extract 1;* ***PE2,*** *propolis extract 2;* ***Phen****, 1,10-phenanthroline.*

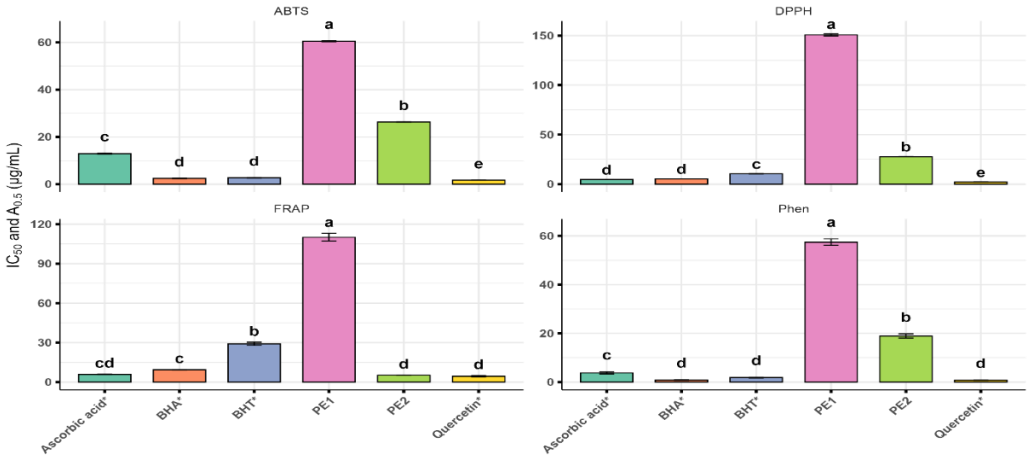


Figure 2.5. IC_{50} (DPPH, ABTS) and $A_{0.5}$ (FRAP, Phen) values of Algerian propolis extracts. Different superscript letters within the same column indicate significant differences ($p < 0.0001$).

The two extracts exhibited markedly different antioxidant performances across all assays ($p < 0.0001$), with PE2 consistently outperforming PE1. In the DPPH assay, PE2 demonstrated approximately a five-fold greater activity than PE1, while its ABTS radical scavenging capacity was nearly two-fold greater. This difference was further accentuated in the reducing power assays, reflecting a stronger electron-donating capacity. In the FRAP assay, PE2 demonstrated a markedly higher reducing capacity, reaching values up to 21-fold greater than PE1. Notably, the activity of PE2 in this assay (5.16 ±0.12 µg/mL) surpassed the reference synthetic antioxidants BHA and BHT, and closely approached the efficacy of the pure flavonoid quercetin and ascorbic acid.

A similar trend was observed in the phenanthroline assay, where PE2 demonstrated higher reducing power than PE1, further supporting its enhanced electron-donating capacity. These findings are consistent with the qualitative and quantitative phytochemical differences identified in the previous characterization sections. The enhanced activity of the Biskra sample is likely associated with its more diverse and concentrated polyphenolic profile, as identified by UHPLC-DAD-ESI/MS. In particular, the higher abundance of phenolic acids and flavonoids may contribute to a broader synergistic effect in radical scavenging capacity.

Compared with existing literature, the studied extracts exhibited higher antioxidant activity than several previously reported Algerian samples (Kouadri et al., 2021), although remaining below the levels described for northeastern regions (Ouahab *et al.*, 2023). The performance of PE2 in the phenanthroline assay is consistent with the findings of Hadjab *et al.* (2024), while its DPPH activity is comparable to that reported by Ayad *et al.* (2024). Notably, the FRAP values obtained in this study exceeded those reported by Teggar *et al.* (2024). These variations highlight the pronounced regional variability in propolis bioactivity, likely influenced by differences in floristic composition and geographical origin, particularly between the Batna and Biskra regions.

Overall, these findings underscore the significant bioactive potential of Algerian propolis, especially the PE2 extract, as a promising natural source of antioxidant compounds.

1.6. Multivariate Correlation and Statistical Integration

To further elucidate the complex interrelationships between the identified phenolic constituents and their corresponding antioxidant properties, a Principal Component Analysis (PCA) was performed. As illustrated in the biplot (Figure 2.6), the first two principal components (F1 and F2) accounted for 87.15% of the total variance, effectively condensing the multi-analytical dataset into a coherent metabolic map. The PCA scores revealed a clear spatial segregation of the samples along the F1 axis (66.93%), with the Biskra extract (PE2) clustering on the positive side and the Batna extract (PE1) grouping on the negative side. Interestingly, the vectors for total phenolic content (TPC), total flavonoid content (TFC), and all antioxidant assays (DPPH, ABTS, FRAP, and Phen) were situated in the negative F1 quadrant, closely associated with the PE1 replicates. This clustering indicates a strong internal correlation between the spectrophotometric values and the radical scavenging capacity, a trend consistent with the observations of Laaroussi *et al.* (2021) and Wang, X., *et al.* (2016). However, the loading plot reveals that the unique bioactivity profile of PE2 is driven by a specific set of high-potency biomarkers rather than just total concentration. Compounds enriched on the positive F1 side, predominantly associated with the Biskra region, included *p*-coumaric acid, caffeic acid cinnamyl ester, *p*-methoxycinnamic acid cinnamyl ester, and chrysin. In contrast, the PE1-specific cluster was characterized by elevated levels of quercetin, kaempferol, and specific caffeic acid derivatives. The second principal component (F2; 20.23%) served to further distinguish the regional signatures, with the positive side correlating with kaempferide and pinobanksin-3-*O*-propionate, and the negative side with quercetin-3-methyl ether. As suggested by Šuran *et al.* (2021), these distributions imply that the antioxidant efficacy of propolis is a result of complex synergistic or antagonistic interactions among its constituents.

Collectively, this multivariate approach confirms that while both geographic origin and altitudinal stress dictate the chemical framework, the presence of specialized phenylpropanoid markers in the arid BWh region provides a distinct metabolic advantage in terms of secondary metabolite diversity and functional potency.

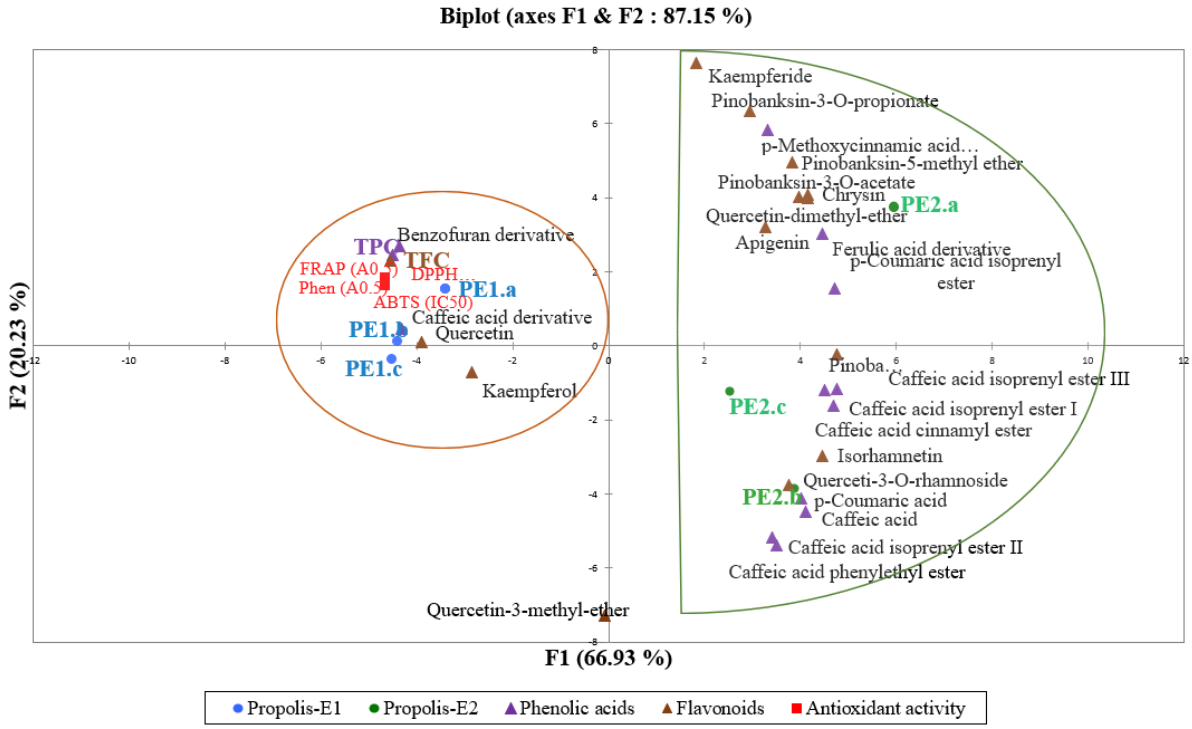


Figure 2.6. Principal component analysis (PCA) biplot showing the distribution of bioactive compounds identified in propolis extracts by UHPLC-DAD-ESI/MS.

***TPC:** Total phenolic content, **TFC:** Total flavonoid content, **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl, **ABTS:** 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), **FRAP:** Ferric reducing antioxidant power, **Phen:** 1,10-Phenanthroline, **PE.1, PE.2, PE.3:** Propolis extract 1(or 2) (three independent replicates).*

2. Characterization of Algerian Honey

2.1. Melissopalynological and Physicochemical Quality Assessment

The botanical identity and commercial quality of the three Algerian honey samples (H1, H2, and H3) were established through microscopic pollen analysis and physicochemical screening. As summarized in Tables 2.7 and 2.8, all measured parameters complied with both the Codex Alimentarius and the Algerian Standard NA 15304 (2016), confirming the freshness, maturity, and proper post-harvest processing of the samples. Pollen analysis enabled clear botanical classification. H2 (Biskra) was confirmed as a monofloral jujube honey due to the dominance of Ziziphus lotus pollen (>45%), aligning with established Algerian thresholds (Mekious *et al.*, 2022; Zerrouk *et al.*, 2021). This classification is further supported by its positive optical rotation (+2.52°),

a critical discriminating variable for authenticating unifloral *Ziziphus* status (Haderbache *et al.*, 2013), whereas H1 (–6.15°) and H3 (–5.58°) exhibited negative values typical of multifloral honeys. Accordingly, H1 (Skikda) and H3 (Laghouat) were classified as multifloral honeys, reflecting the botanical diversity of their respective regions. H1 showed a richer and more diverse pollen spectrum (Fabaceae, Asteraceae, Apiaceae), consistent with coastal vegetation, while H3 displayed a more restricted profile typical of steppe environments dominated by Fabaceae and Asteraceae (Abdellah *et al.*, 2020; Homrani *et al.*, 2020).

The physicochemical parameters further highlighted differences among samples. The pH values of H1 (3.99) and H2 (4.10) fell within the typical range reported for Algerian honeys (3.65–4.35) and were consistent with previous studies (Haderbache *et al.*, 2013; Makhloufi *et al.*, 2010). In contrast, H3 exhibited an unusually high pH (6.26), exceeding commonly reported values (3.7–4.78) (Homrani *et al.*, 2020; Nair *et al.*, 2017). This elevated pH, combined with its low free acidity (9.5 meq/kg), suggests a distinct chemical profile, potentially influenced by specific botanical sources or mineral composition.

Free acidity values (9.5–43.5 meq/kg) remained within the acceptable limit (≤ 50 meq/kg), confirming good quality and absence of fermentation (Makhloufi *et al.*, 2010). Moisture contents (14.0–16.8%) were also within recommended ranges and indicative of proper honey maturation, with the lower value observed in H2 reflecting higher stability and shelf-life potential (Homrani *et al.*, 2020; Haouam *et al.*, 2016). Similarly, HMF levels (4.9–14.82 mg/kg) were well below the regulatory threshold, confirming freshness and appropriate storage conditions (Amessis-Ouchemoukh *et al.*, 2021; Makhloufi *et al.*, 2010).

Electrical conductivity values further reflected the influence of botanical and geographical origin. H1 showed a relatively high conductivity (1.026 mS/cm), exceeding the typical limit for floral honeys (0.8 mS/cm), which may indicate a higher mineral content or the presence of honeydew-related compounds (Haouam et al., 2016; *Moussa et al.*, 2012). In contrast, H2 (0.459 mS/cm) and H3 (0.512 mS/cm) displayed values consistent with floral honeys from semi-arid environments (Zerrouk *et al.*, 2021).

Overall, these variations highlight the strong influence of regional flora and environmental conditions on the physicochemical characteristics of Algerian honey.

Table 2.7. Melissopalynological Results of Algerian Honey Samples.

Honey sample	Pollen Frequency Classes			
	Dominant pollens (> 45%)	**Accompanying pollens (16% - 45%)**	**Minor pollens (3% - 16%)**	**Very minor or isolated pollens (< 3%)**
H1	–	-Fabaceae; -Asteraceae (*Cirsium, Sonchus*); -Apiaceae (wild umbellifers); -Boraginaceae (*Echium*-type)	-Fabaceae (*Anthyllis lotoïdes*); -Brassicaceae (colza); - Apiaceae (*Pimpinella*-type); -Balsaminaceae; Cruciferae; *Eucalyptus* spp.; -*Citrus* spp.; -*Rosmarinus officinalis*	-*Eucalyptus* spp.; -*Sambucus* spp.; -Caryophyllaceae; -*Pinus* sp.; -Rosaceae; -Oleaceae (*Olea europaea*); -Poaceae; -Lamiaceae
H2	Rhamnaceae (*Ziziphus lotus*)	-*Eucalyptus* spp.; -Fabaceae; -Asteraceae (*Artemisia herba-alba*); -Apiaceae (umbellifers)	*Arctium* (burdock); Poaceae (anemophilous grasses); Brassicaceae (*Brassica*); Lamiaceae (thyme); Oleaceae (*Olea europaea*); Boraginaceae (*Echium*)	-Cruciferae; -Asteraceae; -Apiaceae; -*Rosmarinus*-type
H3	–	-Fabaceae; -Asteraceae	-Euphorbiaceae (*Euphorbia*); -Rosaceae; -Brassicaceae (Cruciferae); -Lamiaceae	-*Eucalyptus* spp.; -Apiaceae (umbellifers); - Boraginaceae (*Echium*); -*Arctium* (burdock); -Euphorbiaceae

-Percentages are based on a minimum count of 800 grains per sample. ***Note:*** *Pollen frequency classes are assigned individually to each pollen type according to its relative frequency among the total pollen grains counted. The percentages are therefore not additive and should not be summed across pollen types or frequency classes.*

Table 2.8. Physicochemical Quality Parameters of Algerian Honey Samples.

Parameter	H1	H2	H3	Standard limit[1]
pH	3.99 ± 0.04	4.10 ± 0.02	6.26 ± 0.05	3.2–4.5
Free acidity (meq/kg)	43.5 ± 0.9	25.5 ± 0.4	9.5 ± 0.5	≤ 50
Moisture (%)	15.2 ± 0.2	14.0 ± 0.15	16.8 ± 0.2	≤ 20
HMF (mg/kg)	11.1 ± 0.3	14.82 ± 0.3	4.9 ± 0.2	≤ 40
Electrical conductivity (µS/cm)	1.026 ± 2	0.459 ± 7	0.512 ± 7	≤ 0.8 (floral)[2]
Optical rotation (°)	–6.15 ± 0.05	+2.52 ± 0.08	–5.58 ± 0.03	–

-[1] Based on (NA 15304, 2016) and Codex Alimentarius (2001) standards for honey.

[2] A value > 0.8 mS/cm typically indicates the presence of honeydew honey or mineral-rich floral types from specific bioclimatic zones. All results are expressed as Mean ± Standard Deviation (n=3). Electrical conductivity and optical rotation were measured at 20°C.

2.2. Spectrophotometric Determination of TPC and TFC

Quantitative screening of bioactive markers revealed significant variability in total phenolic content (TPC) and total flavonoid content (TFC) among the three honey samples (Table 2.9). Sample H1 exhibited the lowest values (368.32 mg GAE/100 g for TPC and 55.75 mg QE/100 g for TFC), whereas H2 and H3 showed markedly higher phenolic levels (455.92 and 459.79 mg GAE/100 g, respectively). Notably, H3 exhibited the highest flavonoid concentration (89.12 mg QE/100 g), significantly exceeding both H1 and H2 ($p < 0.05$).

Overall, these values are considerably higher than the ranges previously reported for Algerian honeys (22–122 mg GAE/100 g for TPC and 0.07–33.49 mg QE/100 g for TFC) (Bereksi-Reguig *et al.*, 2024; Haderbache *et al.*, 2013). This variation is primarily linked to botanical origin. Sample H2, identified as unifloral *Ziziphus lotus* honey, is consistent with the well-documented phenolic richness of this species (Haderbache *et al.*, 2013). Likewise, H3, a multifloral honey from a semi-arid steppe environment, likely derives its elevated phenolic and flavonoid content from the diversity of contributing plant families, particularly Fabaceae and Asteraceae (Harbane *et al.*, 2024).

Interestingly, no direct relationship was observed between electrical conductivity and phenolic content. Despite exhibiting the highest electrical conductivity (1.026 mS/cm), H1 contained the lowest TPC and TFC values. This observation suggests that the factors governing mineral enrichment, such as soil salinity in coastal regions or the presence of honeydew markers, are distinct from the biosynthetic pathways responsible for secondary metabolite accumulation. These findings reinforce that botanical origin exerts a more decisive influence on the phenolic composition and bioactive potential of Algerian honeys than mineral content alone (Alvarez-Suarez *et al.*, 2017), providing a robust quantitative foundation for the high-resolution molecular fingerprinting discussed in the following section.

Table 2.9. Total Polyphenols and Flavonoids Contents of Algerian Honey Samples (mg/100g) Expressed as Mean ± SD.

Sample	Polyphenols (mg/100g)	Flavonoids (mg/100g)
H1	368.32 ± 1.55^{a}	55.75 ± 1.14^{a}
H2	455.92 ± 1.05^{b}	60.88 ± 0.71^{b}
H3	459.79 ± 7.48^{b}	89.12 ± 1.03^{c}

-Statistical analysis was performed using one-way ANOVA followed by Tukey's HSD multiple comparison test ($p < 0.05$). Results are expressed as mean ± standard deviation (SD, $n = 3$). Superscript letters (a–c) within the same column indicate significant differences: values sharing the same letter are not significantly different, whereas values with different letters are significantly different.

2.3. Phenolic Compound Identification by UPLC–ESI–MS/MS

The high-resolution molecular landscape of the honey samples was elucidated through UPLC–ESI–MS/MS analysis, which successfully identified and quantified 17 bioactive metabolites across several chemical families (Table 2.10). Representative total ion chromatograms (TICs) are presented in Appendix C. All three honeys share a highly conserved chemical profile. As illustrated in Figure 2.7(A), the samples are dominated by phenolic acids (4–5 compounds) and flavonoids (5 compounds), with additional contributions from stilbenes, terpenes, vitamins, and carotenoids. The Venn diagram (Figure 2.7 (B)) further confirms this stability, showing that 88% of detected metabolites

(15 compounds) are common to all three samples. This high degree of overlap suggests a common functional background for Algerian honeys, likely derived from ubiquitous regional taxa such as *Eucalyptus* and Asteraceae (Haderbache *et al.*, 2013).

Despite this shared core, significant quantitative variations reflect the specific botanical origins established before. Flavonoids (epicatechin, quercetin, luteolin, naringenin, rutin) and hydroxycinnamic acids (caffeic, p-coumaric, sinapic) were detected in all samples but at different concentrations. H1 showed the highest enrichment of resveratrol (1.95 µg/g), kojic acid (10.25 µg/g), and 2-methoxybenzoic acid (6.09 µg/g). Conversely, the arid-zone samples H2 and H3 displayed elevated levels of oleuropein (3.10 and 3.85 µg/g, respectively), sinapic acid, and riboflavin. The presence of oleuropein is particularly significant. As a secoiridoid typically associated with olive-derived products, its enrichment in H2 and H3 correlates with *Olea europaea* pollen detected in the sediment, highlighting the direct impact of local flora on the honey's chemical fingerprint (Bereksi-Reguig *et al.*, 2024; Harbane *et al.*, 2024).

These quantitative differences suggest that honeys from semi-arid regions (H2 and H3) tend to accumulate greater quantities of specific phenolic compounds compared to the humid coastal sample (H1), possibly as a protective mechanism against environmental stressors (Alvarez-Suarez *et al.*, 2017; Meda *et al.*, 2005). While the three honeys share a common metabolic core, the specific quantitative enrichment of high-potency molecules in H2 and H3 may support their enhanced functional and therapeutic potential.

(A) **(B)**

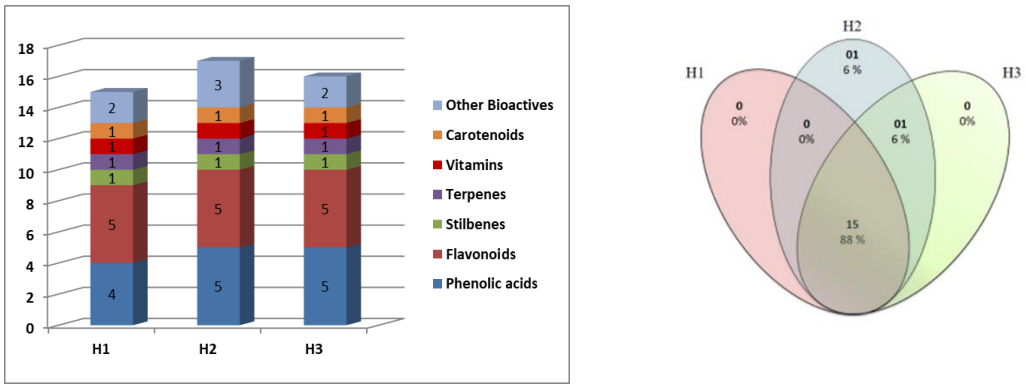


Figure 2.7. Comparative Metabolomic Analysis of Algerian Honey Samples (H1-H3), **(A)** LC–MS/MS-based distribution of major bioactive families showing the structural dominance of phenolic acids and flavonoids; **(B)** Venn diagram illustrating the highly conserved metabolite profile across the three geographical regions.

Table 2.10. Quantification of Diverse Bioactive Metabolites in Algerian Honey Samples (H1–H3) by UPLC–ESI–MS/MS.

Analyte	Formula	MW	ESI Charge	RT (min)	H1 (µg/g)	H2 (µg/g)	H3 (µg/g)	LOD (ppb)	LOQ (ppb)	R^2
Epicatechin	C15H14O6	290.27	(+)	6.83	-	0.08	0.06	50.91	154.00	0.985
Quercetin	C15H10O7	302.23	(+)	4.25	0.62	0.08	0.20	60.53	183.44	0.999
Resveratrol	C14H12O3	228.24	(+)	4.36	**1.95**	0.50	0.30	20.29	61.48	0.996
Luteolin	C15H10O6	286.24	(+)	4.60	0.19	0.41	0.39	6.87	20.82	0.996
Naringenin	C15H12O5	272.25	(+)	4.18	0.22	0.03	0.13	2.05	6.20	0.965
Oleuropein	C30H48O3	540.50	(+)	6.03	**1.10**	**3.10**	**3.85**	125.37	379.92	0.993
Riboflavin	C17H20N4O6	376.40	(+)	6.47	0.41	0.75	1.00	7.32	22.19	0.989
Curcumin	C21H20O6	368.40	(+)	3.73	-	0.01	-	0.17	0.50	0.992
Rutin	C27H30O16	610.50	(+)	4.25	0.15	0.05	0.04	2.42	7.32	0.998
β-Carotene	C40H56	536.87	(+)	6.41	0.03	0.01	0.06	5.12	16.10	0.982
2-Methoxybenzoic Acid	C8H8O3	152.15	(+)	4.24	6.09	**9.99**	1.84	70.47	213.53	0.956
Kojic Acid	C6H6O4	142.11	(+)	3.59	**10.25**	4.26	0.86	149.62	453.38	0.948
Oleanolic Acid	C30H48O3	456.70	(+)	6.26	0.04	0.03	0.02	4.18	12.66	0.925
Sinapic Acid	C11H12O5	224.21	(+)	3.90	0.22	0.87	1.01	3.58	10.84	0.997
Caffeic Acid	C9H8O4	180.16	(–)	4.15	0.03	0.02	0.01	2.27	6.88	0.968
***p*-Coumaric Acid**	C9H8O3	164.16	(–)	3.78	0.05	0.16	0.05	0.61	1.84	0.996
Vanillic Acid	C8H8O4	168.15	(–)	3.72	0.06	0.18	0.10	1.92	5.82	0.989

-RT, *retention time;* ***H1,*** *honey sample 1;* ***H2,*** *honey sample 2;* ***H3,*** *honey sample 3;* ***LOD,*** *limit of detection;* ***LOQ,*** *limit of quantification;* ***R^2,*** *coefficient of determination;* ***(-)****, not detected.*

IV. Conclusion: From Chemical Fingerprints to Bioactive Potential

The phytochemical characterization of Algerian honey and propolis conducted in this study highlights the profound influence of botanical and bioclimatic diversity on their chemical composition. Our analysis revealed a substantial overlap in identified metabolites, particularly phenolic acids and flavonoids. Notably, total phenolic and flavonoid content did not directly correlate with antioxidant activity, indicating that qualitative chemical richness, rather than total abundance, determines bioactivity. This was clearly demonstrated by the Biskra propolis sample (PE2), which exhibited superior activity compared to PE1, despite having lower TPC/TFC values, achieving performances comparable to, and in some assays exceeding, those of standard antioxidants such as ascorbic acid and quercetin.

Despite the shared metabolic background, clear differences were observed in metabolite concentration and physicochemical properties. Honey represents a predominantly water-soluble matrix with relatively low concentrations of individual phenolic compounds, whereas propolis constitutes a more concentrated, resinous source of bioactive metabolites, particularly lipophilic compounds. Variations between samples further suggest that environmental conditions, including aridity and solar exposure, influence the accumulation of secondary metabolites.

From a compositional perspective, the bioactive potential of Algerian bee products appears strongly linked to their geographical and botanical origin. Skikda honey (H1) was characterized by the presence of compounds such as kojic acid and 2-methoxybenzoic acid, while Biskra honey (H2) exhibited a distinct *Ziziphus lotus* signature. Batna propolis (PE1) showed a diterpene-associated profile, whereas Biskra propolis (PE2) revealed two previously unreported compounds: *p*-methoxycinnamic acid cinnamyl ester (reported here for the first time in Algerian propolis) and 1,3-*O*-caffeoyl dihydrocaffeoylglycerol (reported for the first time in propolis worldwide).

Overall, this integrative phytochemical profiling demonstrates that regional origin is a key determinant of chemical composition and suggests that bioactivity is more closely related to specific molecular signatures than to general physicochemical parameters. These findings provide a solid foundation for subsequent investigations into the antibacterial properties of these products and their relevance against mastitis-associated pathogens.

Chapter 2. Antibacterial and Synergistic Potential of Algerian Honey and Propolis Against Bovine Mastitis Pathogens.

I. Introduction

Antimicrobial resistance in bovine mastitis poses a critical global issue that crosses international boundaries and significantly threatens the sustainability of the dairy sector. Pathogens, such as *S. aureus* and *E. coli*, increasingly exhibit multidrug-resistant (MDR) phenotypes, necessitating the rigorous validation of non-conventional therapeutic agents (Tahar *et al.*, 2020; Saidi *et al.*, 2019). In Algeria, the urgency is particularly acute: *S. aureus* isolates have demonstrated 100% resistance to penicillin, while 76.9% of *E. coli* strains are now classified as "Critical Priority" ESBL-producers (WHO, 2024; Tahar *et al.*, 2020; Saidi *et al.*, 2019). The rising prevalence of these resistant pathogens underscores the urgent need for sustainable alternatives to conventional therapy.

In this context, Algerian beehive products, specifically honey and propolis, represent a diverse source of bioactive compounds, the antimicrobial efficacy of which is intrinsically linked to their unique botanical origins (El-Guendouz *et al.*, 2019; Bankova, 2005). This chapter assesses the antibacterial and synergistic potential of these extracts against a panel of clinical bovine mastitis isolates comprising five clinical isolates each of *S. aureus*, *E. coli*, *Klebsiella* spp., *S. uberis*, and *S. dysgalactiae* (25 isolates in total). By evaluating multiple clinical isolates per species rather than relying solely on reference strains, this study captures a degree of intra-species variability, thereby providing a broader initial assessment of the antibacterial activity of Algerian honey and propolis against clinically relevant bovine mastitis pathogens.

The experimental workflow begins with antibiogram profiling to determine the antibiotic resistance phenotypes of the clinical isolates. The antibacterial activity of Algerian honey and propolis extracts is then initially evaluated using the agar well diffusion assay by measuring inhibition zones, providing a preliminary qualitative assessment of the activity of the tested extracts. Their antibacterial potency is subsequently quantified by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) using standardized broth microdilution techniques (Aamer *et al.*, 2014). Finally, a checkerboard assay is employed to investigate the synergistic interaction between honey and propolis. By evaluating the combined activity of these natural extracts, this study aims to identify synergistic combinations with enhanced antibacterial efficacy against bovine mastitis pathogens, potentially contributing to alternative therapeutic strategies for mastitis control (Mikniene *et al.*, 2025; Hareidy *et al.*, 2021; Benhanifia *et al.*, 2020).

I- Materials & Methods

1. Preparation of Propolis and Honey Extracts

1.1. Honey

The honey stock solution was prepared at a concentration of 75% (w/v). Briefly, 37.5 g of honey were gradually solubilized in Mueller-Hinton Broth (MHB) under gentle magnetic stirring at room temperature until complete dissolution. The final volume was adjusted to 50 mL in a volumetric flask. To avoid thermal degradation of heat-sensitive phenolic compounds, the solution was filter-sterilized using a 0.22 µm membrane filter (syringe filter). The stock solution was stored at 4°C and freshly prepared every 3 days to ensure stability.

1.2. Propolis

The ethanolic propolis extracts (EEP) used in the antibacterial assays were those prepared and lyophilized as described in Chapter 2. The resulting extracts were stored at 4 °C until use.

2. Bacterial Strains and Growth Conditions

The clinical panel used in this study comprised 25 wild-type isolates obtained from the Mastitis Pathogen Culture Collection (MPCC) of the Canadian Bovine Mastitis and Milk Quality Research Network (Dufour *et al.*, 2019). These isolates represent 5 of the most prevalent etiological agents of bovine mastitis in Canada, distributed as follows: *S. aureus* (n=5), *S. uberis* (n=5), *S. dysgalactiae* (n=5), *E. coli* (n=5), and *Klebsiella* spp. (n=5). In addition, two reference strains, *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), were used as quality controls to validate the susceptibility testing protocols.

Cryopreserved isolates (stored at –80°C in BHI/20% glycerol) were revived on blood agar for 24 hours at 37°C prior to each assay. Bacterial inocula were prepared in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2009; M02-A10) guidelines. Pure colonies from 18-24 hour cultures were suspended in 0.85% (w/v) sterile saline solution and adjusted to a turbidity of 0.5 McFarland standard. This standardization corresponds to a spectrophotometric absorbance (OD_{600}) of 0.08-0.13, yielding an approximate bacterial density of 1.5×10^8 CFU/mL. These standardized suspensions served as the working inocula for all subsequent antimicrobial susceptibility assays.

3. Antimicrobial Susceptibility Profiling (Antibiogram)

The antimicrobial susceptibility of the clinical panel was determined by Minimum Inhibitory Concentrations (MICs) using the Thermo Scientific™ Sensititre™ ARIS™ 2X semi-automated system (TREK Diagnostic Systems, Thermo Fisher Scientific). All procedures followed CLSI M100 and VET01S standards (CLSI, 2020). The experimental workflow followed the manufacturer's standardized sequence: plate selection, inoculum preparation, inoculation, incubation, reading, and interpretation.

3.1. Select Plate (Species-Specific 96-Well Sensititre™ Plates)

Isolates were evaluated using three species-specific plates to ensure clinical relevance to bovine mastitis:

-GPALL1F (Gram-Positive Plate): Utilized for *S. aureus*. This panel covers 20 antimicrobials, including glycopeptides (vancomycin), oxazolidinones (linezolid), lipopeptides (daptomycin), glycylcyclines (tigecycline), and β-lactams (oxacillin with 2% NaCl, penicillin). Notably, this plate includes specialized screening for inducible clindamycin resistance (D-test) and a cefoxitin screen for presumptive MRSA detection.

-CMV1AMAF (Veterinary Mastitis Plate): Utilized for *S. uberis* and *S. dysgalactiae*. This plate is specifically designed for bovine mastitis pathogens, featuring lincosamides (pirlimycin), third-generation cephalosporins (ceftiofur), combination therapy (penicillin/novobiocin), alongside standard tetracyclines and sulphadimethoxine.

-BOPO7F (Bovine Plate): Utilized for *E. coli* and *Klebsiella* spp.. This panel evaluates susceptibility to fluoroquinolones (enrofloxacin, danofloxacin), macrolides (tilmicosin, tulathromycin, tildipirosin, tylosin, gamithromycin), aminoglycosides (gentamicin, neomycin, spectinomycin), and phenicols (florfenicol).

The complete composition of each Sensititre™ plate, including all antimicrobial agents tested and their corresponding concentration ranges, is provided in Appendix D.

3.2. Inoculum Preparation

Bacterial suspensions were adjusted to a 0.5 McFarland standard using the Sensititre™ Nephelometer and diluted in Sensititre™ demineralized water according to the manufacturer's instructions. Each well of the selected plate received 100 µL of the standardized inoculum via the Sensititre™ AIM™ Automated Inoculator to ensure precise volumetric delivery. Plates were placed in the ARIS™ 2X automated incubator at 35–37°C for 18–24 hours (20–24 hours for *Streptococcus* spp.).

3.3. Reading and Interpretation

MIC endpoints were visualized using the Sensititre™ Vizion™ Digital MIC Viewing System and automatically recorded by the ARIS™ 2X software. MIC values were interpreted according to CLSI breakpoints (M100, VET01S) and classified as Susceptible (S), Intermediate (I), or Resistant (R). For *S. aureus*, a cefoxitin MIC ≥ 4 µg/mL indicated presumptive MRSA; a D-test was performed to identify inducible clindamycin resistance.

4. Antibacterial Susceptibility Testing: Inhibition Zones and MIC/MBC

4.1. Agar Diffusion Assay (Zone of Inhibition)

The antimicrobial potential of honey and propolis was assessed using the disc diffusion (Kirby-Bauer) method (Barry *et al.*, 1979). Bacterial inocula were standardized to approximately 1.5×10^8 CFU/mL (0.5 McFarland) and spread uniformly over the surface of Mueller-Hinton Agar (MHA) plates using sterile swabs. For *Streptococcus* isolates, the medium was supplemented with 5% defibrinated sheep blood.

-Honey Preparation: Wells (8 mm diameter, 3 mm depth) were prepared in the agar and subsequently filled with 100 µL of a 75% (w/v) honey solution.

-Propolis Preparation: Sterile discs were impregnated with 20 µL of the ethanolic extract of propolis.

-Controls: Penicillin (6 µg) and nalidixic acid (30 µg) discs served as positive controls for Gram-positive and Gram-negative bacteria, respectively. To ensure the observed inhibition was attributable to the specific bioactive components of the samples, 70% ethanol and a 60% (w/v) synthetic sugar solution were employed as negative controls to account for solvent interference and osmotic pressure.

The plates were incubated at 37°C for 24 hours. Following incubation, the dishes were carefully examined to detect any zones of inhibition around the wells and discs. The diameters

of the zones of inhibition were measured using a caliper and recorded in millimeters. For the *Streptococcus* plates containing blood, measurements were performed from the upper surface of the medium, illuminated with reflected light and with the lids removed, to ensure accurate visualization of the inhibition zones.

4.2. Broth Microdilution Assay (MIC/MBC)

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined using the broth microdilution method in sterile 96-well microplates. The experimental design was conducted in accordance with the CLSI M31-A3 (Vol. 28 No. 8) guidelines for antimicrobial susceptibility testing of bacteria isolated from animals. All tests were performed in triplicate.

The MIC of honey was determined according to the method described by Tan *et al.* (2009) with slight modifications. A 75% (w/v) honey stock solution was prepared in Mueller-Hinton Broth (MHB), followed by two-fold serial dilutions to obtain concentrations ranging from 0.87% to 56% (w/v). Subsequently, 100 µL of each honey sample was transferred to the microplate wells, followed by the addition of 100 µL of bacterial inoculum. The inoculum was prepared by adjusting a bacterial suspension to a 0.5 McFarland standard (approximately 1.5 x 10^8 CFU/mL) and performing serial dilutions in MHB to achieve a final concentration of approximately 5 x 10^5 CFU/mL in each well, in accordance with CLSI guidelines.

The MIC of propolis was determined according to the method described by Bedaida *et al.* (2020). The EEP was prepared at a stock concentration of 4.5 mg/mL in 70% ethanol. Two-fold serial dilutions were prepared in MHB to obtain concentrations ranging from 300 to 9.375 µg/mL. Subsequently, 20 µL of each propolis sample was transferred to the microplate wells, followed by the addition of 280 µL of bacterial inoculum.

For each assay, control wells included: 1) Negative control: MHB only (sterility control), 2) Growth control: MHB + bacterial inoculum (no sample), 3) Sample control: MHB + sample (no inoculum).

Microplates were incubated at 37°C for 18-24 hours. Bacterial growth was identified by macroscopic observation of turbidity and the formation of a visible bacterial pellet at the base of the wells. For *Streptococcus* isolates, visual detection was challenging due to the color of the blood supplement. Therefore, a specialized medium, Bacto™ Todd Hewitt Broth, was used, and microplates were re-incubated for an additional 18 hours at 37°C. The MIC was

defined as the lowest concentration of honey or propolis that completely inhibited visible bacterial growth.

To determine the MBC, 10 µL from three separate wells (corresponding to the MIC concentration and two higher concentrations) were subcultured onto blood agar plates. Plates were incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration that killed ≥ 99.9% of the initial bacterial inoculum (i.e., no visible growth on subculture).

5. Preparation of Binary Combinations and Checkerboard Assay

To evaluate interactive effects between beehive products, binary combinations were formulated by pairing the most potent honey sample (H1, selected based on its lowest individual MIC values) with each propolis extract (PE1 and PE2), yielding two binary test combinations: PE1H1 and PE2H1.

Synergistic interactions were evaluated using the broth microdilution checkerboard technique, adapted from AL-Ani *et al.* (2018) with slight modifications. The assay was performed in sterile 96-well microplates to determine the Fractional Inhibitory Concentration Index (FICI). Each well contained a total reaction volume of 200 µL, consisting of:

-50 µL of the honey-propolis mixture: prepared from stock solutions of 75% (w/v) for honey and 40.00 mg/mL propolis.

-50 µL of 4x-concentrated MHB (prepared at four times the standard concentration; the addition of 50 µL to a final volume of 200 µL resulted in a final 1× MHB concentration): added to maintain an appropriate nutrient concentration for bacterial growth in the final assay mixture.

-100 µL of bacterial inoculum: adjusted to achieve the target final bacterial density.

The interaction between the two agents was quantified by calculating the FICI using the following formula:

$$FICI = \frac{MIC_{A(comb)}}{MIC_{A(alone)}} + \frac{MIC_{B(comb)}}{MIC_{B(alone)}}$$

Where MIC_A and MIC_B represent the Minimum Inhibitory Concentrations of the honey and propolis samples, respectively. The resulting indices were interpreted according to standard synergy scales: Synergy: FICI ≤ 0.5, Additivity: 0.5 < FICI ≤ 1.0, Indifference: 1.0 < FICI ≤ 4.0, Antagonism: FICI > 4.0. All tests were performed in triplicate.

6. Statistical Analysis

Statistical analyses were performed using R (version 4.5.2) and XLSTAT 2016. Data were expressed as the mean ± standard deviation of three independent replicates (n=3).

6.1. Comparative Analysis of Antibacterial Activity

To evaluate the efficacy of hive products (H1, H2, H3, PE1, PE2) and the reference antibiotic, inhibition zone diameters were analyzed using one-way Analysis of Variance (ANOVA). Normality and homoscedasticity were assessed via residual analysis; where assumptions were violated, the Kruskal–Wallis test was employed. Post-hoc comparisons were conducted using Tukey's Honestly Significant Difference (HSD) test to control the family-wise error rate.

6.2. Synergy and Interaction Analysis

Minimum Inhibitory Concentrations (MICs) were compared between individual treatments and binary mixtures. To evaluate synergistic potential, the top-performing honey sample (H1), which demonstrated the lowest baseline MICs, was selected for combination with the two propolis extracts (PE1 and PE2), yielding PE1H1 and PE2H1. The significance of dose reductions in combinations versus single treatments (e.g., H1 vs. PE1H1) was assessed using paired *t*-tests (or Wilcoxon signed-rank tests for non-parametric data). To evaluate differences in Fractional Inhibitory Concentration Index (FICI) values between the two combinations across all isolates, Wilcoxon signed-rank tests were applied. Furthermore, the distribution of interaction categories (synergy, additive, indifference, and antagonism) was analyzed using Pearson's chi-squared (χ^2) test on contingency tables

6.3. Multivariate Characterization

To visualize the relationship between chemical composition and biological activity, Principal Component Analysis (PCA) was performed. The data matrix included mean inhibition diameters, MIC values, Total Phenolic Content (TPC), and Total Flavonoid Content (TFC). Variables were centered and scaled prior to analysis. Biplots were generated to identify correlations between phytochemical markers and isolate susceptibility, specifically focusing on the clustering of Gram-positive versus Gram-negative phenotypes.

II- Results & Discussion

1. Antibiogram Profiling of Clinical Mastitis Isolates

The antimicrobial susceptibility profiles of 25 clinical mastitis isolates are summarized in Table 3.1, revealing species-specific resistance patterns with potential therapeutic implications.

All *E. coli* isolates in this study were susceptible to ampicillin and ceftiofur, indicating preserved susceptibility to these specific β-lactam agents within the tested panel. However, isolate *E. coli* 73 exhibited a multidrug-resistant (MDR) phenotype, with co-resistance to gentamicin, tetracycline, and trimethoprim/sulfamethoxazole, a pattern frequently associated with mobile genetic elements carrying multiple resistance genes (Kerro Dego, 2020).

For *Klebsiella* spp., 80% of isolates were resistant to ampicillin, reflecting the intrinsic chromosomal β-lactamase constitutively expressed by this genus (Stock and Wiedemann, 2001). All isolates remained susceptible to gentamicin and tetracycline, consistent with recent surveys (Naranjo-Lucena & Slowey, 2022; Bolte *et al.,* 2020).

All *S. aureus* isolates were susceptible to oxacillin, confirming a methicillin-susceptible *S. aureus* (MSSA) phenotype, and retained susceptibility to most tested agents including vancomycin, daptomycin, and fluoroquinolones. Notably, *S. aureus* isolate 47 showed resistance to linezolid, a last-resort oxazolidinone rarely used in veterinary practice, raising concerns about potential horizontal gene transfer (Chowdhury *et al.*, 2025).

Streptococcus uberis emerged as the most resistant pathogen in this study. Three isolates were resistant to multiple β-lactams (ampicillin, oxacillin, penicillin), a finding increasingly reported and potentially mediated by alterations in penicillin-binding proteins (PBPs) (Naranjo-Lucena & Slowey, 2022). Pirlimycin resistance was observed in 40% of isolates, typically mediated by *erm*(A) genes, which significantly limits intramammary treatment options, as pirlimycin is a lincosamide specifically approved for this route (Schwarz *et al.*, 2004). Tetracycline resistance and intermediate susceptibility were also detected, which are commonly associated with *tet*(K), *tet*(L), and *tet*(M) genes (Schwarz *et al.*, 2004). The observed heterogeneity in resistance profiles among *S. uberis* isolates highlights the potential usefulness of targeted susceptibility testing to inform antimicrobial selection, particularly in recurrent or non-responsive cases, while its interpretation should consider practical constraints and the imperfect correlation between *in vitro* susceptibility and clinical treatment outcomes.

Streptococcus dysgalactiae isolates were uniformly susceptible to all β-lactams, erythromycin, and pirlimycin. However, all isolates showed intermediate susceptibility to tetracycline, suggesting an emerging MIC shift toward resistance, likely linked to *tet(M)* selection pressure from historical antibiotic use in livestock (Naranjo-Lucena & Slowey, 2022).

The high frequency of NOINTP (no interpretation) results reflects a recognized limitation of current CLSI panels in veterinary antimicrobial susceptibility testing, highlighting the urgent need for updated, species-specific breakpoints for bovine mastitis pathogens.

2. Evaluation of the Antibacterial Efficacy of Propolis and Honey Against Mastitis Isolates

To our knowledge, this is the first study to investigate the antibacterial and synergistic potential of Algerian honey and propolis against clinical isolates of *S. uberis*, *S. dysgalactiae*, and *Klebsiella* spp. from bovine mastitis. Although the general antimicrobial properties of Algerian bee products have been reported against standard pathogens, limited data exist on their efficacy against these specific mastitis pathogens.

2.1. Comparative Analysis of Growth Inhibition Zones

The inhibition zone diameters recorded for the three honey samples (H1, H2, H3), and two propolis extracts (PE1, PE2) against 25 bovine mastitis isolates are presented in Figures 3.1. Overall, all tested samples demonstrated measurable antibacterial activity, though their inhibition zones were smaller than those of the positive controls (penicillin for Gram-positive: up to 50 mm; nalidixic acid for Gram-negative: up to 30 mm). A clear differential susceptibility was observed between Gram-positive and Gram-negative isolates. Gram-positive pathogens, particularly *S. aureus*, *S. dysgalactiae*, and *S. uberis*, exhibited markedly larger inhibition zones (ranging from approximately 9 to 29 mm for propolis and honey) compared to Gram-negative isolates (*E. coli* and *Klebsiella* spp.), which showed reduced zones (approximately 9 to 17 mm). This differential susceptibility is consistent with the structural barrier posed by the outer membrane of Gram-negative bacteria, which limits the penetration of hydrophobic bioactive compounds such as propolis flavonoids and honey-derived phenolics (Rana *et al.*, 2025).

Among the tested products, H1 generally produced the largest inhibition zones against *S. aureus*, *E. coli*, and *Klebsiella* spp., whereas PE1 and PE2 tended to exhibit greater inhibition

against *S. uberis* and *S. dysgalactiae*. However, relative performance varied among individual isolates, with specific strains showing larger zones in response to other products. These findings support the antibacterial potential of honey and propolis against major bovine mastitis pathogens, while highlighting species- and strain-dependent differences in susceptibility (Rana *et al.*, 2025; Zulhendri *et al.*, 2021).

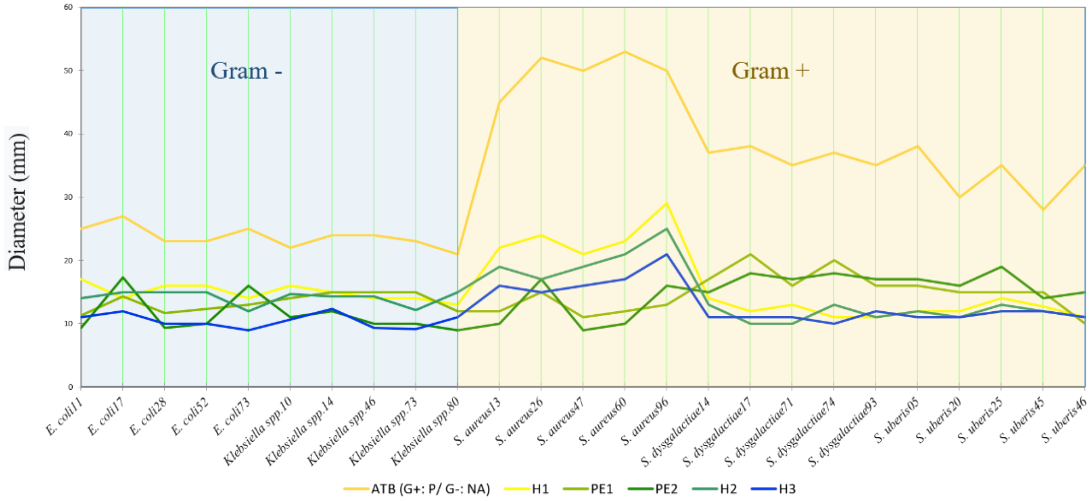
Table 3.1. Antimicrobial Susceptibility Profiles of Gram+ and Gram- Bacteria Isolated from Bovine Mastitis (n=25).

Antibiotic Class	**Antibiotic**	***E. coli* (n=5)**					***Klebsiella* spp. (n=5)**					***S. aureus* (n=5)**					***S. dysgalactiae* (n=5)**					***S. uberis* (n=5)**				
		11	*17*	*28*	*52*	*73*	*10*	*14*	*46*	*73*	*80*	*13*	*26*	*47*	*60*	*96*	*14*	*17*	*71*	*74*	*93*	*05*	*20*	*25*	*45*	*46*
β-lactams	**Ampicillin**	S	S	S	S	S	S	**R**	**R**	**R**	I	N	N	N	N	N	S	S	S	S	S	S	**R**	**R**	**R**	S
	Ceftiofur	S	S	S	S	S	N	N	N	N	N	-	-	-	-	-	S	S	S	S	S	S	S	S	S	S
	Cephalothin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	S	S	S	S	S
	Oxacillin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	N	N	N	N	N	**R**	**R**	**R**	**R**	**R**
	Penicillin	N	N	N	N	N	N	N	N	N	N	S	S	S	S	S	S	S	S	S	S	S	**R**	**R**	**R**	S
	Penicillin/Novobiocin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	S	S	S	S	S
	Cefoxitin (screen)	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-
Aminoglycosides	**Gentamicin**	S	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
	Neomycin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Spectinomycin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	High-level Gentamicin (500 µg/mL)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	High-level Streptomycin (1000 µg/mL)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tetracyclines	**Tetracycline**	S	S	S	S	**R**	S	S	S	S	S	S	S	S	S	S	I	I	I	I	I	S	**R**	S	I	**S**
	Tigecycline	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-
Fluoroquinolones	Ciprofloxacin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
	Levofloxacin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
	Moxifloxacin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
	Danofloxacin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Enrofloxacin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Antibiotic Class	Antibiotic	*E. coli* (n=5)					*Klebsiella* spp. (n=5)					*S. aureus* (n=5)					*S. dysgalactiae* (n=5)					*S. uberis* (n=5)				
		11	*17*	*28*	*52*	*73*	*10*	*14*	*46*	*73*	*80*	*13*	*26*	*47*	*60*	*96*	*14*	*17*	*71*	*74*	*93*	*05*	*20*	*25*	*45*	*46*
Macrolides	Erythromycin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
	Gamithromycin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tildipirosin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tilmicosin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tulathromycin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Tylosin	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lincosamides	Clindamycin	N	N	N	N	N	N	N	N	N	N	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
	Pirlimycin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	S	**R**	S	**R**	S
	D-test 1	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
	D-test 2	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
Phenicols	**Chloramphenicol**	-	-	-	-	-	-	-	-	-	-	I	I	I	S	I	-	-	-	-	-	-	-	-	-	-
	Florfenicol	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-				
Glycopeptides	Vancomycin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
Lipopeptides	Daptomycin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
Oxazolidinones	**Linezolid**	-	-	-	-	-	-	-	-	-	-	S	S	**R**	S	S	-	-	-	-	-	-	-	-	-	-
Rifamycins	Rifampin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
Nitrofurans	Nitrofurantoin	-	-	-	-	-	-	-	-	-	-	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-
Streptogramins	Quinupristin/Dalfopristin	-	-	-	-	-	-	-	-	-	-	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-
Pleuromutilins	Tiamulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-				
Sulfonamides	Sulphadimethoxine	N	N	N	N	N	N	N	N	N	N	-	-	-	-	-	N	N	N	N	N	N	N	N	N	N
Combination	**Trimethoprim/Sulfamethoxazole**	S	S	S	S	**R**	N	N	N	N	N	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-

*- **S**: susceptible; **I**: intermediate; **R**: resistant; **N**: no interpretation (MIC measured but no CLSI breakpoints available); **(-)**: antimicrobial not included on the corresponding Sensititre™ plate. Note: Numbers listed in the sub-header (e.g., 11, 17, 28, 52, 73) correspond to individual bacterial isolate IDs within each species group (n = 5 per species): Escherichia coli, Klebsiella* spp.*, Staphylococcus aureus, Streptococcus dysgalactiae, and Streptococcus uberis. The complete list of antimicrobial agents included in each Sensititre™ plate and their corresponding concentration ranges is provided in Appendix D.*

(A)



(B)

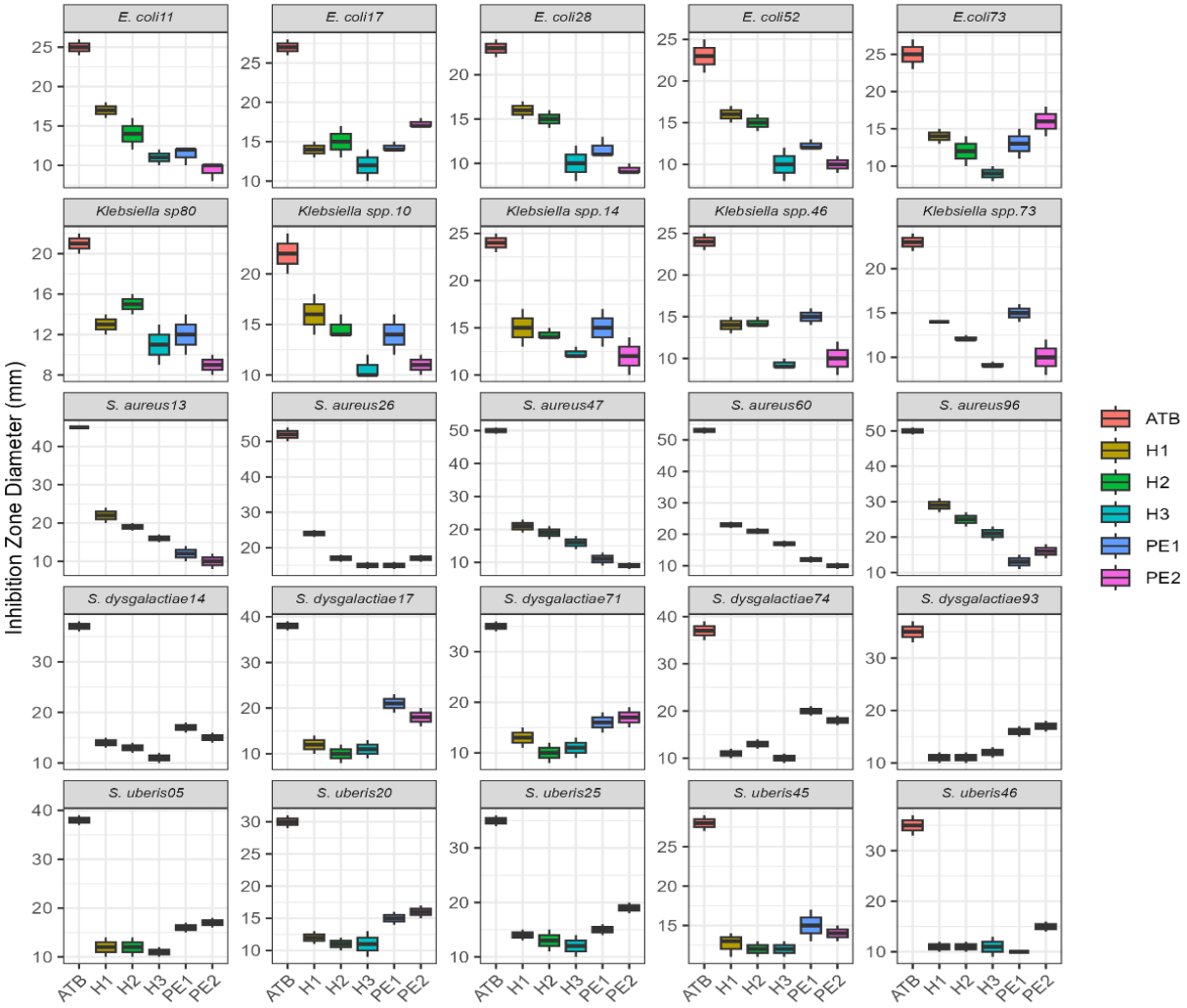


Figure 3.1. (A) Inhibition Zone Diameters of Tested Products Against Gram-Negative and Gram-Positive Bovine Mastitis Isolates. **(B)** Distribution of Inhibition Zone Diameters Across Bovine Mastitis Bacterial Strains *(Escherichia coli, Klebsiella* spp., *Staphylococcus aureus, Streptococcus dysgalactiae, and Streptococcus uberis). **ATB:** standard antibiotic control; **H1, H2, H3:** honey samples; **PE1, PE2:** propolis extracts; **P:** penicillin; **NA:** nalidixic acid. Negative controls, including ethanol (solvent control) and sugar solution, produced no visible zone of inhibition (0 mm) across all tested isolates.*

2.2. Determination of Minimum Inhibitory (MIC) and Bactericidal (MBC) Concentrations of Propolis and Honey

The Minimum Inhibitory Concentrations (MIC) of honey (H1, H2, H3), propolis (PE1, PE2), and their combinations (PE1H1, PE2H1) against 25 bovine mastitis isolates are presented in Table 3.2 and Figure 3.2. Results are expressed as mean values from duplicate experiments. Notably, MBC values were consistently equal to MIC values across all tested products and isolates (MBC/MIC ratio = 1), indicating a strictly bactericidal rather than bacteriostatic effect. Therefore, only MIC results are reported.

The three honey samples exhibited interesting antibacterial activity, with MIC ranging from 0.44% to 56% (w/v). Among these, H1 consistently demonstrated the highest potency, exhibiting the lowest MIC values across all tested bacterial species, followed by H2 and H3 (Table 3.2). The superior efficacy of H1, identified as honeydew honey ("forest honey" by local apiarists), can be attributed to its low pH (3.99), low moisture content (15.2%), and rich LC-MS/MS profile (Chapter 2), aligning with previous reports on Algerian honey from Mediterranean flora (Homrani *et al.*, 2020). The antibacterial mechanism of honey is multifactorial. Its low water content (13–23%) creates a hyperosmotic environment that inhibits microbial growth, while low pH and high sugar concentration exert additional osmotic pressure on bacterial cells (Bava *et al.*, 2025; Vică *et al.*, 2021). Furthermore, honey generates hydrogen peroxide via the enzyme glucose oxidase during glucose oxidation, which irreversibly damages bacterial DNA and cell membranes (Bava *et al.*, 2025; Bouacha *et al.*, 2023). The complex composition of honey, including organic acids, enzymes, and secondary metabolites that originate from botanical source, acts synergistically, making honey a multifunctional antimicrobial agent with a low propensity for inducing bacterial resistance (Bava *et al.*, 2025; Bouacha *et al.*, 2023; Vică *et al.*, 2021).

Against *E. coli* isolates, H1 MIC ranged from 2.62–3.50%, while H2 and H3 showed substantially higher values (14–28% and 28–56%, respectively). These results fall within the expected range for Algerian honey yet remain lower, indicating greater potency, than those reported in recent regional studies (Bereksi-Reguig, 2024; Bouacha *et al.*, 2023). For *Klebsiella* spp., H1 MIC ranged from 3.5–5.3%, compared to 14–28% for H2 and 28% for H3.

Among the Gram-positive pathogens, *S. aureus* isolates exhibited the highest susceptibility. H1 demonstrated exceptional potency, with MIC values as low as 0.44% (isolate 60), while H2 and H3 showed higher MICs ranging from 2.6% to 14% and 14% to 21%, respectively, though these remain lower compared to previous studies (Bereksi-Reguig, 2024; Bouacha *et*

al., 2023; Bouacha *et al.*, 2020). In contrast, *S. dysgalactiae* and *S. uberis* displayed higher MIC values compared to *S. aureus*. For *S. dysgalactiae*, H1 MIC ranged from 3.5% to 28%, while H2 and H3 ranged from 10.5% to 56%. For *S. uberis*, honey samples exhibited MIC values ranging from 10.5% to 56%.

MIC determinations for honey against bovine mastitis isolate demonstrated antimicrobial activity even at dilutions of 1/33.

Propolis samples PE1 and PE2 displayed distinct antibacterial activity patterns across the tested bacterial species (Table 3.2). PE2 consistently demonstrated lower MIC values than PE1, correlating with its qualitative and quantitative phytochemical richness. Against *E. coli* isolates, PE1 exhibited a consistent MIC of 225 µg/mL, while PE2 showed significantly higher potency with MIC values ranging from 100 to 109 µg/mL. These results align with previous reports on Algerian propolis (Segueni *et al.*, 2020) and are consistent with the broader antibacterial activity of propolis reported in the literature (Przybyłek & Karpiński, 2019). A striking difference in efficacy was observed against *Klebsiella* spp.: while PE1 showed limited activity with a consistent MIC of 300 µg/mL, PE2 demonstrated potent antibacterial action with MIC values ranging from 116.9 to 118.8 µg/mL.

Against *S. aureus*, both propolis samples exhibited high antibacterial activity. PE1 and PE2 demonstrated MIC values of 56.25 µg/mL and 20.08–25.66 µg/mL, respectively. These results are comparable to, and in some instances exceed, the potency reported in previous Algerian studies (Bedaida *et al.*, 2020; Bouacha *et al.*, 2020; Segueni *et al.*, 2020) as well as findings summarized in a comprehensive literature review (Przybyłek & Karpiński, 2019).

Regarding the streptococci, PE1 showed MIC values ranging from 37.5 to 300 µg/mL for *S. dysgalactiae* and a consistent 300 µg/mL for *S. uberis*. In contrast, PE2 demonstrated superior activity, with lower MICs ranging from 59.46 to 102.96 µg/mL for *S. dysgalactiae* and 81.27 to 124.4 µg/mL for *S. uberis*. Notably, despite the resistance of *S. uberis*, PE2 maintained significant inhibitory activity, suggesting its potential as an effective agent against recalcitrant streptococcal mastitis.

The observed antibacterial activity of propolis can be attributed to its complex chemical composition, which makes the precise identification of a single mechanism of action difficult. However, available evidence suggests that propolis exerts its antibacterial effects through multiple pathways. These include disruption of bacterial cell membrane integrity, increased membrane permeability, inhibition of ATP production, and interference with nucleic acid synthesis (RNA and DNA) (Zulhendri *et al.*, 2021). In addition, several bioactive constituents,

particularly flavonoids, are known to alter membrane permeability and compromise cellular functions (El-Guendouz *et al.*, 2021; Bedaida *et al.*, 2020).

From a physicochemical perspective, the activity of propolis may also be linked to the presence of negatively charged compounds associated with mobile protons, contributing to its acidic nature. These protons can interact with bacterial cell surfaces, disrupting electrostatic stability, increasing membrane permeability, and ultimately leading to cell death. This mechanism may partly explain the generally higher susceptibility of Gram-positive bacteria compared to Gram-negative ones, as the latter possess a more complex outer membrane and higher surface charge, which can limit the penetration of active compounds (Zulhendri *et al.*, 2021).

Overall, the MIC values obtained in this study confirm the strong antimicrobial potential of propolis against bovine mastitis pathogens, even at relatively low concentrations.

The synergistic potential of the PE1H1 and PE2H1 combinations, prepared as described in Section 5 of the Materials and Methods, was subsequently evaluated across the tested isolates (Table 3.2 and Figure 3.2). Against *E. coli* and *Klebsiella* spp., the propolis concentration required at the inhibitory endpoint of the combination was 250 µg/mL for PE1 and 125 µg/mL for PE2, compared with their respective standalone MICs of 225 µg/mL and 101–110 µg/mL. Conversely, the concentration of the honey component was markedly reduced, reaching 1.87% in PE1H1 and 0.94% in PE2H1, compared with 2.6–3.5% for H1 alone. These differences indicate an asymmetric contribution of the two components to growth inhibition within the tested combinations, with a reduction in the concentration of honey required at the inhibitory endpoint, whereas no corresponding reduction was observed for propolis.

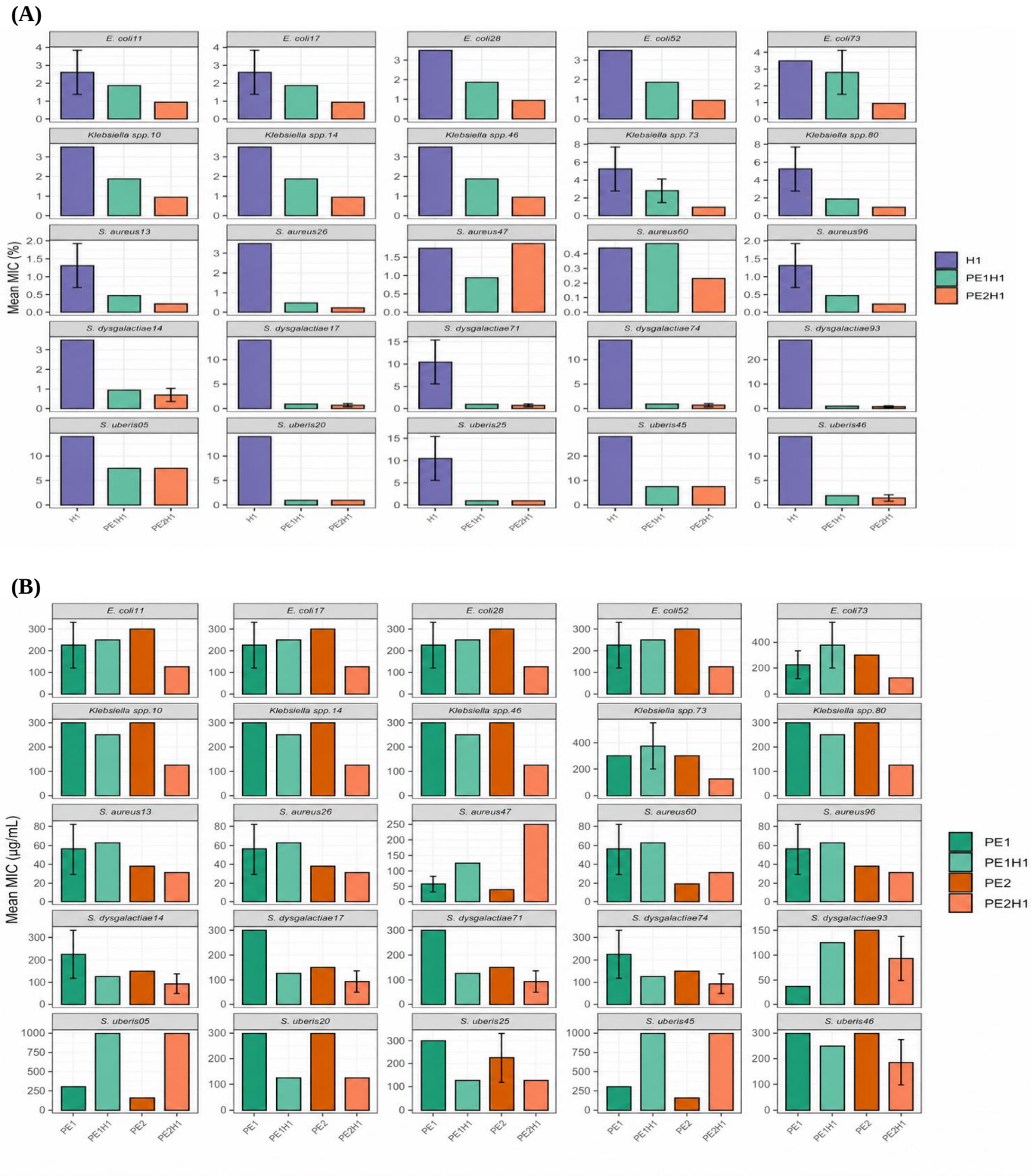


Figure 3.2. Comparative analysis of Minimum Inhibitory Concentrations (MIC) values of honey, propolis, and their combinations. **(A)** MIC values of H1 compared with H1 combined with PE1 or PE2. **(B)** MIC values of PE1 and PE2 compared with their respective combinations with H1. *Expressed as µg/mL for propolis and percentage (w/v) for honey. Results represent mean values of triplicate experiments. Note: missing error bars indicate identical MIC replicates.*

Table 3.2. Minimum Inhibitory Concentrations (MIC) of Individual and Combined Honey and Propolis Against Clinical Bovine Mastitis Isolates.

Isolate	H1 (%)	H2 (%)	H3 (%)	PE1 (µg/mL)	PE2 (µg/mL)	PE1H1	PE2H1
Gram-negative							
***E. coli* 11**	**2.62** ± 1.24	14 ± 0.00	28 ± 0.00	225 ± 106.1	100.6 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***E. coli* 17**	**2.62** ± 1.24	28 ± 0.00	56 ± 0.00	225 ± 106.1	109.6 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***E. coli* 28**	**3.5** ± 0.00	21 ± 9.90	28 ± 0.00	225 ± 106.1	102.3 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***E. coli* 52**	**3.5** ± 0.00	14 ± 0.00	28 ± 0.00	225 ± 106.1	100.8 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***E. coli* 73**	**3.5** ± 0.00	14 ± 0.00	28 ± 0.00	225 ± 106.1	100.8 ± 0.00	375 ± 176.8 & 2.81 ± 1.0	125 ± 0.0 & 0.94 ± 0.0
***Klebsiella* spp. 10**	**3.5** ± 0.00	21 ± 9.90	28 ± 0.00	300 ± 0.00	118.4 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***Klebsiella* spp. 14**	**3.5** ± 0.00	14 ± 0.00	28 ± 0.00	300 ± 0.00	116.9 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***Klebsiella* spp. 46**	**3.5** ± 0.00	21 ± 9.90	28 ± 0.00	300 ± 0.00	118.4 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***Klebsiella* spp. 73**	5.25 ± 2.47	21 ± 9.90	28 ± 0.00	300 ± 0.00	118.8 ± 0.00	375 ± 176.8 & 2.81 ± 1.0	125 ± 0.0 & 0.94 ± 0.0
***Klebsiella* spp. 80**	5.25 ± 2.47	14 ± 0.00	28 ± 0.00	300 ± 0.00	117.3 ± 0.00	250 ± 0.0 & 1.87 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
Gram-positive							
***S. aureus* 13**	**1.31** ± 0.62	**2.62** ± 1.24	14 ± 0.00	56.25 ± 26.5	**21.25** ± 0.00	62.5 ± 0.0 & 0.47 ± 0.0	31.2 ± 0.0 & 0.23 ± 0.0
***S. aureus* 26**	**3.5** ± 0.00	14.0 ± 0.00	21 ± 9.90	56.25 ± 26.5	**25.66** ± 0.00	62.5 ± 0.0 & 0.47 ± 0.0	31.2 ± 0.0 & 0.23 ± 0.0

Isolate	H1 (%)	H2 (%)	H3 (%)	PE1 (µg/mL)	PE2 (µg/mL)	PE1H1	PE2H2
***S. aureus* 47**	**1.75** ± 0.00	**5.25** ± 2.47	14 ± 0.00	56.25 ± 26.5	**21.91** ± 0.00	125 ± 0.0 & 0.94 ± 0.0	250 ± 0.0 & 1.87 ± 0.0
***S. aureus* 60**	**0.44** ± 0.00	**3.50** ± 0.00	21 ± 9.90	56.25 ± 26.5	**20.08** ± 0.00	62.5 ± 0.0 & 0.47 ± 0.0	31.2 ± 0.0 & 0.23 ± 0.0
***S. aureus* 96**	**1.31** ± 0.62	**2.62** ± 1.24	14 ± 0.00	56.25 ± 26.5	**21.25** ± 0.00	62.5 ± 0.0 & 0.47 ± 0.0	31.2 ± 0.0 & 0.23 ± 0.0
***S. dysgalactiae* 14**	**3.5** ± 0.00	10.5 ± 4.95	56 ± 0.00	225 ± 106.1	84.64 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	93.7 ± 44.2 & 0.70 ± 0.3
***S. dysgalactiae* 17**	14 ± 0.00	10.5 ± 4.95	56 ± 0.00	300 ± 0.00	102.9 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	93.7 ± 44.2 & 0.70 ± 0.3
***S. dysgalactiae* 71**	10.5 ± 4.95	14 ± 0.00	10.5 ± 4.95	300 ± 0.00	93.21 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	93.7 ± 44.2 & 0.70 ± 0.3
***S. dysgalactiae* 74**	14 ± 0.00	10.5 ± 4.95	56 ± 0.00	225 ± 106.1	86.89 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	93.7 ± 44.2 & 0.70 ± 0.3
***S. dysgalactiae* 93**	28 ± 0.00	56 ± 0.00	56 ± 0.00	37.5 ± 0.00	59.46 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	93.7 ± 44.2 & 0.70 ± 0.3
***S. uberis* 05**	14 ± 0.00	10.5 ± 4.95	28 ± 0.00	300 ± 0.00	96.96 ± 0.00	1000 ± 0.0 & 7.5 ± 0.0	1000 ± 0.0 & 7.5 ± 0.0
***S. uberis* 20**	14 ± 0.00	10.5 ± 4.95	56 ± 0.00	300 ± 0.00	124.4 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***S. uberis* 25**	10.5 ± 4.95	21 ± 9.90	56 ± 0.00	300 ± 0.00	115.2 ± 0.00	125 ± 0.0 & 0.94 ± 0.0	125 ± 0.0 & 0.94 ± 0.0
***S. uberis* 45**	28 ± 0.00	21 ± 9.90	56 ± 0.00	300 ± 0.00	108.2 ± 0.00	1000 ± 0.0 & 7.5 ± 0.0	1000 ± 0.0 & 7.5 ± 0.0
***S. uberis* 46**	14 ± 0.00	28 ± 0.00	56 ± 0.00	300 ± 0.00	81.27 ± 106.1	250 ± 0.0 & 1.87 ± 0.0	187.5 ± 88.4 & 1.41 ± 0.7

-Values represent the Mean Minimum Inhibitory Concentration (MIC) ± Standard Deviation (SD) from three independent replicates (n=3). Note: MIC values are expressed as % (w/v) for honey and µg/mL for propolis extracts. For combinations, MIC values are reported as the concentrations of propolis extract (µg/mL) and honey (% w/v) simultaneously present in the well at the MIC endpoint.

However, a marked increase in the required concentration was observed for the more resistant isolates, specifically *E. coli* 73 and *Klebsiella* spp. 73. For these strains, the PE1H1 combination required a higher dose of 375 µg/mL + 2.81% to achieve inhibition. This indicates that the PE1H1 mixture is less effective against highly resistant phenotypes compared to PE2H1, which maintained its potency at lower levels.

In Gram-positive bacteria, against *S. aureus*, the combinations demonstrated a distinct interaction pattern where the honey component exhibited a significant increase in potency. PE1H1 (62.5 µg/mL + 0.47%) and PE2H1 (31.2 µg/mL + 0.23%) achieved inhibition at honey concentrations substantially lower than the individual H1 MIC (0.44–1.31%) for most isolates. This was accompanied by a decrease in propolis potency, which was required at concentrations higher than its individual MIC (56.25 µg/mL for PE1; 21.25–25.66 µg/mL for PE2).

For the linezolid-resistant isolate *S. aureus* 47, a different trend was observed: PE1H1 required 125 µg/mL + 0.94% honey, while PE2H1 required 250 µg/mL + 1.87% honey. Although these values reflect strain-specific variation and a relative decrease in potency compared with the more susceptible isolates, both combinations retained notable inhibitory activity against this isolate.

Against *S. dysgalactiae*, both combinations demonstrated effective inhibition. PE1H1 (125 µg/mL + 0.94%) achieved inhibition at concentrations substantially lower than the individual MICs of its components. PE2H1 showed a similar trend, maintaining efficacy at 93.7 µg/mL + 0.70%, though this was slightly higher than the baseline concentrations observed for the PE1H1 mixture.

For *S. uberis*, notable heterogeneity in response was observed, reflecting the variable resistance profiles identified in the initial antibiogram. Isolates 5 and 45 required higher combination concentrations (1000 µg/mL + 7.5%) for both PE1H1 and PE2H1. In contrast, isolates 20, 25, and 46 were much more susceptible, with inhibitory thresholds ranging from 125 to 250 µg/mL + 0.94% to 1.87%. This indicates that the efficacy of honey-propolis combinations against *S. uberis* is strain-dependent.

Overall, these results highlight that the antibacterial performance of propolis–honey combinations is not simply additive but varies according to bacterial species, strain, and

resistance level. This variability suggests the presence of complex interactions between their bioactive constituents, which may differentially influence bacterial susceptibility.

3. Multivariate Analysis of Antibacterial Activity in Relation to Total Phenolic and Flavonoid Contents (PCA)

Given the observed variability in antibacterial activity among samples, Principal Component Analysis (PCA) was conducted to investigate whether total phenolic (TPC) and flavonoid content (TFC) correlated with antibacterial efficacy. The PCA biplot (Figure 3.3) explained 85.47% of the total variance (F1: 57.89%; F2: 27.58%).

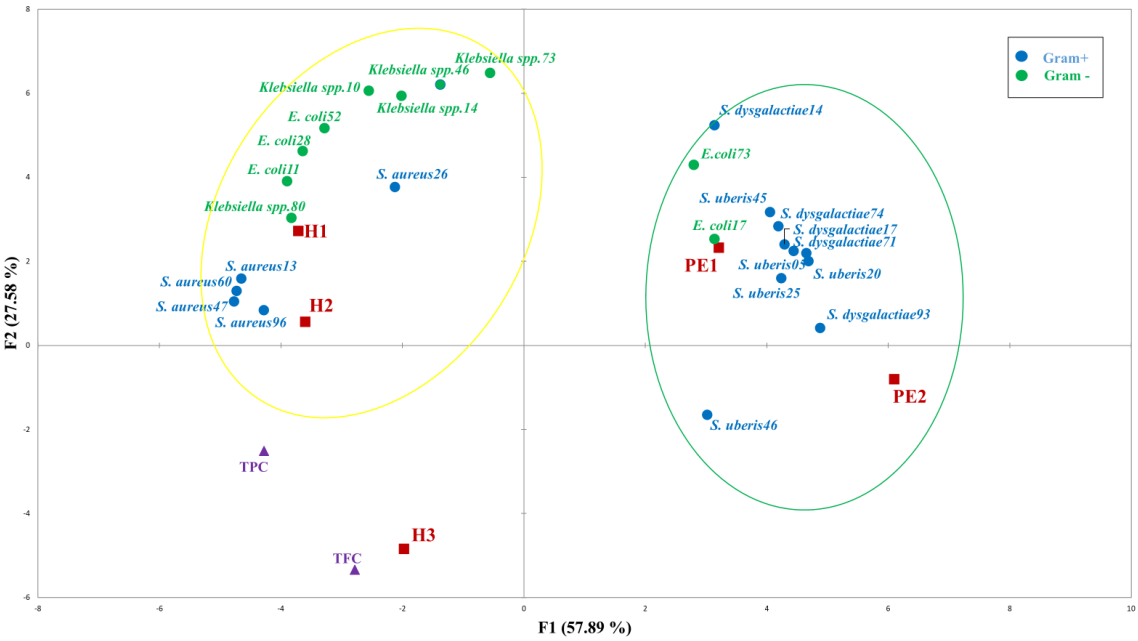


Figure 3.3. Principal Component Analysis (PCA) of Antibacterial Activity and Phytochemical Parameters (TPC and TFC) of Honey and Propolis Samples Against Mastitis Isolates.

The analysis reveals that antibacterial activity is not correlated with total phenolic or flavonoid content. H1 and PE2, despite having lower TPC/TFC values than H2, H3, and PE1, are positioned closer to the most susceptible isolates (*S. aureus*, *E. coli*, *Klebsiella* spp.), confirming that qualitative phytochemical richness, rather than total phenolic quantity, determines antibacterial efficacy (Chapter 2). This observation is supported by chemical profiling, which showed that H1 contained higher concentrations of specific

bioactive compounds, particularly kojic acid (10.25 µg/g in H1 vs. 4.26 µg/g in H2 and 0.86 µg/g in H3). Kojic acid, in particular, has been reported to exert antibacterial effects through metal ion chelation, disruption of membrane integrity, and interference with bacterial DNA, ultimately leading to cell death (Zilles *et al.*, 2022). Similarly, flavonoids such as quercetin and luteolin are known to inhibit key bacterial enzymes, including DNA gyrase, thereby impairing replication and cellular functions (Elmuhayaoui, 2020). Phenolic acids such as p-coumaric and caffeic acids further contribute through membrane destabilization and inhibition of energy metabolism (Almasaudi, 2020).

A clear separation was observed between bacterial groups. Gram-negative isolates (*E. coli* and *Klebsiella* spp.) clustered with honey samples, while Gram-positive isolates (*S. aureus*, *S. dysgalactiae*, *S. uberis*) were more closely associated with propolis, particularly PE2. This differential clustering likely reflects structural differences in bacterial cell envelopes: the outer membrane of Gram-negative bacteria may limit the penetration of certain propolis compounds, whereas Gram-positive bacteria remain more susceptible to flavonoid-rich matrices. Notably, PE2 contained a wider diversity of active flavonoids and phenolic esters, including pinobanksin derivatives, chrysin, apigenin, kaempferol, and caffeic acid esters which are known to act through multiple mechanisms such as membrane disruption, inhibition of nucleic acid synthesis, alteration of cytoplasmic membrane function, and reduction of biofilm formation (Elmuhayaoui, 2020).

Some of these compounds, including apigenin and quercetin, exhibit multi-target antibacterial potential. Apigenin inhibits DNA gyrase and has shown synergistic effects when combined with β-lactams against methicillin-resistant *S. aureus*, while quercetin binds to the DNA gyrase subunit of *E. coli* and modulates bacterial resistance to β-lactam antibiotics, highlighting the multi-target potential of these flavonoids (Almasaudi, 2020; Elmuhayaoui *et al.*, 2020).

Notably, PE2 positioned as an outlier on the F1 axis, strongly associated with the inhibition of *S. dysgalactiae* and *S. uberis*, highlighting its distinct mechanism of action against streptococcal species. The dispersion of these isolates across the biplot reflects their strain-dependent variability and higher MIC values, consistent with their multidrug-resistant profiles. Overall, these findings indicate that antibacterial activity is

governed by the presence of specific bioactive molecules and their synergistic interactions rather than by total phenolic or flavonoid content alone, supporting the concept of a multi-target mode of action for both honey and propolis.

The clustering patterns observed in the PCA biplot also reflect the influence of geographical origin, climatic conditions, and botanical sources on antibacterial activity. H1 (humid Mediterranean) and PE2 (hot desert) were positioned closer to the most susceptible isolates, whereas H2 and H3 (semi-arid/steppe) and PE1 (humid subtropical) were less associated with antibacterial potency. This suggests that environmental factors shape the qualitative composition of bioactive compounds, thereby influencing biological activity, as widely reported (El-Guendouz *et al.*, 2019; Bankova, 2005).

4. Evaluation of Fractional Inhibitory Concentration Index (FICI) of Propolis–Honey Combinations

Transitioning from MIC determination to the checkerboard assay, the evaluation of FICI values revealed that the interactions between propolis–honey combinations (PE1H1 and PE2H1) varied markedly depending on bacterial species and strain (Figure 3.3). Overall, most interactions were classified as indifferent, particularly for Gram-negative isolates (*E. coli* and *Klebsiella* spp.), where FICI values were predominantly > 1. This indicates that combining honey with propolis did not consistently enhance antibacterial efficacy compared to individual treatments.

Regarding interaction patterns, a notable divergence was observed between the two combinations. Despite PE2 exhibiting superior standalone activity (lower individual MICs), the PE1H1 mixture demonstrated more frequent additive and synergistic interactions, particularly against *Klebsiella* spp., *S. dysgalactiae*, and *S. uberis*. True synergy (FICI ≤ 0.5) was achieved with PE1H1 against *S. dysgalactiae* (isolates 17 and 71) and *S. uberis* (isolates 20 and 25). This is highly significant given the multidrug-resistant (MDR) profile of *S. uberis* to β-lactams, lincosamides, and tetracyclines tested in the antibiogram (Section 1). These findings support previous evidence that hive product potentiation is not simply the sum of individual potencies but depends on the

specific chemical compatibility between the honey and propolis matrices (Sánchez-Martín *et al.*, 2025; Eslami *et al.*, 2016).

Regarding antagonistic interactions, the PE2H1 combination exhibited antagonism (FICI > 4) against the linezolid-resistant *S. aureus* 47 and *S. uberis* isolates 5 and 45. This correlates with the MIC data, where PE2 required a higher concentration in the mixture than alone (e.g., from ~100 µg/mL to 125 µg/mL). As suggested by Sánchez-Martín *et al.* (2025), the efficacy of honey–propolis combinations depends heavily on the specific pairing. In this case, the high standalone potency of PE2 appears to be partially inhibited by the H1 matrix. Nevertheless, the mixture remained active, albeit at a higher propolis concentration.

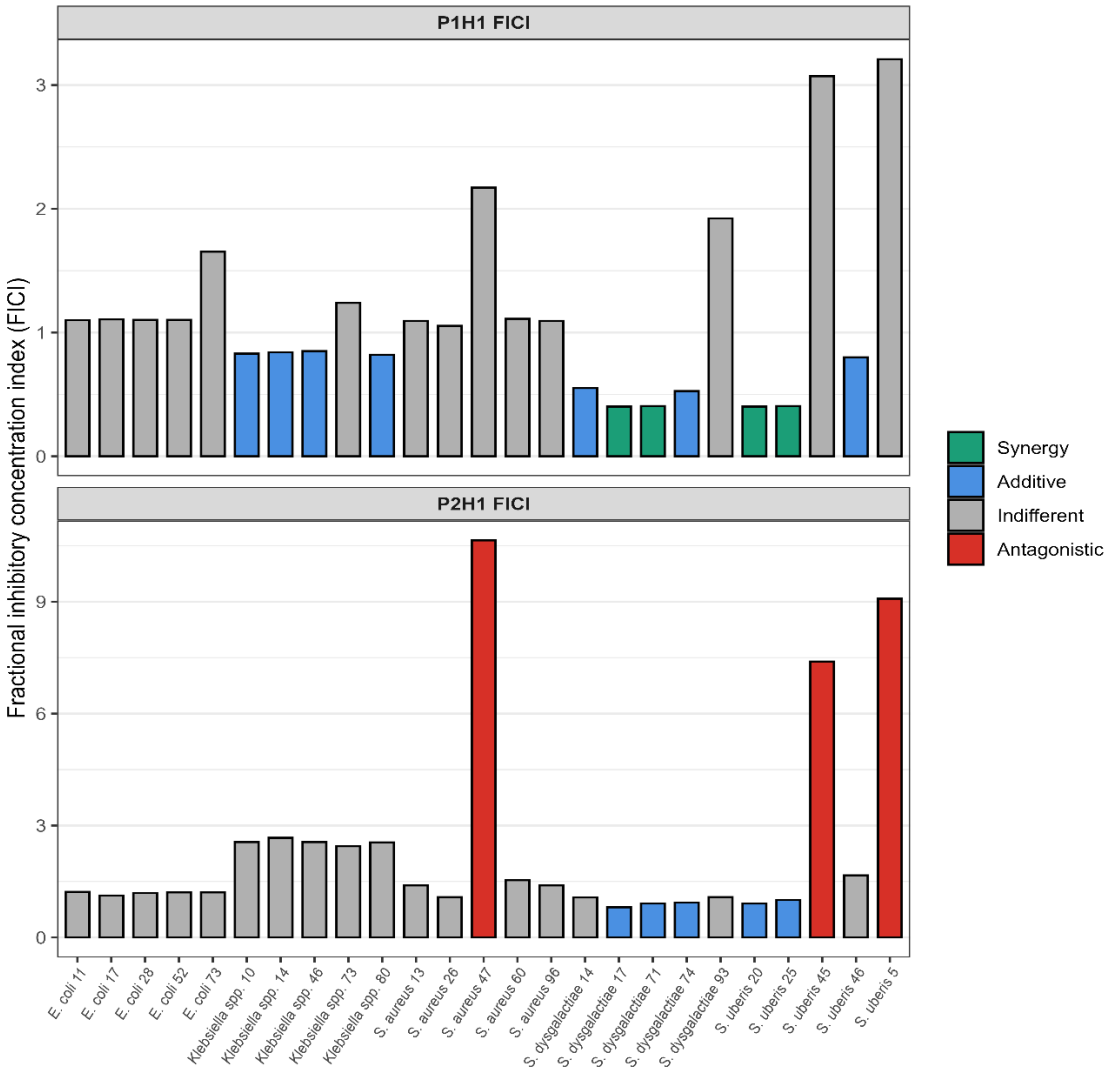


Figure 3.4. Fractional Inhibitory Concentration Index (FICI) of Honey-Propolis Combinations Against Bovine Mastitis Isolates (PE1H1 vs. PE2H1).

-The bar graphs show FICI values for 25 mastitis-associated bacterial species when honey and propolis are combined under two experimental conditions (PE1H1 and PE2H1). The FICI categorizes interactions as: synergy (green, FICI ≤ 0.5), additive (blue, 0.5 < FICI ≤ 1), indifferent (gray, 1 < FICI ≤ 4), and antagonistic (red, FICI > 4).

This apparent paradox, where PE1H1 showed more synergy than PE2H1 despite PE2 being more potent alone, can be explained by the fact that the lower intrinsic potency of PE1 leaves greater scope for enhancement when combined with strong honey, whereas the already high activity of PE2 leaves little additional effect to be gained from combination, a phenomenon where strong standalone honey activity can mask synergistic contributions with propolis (Postali *et al.*, 2022).

With respect to overcoming conventional antibiotic resistance, a major highlight of this study is the lack of cross-resistance between conventional antibiotics and bee products. MDR *E. coli* 73 (resistant to gentamicin, tetracycline, and trimethoprim/sulfamethoxazole) and linezolid-resistant *S. aureus* 47 remained highly susceptible to both individual products and their combinations. This confirms that bee products operate through mechanisms distinct from conventional antibiotics (Rana *et al.*, 2025; Bouchelaghem, 2022).

Regarding strain-dependent variability, the elevated FICI values for *S. aureus* 47 and *S. uberis* (isolates 5 and 45) corresponded with the high phenotypic resistance observed in the initial antibiogram. This suggests that certain intrinsic resistance factors, such as thickened peptidoglycan layers or altered cell wall permeability, may provide partial protection against natural product diffusion, necessitating higher therapeutic doses to achieve bactericidal effects (Naranjo-Lucena & Slowey, 2022).

In summary, these findings demonstrate that while honey and propolis are individually effective against bovine mastitis pathogens, their combination does not systematically guarantee a synergistic improvement in activity. In specific cases, the matrix may even reduce the contribution of the more potent component through competitive inhibition. Nevertheless, even in antagonistic cases, the combinations remained effective against the tested isolates, albeit at higher propolis concentrations. The observation that PE1H1 (utilizing a less intrinsically potent propolis) yielded more frequent additive and synergistic interactions than PE2H2 highlights that clinical efficacy depends not only on individual potency but also on the specific chemical compatibility between the components and their interaction with the unique physiology of the target pathogen.

IV. Conclusion: From Phenotypic Susceptibility to Synergistic Potential

This chapter systematically evaluated the antibacterial activity and interaction potential of Algerian honey and propolis against a panel of clinical bovine mastitis isolates. The findings suggest that antibacterial efficacy was more closely associated with qualitative phytochemical composition than with total phenolic content alone. Importantly, the multidrug-resistant *E. coli* and linezolid-resistant *S. aureus* isolates tested remained susceptible to the bee products, suggesting no apparent cross-resistance with the antibiotic-resistance phenotypes represented in this panel.

Bioactivity profiling established that the individual propolis extracts possessed high standalone antibacterial potency. By contrast, the evaluation of binary combinations revealed an asymmetric concentration pattern between the two components: within the inhibitory combinations, the concentration of propolis generally remained comparable to, or exceeded, its individual MIC, whereas honey was present at substantially lower concentrations than those required when tested alone. This pattern indicates that combining the two products did not produce a uniform improvement over their individual activities, but rather generated distinct interaction profiles across the tested pathogens and isolates. Consequently, the results support a pathogen-tailored strategy rather than a single universal formulation. Among the individual products, PE2 and H1 showed the most favorable antibacterial profiles (consistent with their distinctive phytochemical profiles), whereas the binary combinations PE2H1 and PE1H1 may represent complementary options in specific pathogen–product contexts where favorable interactions were observed.

Thus, the principal outcome of this chapter is not the identification of a single universally optimal formulation, but the recognition that the relative value of an individual bee product or a binary combination depends on the interplay between phytochemical composition, formulation matrix, and bacterial target.

While these *in vitro* findings characterize the phenotypic antibacterial responses of the tested bee products, they do not fully explain the molecular interactions underlying their activity. The subsequent chapter therefore investigates the identified bioactive constituents using complementary *in silico* approaches. Molecular docking will be

employed to predict their interactions with selected bacterial targets, including DNA gyrase and PBP2a, while Keap1 will be examined in relation to the antioxidant activity of the bee products. Complementary ADMET profiling will further provide information on the predicted pharmacokinetic and safety characteristics of the identified constituents.

Chapter 3. Virtual Screening and Pharmacokinetic Analysis of Natural Bioactive Compounds from Algerian Propolis and Hone

I. Introduction

Building upon the experimental findings detailed in previous chapters, this chapter transitions to an *in silico* exploration of the bioactive mechanisms of Algerian propolis and honey. Computational modeling bridges the extensive LC–MS phytochemical library (Chapter 2) with atomic-level insights into ligand–protein interactions that are not directly accessible through conventional *in vitro* assays, thereby providing a molecular basis for interpreting the biological activities observed experimentally. In the context of these complex natural products, the objective is not to identify isolated drug candidates but to improve the mechanistic understanding of how individual bioactive constituents may contribute to the overall antibacterial activity of the crude bee products.

While reactive oxygen species (ROS) play a fundamental role in immune defense, their excessive production relative to antioxidant capacity can lead to oxidative stress (Sies, 2020). In dairy cows, the transition from the dry period to early lactation is associated with increased ROS generation, which may contribute to the inflammatory processes involved in mastitis (Turk *et al.*, 2017). In this context, oxidative imbalance may predispose the mammary tissue to damage and compromise local defense mechanisms. The Keap1–Nrf2 pathway, an intracellular signaling cascade centered on the interaction between Kelch-like ECH-associated protein 1 (Keap1) and nuclear factor erythroid 2-related factor 2 (Nrf2), plays a pivotal role in regulating cellular antioxidant and cytoprotective responses(Crisman *et al.*, 2023).

Although the direct antioxidant activity of propolis is well established, its potential to act as a non-covalent modulator of the Keap1–Nrf2 complex remains less explored. Given the abundance of polyphenols, particularly flavonoids and phenolic acids, known to influence this pathway (Culletta *et al.*, 2024), molecular docking was employed to investigate the interactions between these compounds and the Kelch domain of Keap1. This host-directed perspective complements the antimicrobial findings, as reducing oxidative stress may indirectly support the host's capacity to control infection.

In parallel, the identified compounds from propolis and honey were evaluated for their direct antibacterial potential against selected molecular targets essential for bacterial survival. These simulations are designed to complement the *in vitro* antibacterial findings

reported in Chapter 2, where Algerian propolis and honey demonstrated significant activity against mastitis-associated pathogens, by providing a molecular-level explanation of their mechanisms of action. DNA gyrase, a key enzyme involved in DNA replication, represents a major antibacterial target of quinolones such as nalidixic acid (Rajakumari *et al.*, 2024; Spencer & Panda, 2023; Fàbrega *et al.*, 2009). In addition, penicillin-binding protein 2a (PBP2a), a determinant of β-lactam resistance, was included due to its critical role in antimicrobial resistance mechanisms (Shalaby *et al.*, 2020; My *et al.*, 2011; Guignard *et al.*, 2005). Docking against these targets provides mechanistic insight into the antibacterial activity observed experimentally and enables comparison with the reference antibiotics used in the in vitro assays, namely penicillin and nalidixic acid.

To complement the molecular docking analysis, ADME-Tox (Absorption, Distribution, Metabolism, Excretion, and Toxicity) prediction was performed to evaluate the drug-likeness and predicted pharmacokinetic and safety characteristics of the identified constituents. Together, these complementary computational approaches enable a more comprehensive *in silico* characterization of the bioactive compounds identified in Algerian propolis and honey.

II. Materials and Methods

1. Keap1-Nrf2 Antioxidant Pathway Modeling

The X-ray crystal structure of the KEAP1 Kelch domain (PDB ID: 4L7B), complexed with ML334 [(1S,2R)-2-[(1S)-1-[(1,3-dioxoisoindol-2-yl)methyl]-3,4-dihydro-1H-isoquinoline-2-carbonyl]cyclohexane-1-carboxylic acid)], was selected for molecular docking based on its 2.41 Å resolution and co-crystallization with a reference ligand, which provides an optimal framework for validating our docking protocol. The structure was retrieved from the Protein Data Bank (https://www.rcsb.org/). This structure was prepared using the Protein Preparation Wizard in Maestro (v11.8) with the OPLS3e force field, as per Bourougaa *et al.* (2024). Potential ionization states and partial atomic charges were set at pH 7.0. Geometry was refined via restrained minimization to a Root Mean Square Deviation (RMSD) of 0.3 Å, consistent with Keighobadi *et al.* (2018). Additional validation was performed using the Dock RMSD online tool (https://zhanggroup.org/DockRMSD/). This prepared structure then served as the receptor for grid generation, utilizing standard box dimensions for XP-docking.

The initial 2D structures of the 28 bioactive compounds identified by LC-MS (Table 2.5) were retrieved from the Pub-Chem database (https://pubchem.ncbi.nlm.nih.gov/) or drawing using ChemDraw 23.1 and saved as PDB files. Their corresponding 3D structures were generated and energy-minimized using the LigPrep module of Schrödinger Suite (v11.8).

Finally, the prepared molecules (ML334 (reference ligand) and the 28 bioactive components) were docked into the active site of KEAP1 (PDB ID: 4L7B, https://www.rcsb.org/structure/4L7B) using the extra-precision (XP-docking) feature of Maestro's Glide module. Interactions were visualized using Discovery Studio Visualizer to illustrate the interactions between the bioactive compounds and the KEAP1 active site and to evaluate their potential inhibitory activity, as described by Bourougaa *et al.* (2024).

2. Antibacterial Target Docking (DNA Gyrase and PBP2a)

Molecular docking simulations were performed using AutoDock 4.2 (Morris *et al.*, 2009) to evaluate the binding potential of bioactive compounds identified by LC-MS from propolis and honey against bacterial targets. The crystal structures of DNA gyrase (PDB ID: 4URO, https://www.rcsb.org/structure/4URO) and penicillin-binding protein PBP2a (PDB ID: 3UDI, https://www.rcsb.org/structure/3UDI) were retrieved from the RCSB Protein Data Bank (Berman 2000). These targets were selected due to their essential roles in bacterial DNA replication and cell wall biosynthesis, and their relevance as established antibacterial targets (Rajakumari *et al.*, 2024; Spencer & Panda, 2023; Shalaby et *al.*, 2020)

Prior to docking, all water molecules and co-crystallized ligands were removed from the protein structures, and polar hydrogens were added (Adaika *et al.*, 2025; Bekkar *et al.*, 2025a). Kollman charges were assigned using AutoDock Tools version 1.5.7 (Morris *et al.*, 2009). Ligands identified by LC-MS were retrieved from the PubChem database in SDF format (Kim *et al.*, 2023), energy-minimized using the MMFF94 force field, and converted to PDBQT format with Open Babel (O'Boyle *et al.*, 2011). Gasteiger charges were assigned, and all rotatable bonds were defined.

Docking calculations were performed using the Lamarckian genetic algorithm (LGA). A grid box of 60 × 60 × 60 Å, with grid spacing of 0.375 Å, was centered on the active site of each protein (DNA gyrase: X = −1.311, Y = 0.207, Z = −12.916; PBP2a: X = 34.033, Y = −0.615, Z = 12.352). For each ligand, 100 independent LGA runs were performed, while all other parameters were maintained at default values unless otherwise specified (Samah *et al.*, 2026; Bendaas *et al.*, 2025).

To validate the protocol, co-crystallized ligands were re-docked under the same conditions. The protocol was considered reliable when the RMSD between the docked and crystallographic conformations was less than 2.0 Å (Bekkar *et al.*, 2025b; Lanez *et al.*, 2025). Docking results were analyzed based on binding energy and intermolecular interactions, including hydrogen bonds and hydrophobic contacts, using PyMOL and the Protein-Ligand Interaction Profiler (Schrödinger and DeLano 2020; Salentin *et al.*, 2015).

3. ADME-Tox and Pharmacokinetic Prediction

In silico ADME-Tox and pharmacokinetic properties were evaluated for the compounds identified by LC-MS and prioritized based on their molecular docking results. ADME describes the main processes that influence the behavior of a compound in the body, while toxicity assessment provides information on its potential safety risks. Canonical SMILES (Simplified Molecular Input Line Entry System) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and used as molecular input for the prediction platforms.

SwissADME (http://www.swissadme.ch/) was used to evaluate drug-likeness and predicted oral bioavailability according to Lipinski's Rule of Five and complementary Ghose, Veber, and Egan criteria, based on molecular descriptors including molecular weight, lipophilicity (LogP), hydrogen-bond donors and acceptors, topological polar surface area, molar refractivity, and rotatable bonds (Lipinski, 2004; Veber *et al.*, 2002; Egan *et al.*, 2000). Pharmacokinetic parameters were further predicted using SwissADME and pkCSM, covering absorption (gastrointestinal absorption and Caco-2 permeability), distribution (volume of distribution and blood-brain barrier permeability), metabolism (predicted cytochrome P450 interactions), and excretion (total clearance) (Daina *et al.*, 2017; Pires *et al.*, 2015).

Potential toxicological effects were assessed using ProTox-II (https://tox-new.charite.de/protox_II/), and pkCSM. The evaluated endpoints included acute toxicity and predicted LD_{50} values, hepatotoxicity, cytotoxicity, immunotoxicity, mutagenicity (AMES), and cardiotoxicity associated with hERG inhibition (Banerjee and Ulker, 2022; Pires *et al.*, 2015).

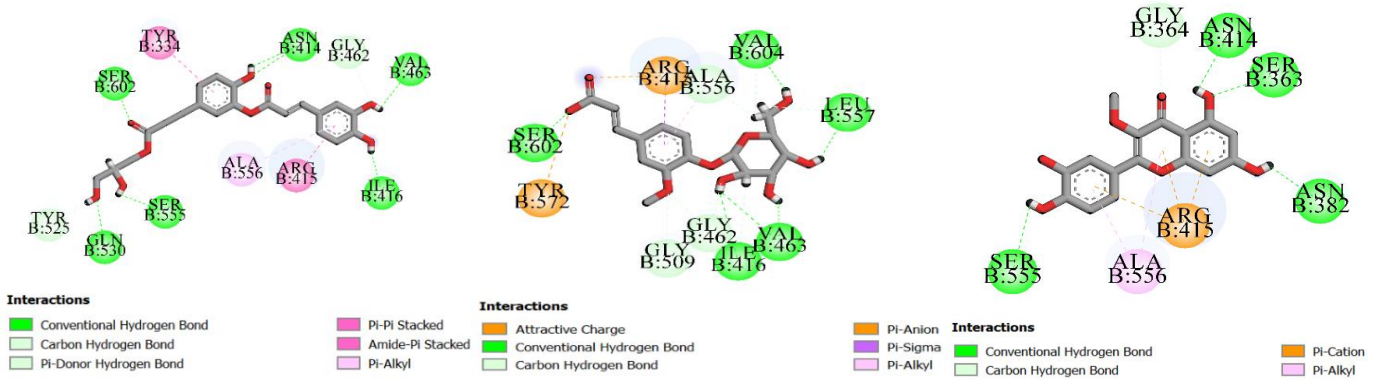
III. Results and Discussion

1. Keap1-Nrf2 Pathway: Docking and ADME-Tox of Propolis Compounds

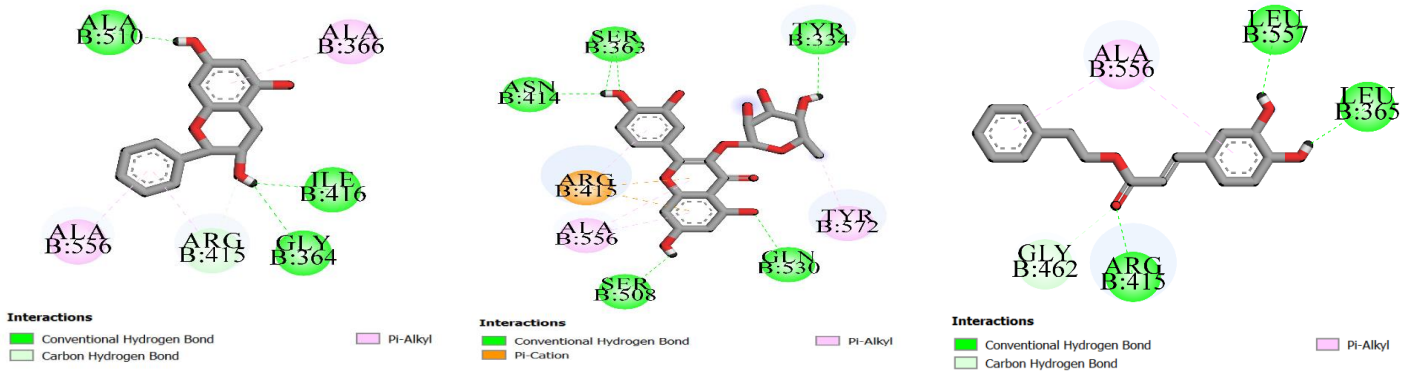
To ensure the reliability of the protein–ligand docking (PLD) outcomes, the root-mean-square deviation (RMSD) was calculated by comparing the predicted ligand conformation with the crystallographic structure (Velázquez-Libera *et al.*, 2020). Our calculated RMSD of 0.683 Å is well within the acceptable threshold of < 2.0 Å, confirming the accuracy and reproducibility of the docking protocol.

Our findings revealed that 9 of the 28 compounds identified from LC-MS analysis exhibited strong affinity for the Keap1 binding site, disrupting the Keap1-Nrf2 complex more effectively than the reference molecule ML334 (Figure 4.1).

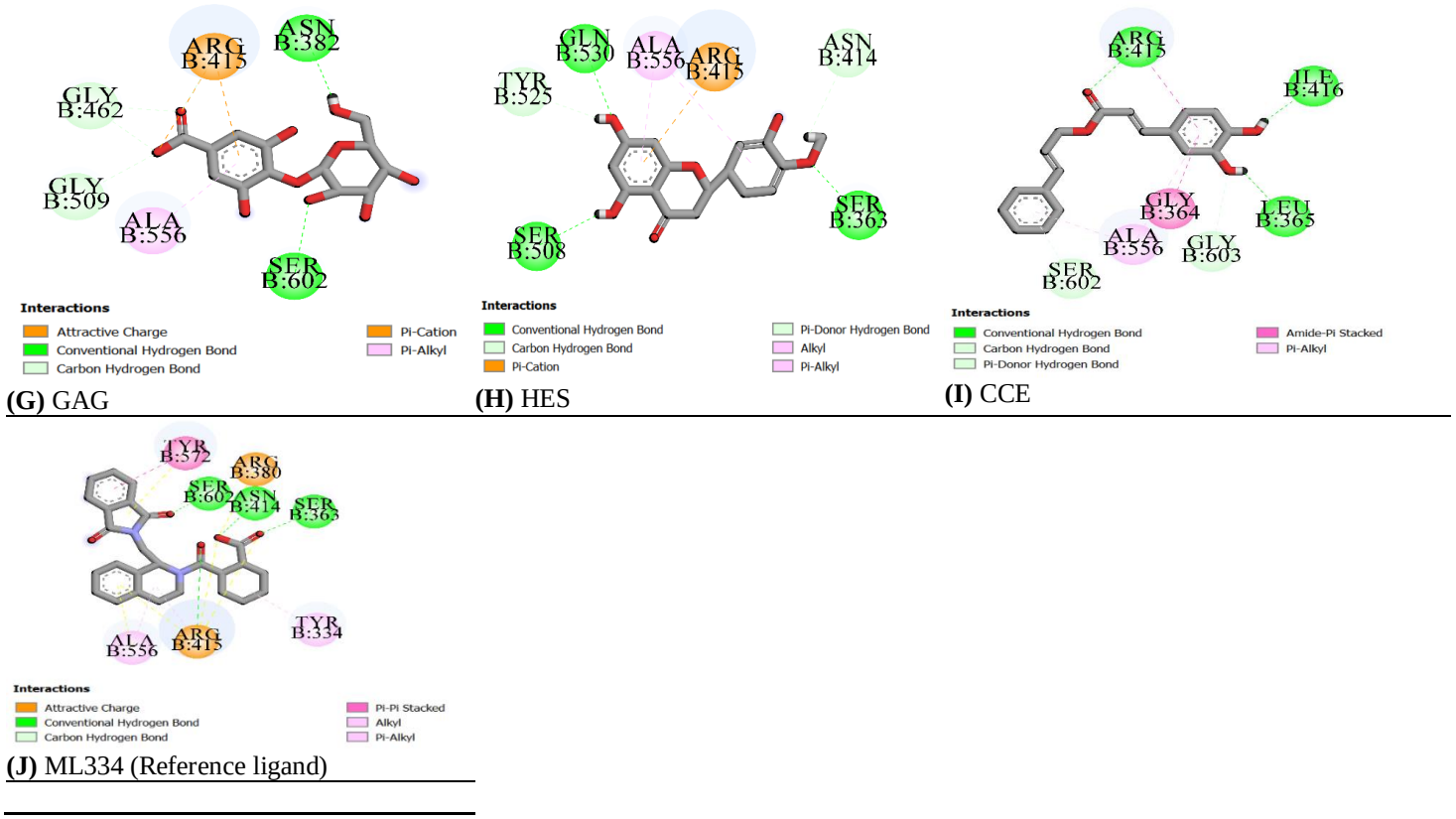
Binding affinity, which quantifies the interaction strength, is characterized by lower binding energy values indicating stronger interactions (Diniyah *et al.*, 2023). The detailed binding affinity results are presented in Table 4.1.



(A) CDG **(B)** FAG **(C)** QME



(D) PIN **(E)** QOR **(F)** CPE



(G) GAG **(H)** HES **(I)** CCE

(J) ML334 (Reference ligand)

Figure 4.1. Two-Dimensional Interaction Diagrams of the Top Propolis Compounds (A–I) And the Reference Inhibitor ML334 (J) Bound to the Keap1 Kelch Domain (PDB ID: 4L7B).

-CDG: *1,3-O-caffeoyl-dihydrocaffeoylglycerol;* ***FAG:*** *ferulic acid 4-O-glucoside;* ***QME:*** *quercetin-3-methyl-ether;* ***PIN:*** *pinobanksin;* ***QOR:*** *quercetin-3-O-rhamnoside;* ***CPE:*** *caffeic acid phenylethyl ester;* ***GAG:*** *gallic acid 4-O-glucoside;* ***HES:*** *hesperetin;* ***CCE:*** *caffeic acid cinnamyl ester;* ***Residues : Ala :*** *Alanine ;* ***Arg :*** *Arginine ;* ***Asn :*** *Asparagine ;* ***Gln :*** *Glutamine ;* ***Gly :*** *Glycine ;* ***Ile :*** *Isoleucine ;* ***Leu :*** *Leucine ;* ***Ser :*** *Serine ;* ***Tyr :*** *Tyrosine ;* ***Val:*** *Valine.*

Table 4.1. The Molecular Docking Binding Affinities and Interaction Profiles of Top Propolis Compounds Against Keap1 (PDB: 4L7B).

Complex (4L7B–Compound)	Binding Affinity (kcal/mol)	Hydrogen Bonds	Distance (Å)	Hydrophobic Interactions	Distance (Å)
CDG	−11.020	Asn414, Gln530, Ser602, Ser555, Asn414, Val463, Ile416, Gly462, Tyr525	[1.81–2.99]	Tyr334, Arg415, Ile416, Ala556,	[4.63–5.15]
FAG	−10.021	Ser602, Ile416, Val463, Leu557, Val604, Gly462, Gly509, Ala556	[1.61–2.81]	Arg415, Ala556	[2.83–3.45]
QME	−8.991	Ser363, Asn414, Asn382, Ser555, Gly364,	[1.72–2.79]	Arg415, Ala556	[5.21–5.42]
PIN	−8.379	Gly364, Ile416, Ala510, Arg415	[1.64–2.99]	Ala336, Arg415, Ala556	[3.62–5.47]
QOR	−8.335	Ser363, Gln530, Ser508, Asn414, Tyr334	[1.91–2.82]	Tyr572, Ala556, Arg415	[4.34–4.89]
CPE	−7.180	Arg415, Leu557, Leu365, Gly462	[1.83–2.78]	Ala556	[4.09]
GAG	−6.971	Ser602, Asn382, Arg415, Gly462, Gly509	[1.83–3.10]	Arg415, Ala556	[3.59–4.77]
HES	−6.990	Ser363, Gln530, Ser508, Asn414, Tyr525	[1.95–2.68]	Ala556	[4.87]
CCE	−7.009	Arg415, Leu365, Ile416, Gly603, Ser602	[1.88–2.97]	Gly364, Leu365, Arg415, Ile416, Ala556,	[4.83–5.03]
ML334	−6.807	Ser363, Asn414, Arg415, Ser602, Arg380	[1.71–3.04]	Arg415, Ala556, Tyr334	[3.48–4.85]

-Binding energies are expressed in kcal/mol; lower values indicate stronger interactions. Hydrogen bond and hydrophobic interaction distances are reported in Angstroms (Å). ***ML334*** *is the reference inhibitor.* ***CDG:*** *1,3-O-caffeoyl-dihydrocaffeoylglycerol;* ***FAG:*** *ferulic acid 4-O-glucoside;* ***QME:*** *quercetin-3-methyl-ether;* ***PIN:*** *pinobanksin;* ***QOR:*** *quercetin-3-O-rhamnoside;* ***CPE:*** *caffeic acid phenylethyl ester;* ***GAG:*** *gallic acid 4-O-glucoside;* ***HES:*** *hesperetin;* ***CCE:*** *caffeic acid cinnamyl ester. Note: The reported distances indicate the spatial proximity between the ligand and the interacting residues. For hydrogen bonds, shorter distances generally indicate more favorable interactions, whereas distances reported for hydrophobic contacts primarily reflect the proximity of non-polar groups within the binding pocket.*

The calculated binding energies ranged from −11.020 to −6.971 kcal/mol. More negative binding-energy values indicate stronger predicted ligand–protein interactions; therefore, all nine bioactive compounds exhibited stronger predicted binding affinities than the ML334 reference molecule (−6.807 kcal/mol). ML334 forms hydrogen bonds and hydrophobic interactions with key amino acid residues, including Ser363, Asn414, Arg415, Ser602, Arg380, Ala556, and Tyr334. These interactions contribute to the stability of the ligand-protein complexes formed between the nine bioactive compounds and the Keap1 binding cavity.

Of the top three complexes presented in Table 4.1, 1,3-*O*-caffeoyl-dihydrocaffeoylglycerol (CDG) showed the strongest predicted affinity for Keap1 (-11.020 kcal/mol). In molecular docking, interaction distances represent the spatial separation between a ligand and the amino acid residues involved in the predicted interaction. For hydrogen bonds, shorter distances generally indicate more favorable interactions, whereas distances reported for hydrophobic contacts indicate the proximity of non-polar groups within the binding pocket. This compound forms hydrogen bonds with Asn414, Gln530, Ser602, Ser555, Val463, Ile416, Gly462, and Tyr525 (distances ranging from 1.81 to 2.99 Å), as well as hydrophobic interactions with Tyr334, Arg415, Ile416, and Ala556 (distances from 4.63 to 5.15 Å). The extensive hydrogen-bonding and hydrophobic interactions observed for CDG may contribute to the stability of its predicted complex with Keap1**.** Ferulic acid 4-O-glucoside (FAG) ranked second with a binding affinity of −10.021 kcal/mol, forming hydrogen bonds with Ser602, Ile416, Val463, Leu557, Val604, Gly462, Gly509, and Ala556 (1.61-2.81 Å), alongside hydrophobic interactions with Arg415 and Ala556 (2.83–3.45 Å). Quercetin-3-methyl-ether (QME, −8.991 kcal/mol) completed the top three, interacting with Ser363, Asn414, Asn382, Ser555, and Gly364 via hydrogen bonds (1.72-2.79 Å), and with Arg415 and Ala556 hydrophobically (5.21-5.42 Å).

These results demonstrate that several propolis-derived polyphenols, particularly caffeoyl glycerides and flavonoid glycosides, are potent non-covalent modulators of the Keap1-Nrf2 pathway, consistent with previous reports on plant-derived Keap1 inhibitors (Culletta *et al.*, 2024; Zhuang *et al.*, 2017).

The ADME-Tox properties of the bioactive compounds from both Algerian propolis extracts are detailed in Table 4.2. The parameters presented provide complementary information on the predicted physicochemical, pharmacokinetic, and toxicity profiles of the identified compounds. Molecular weight (MW) and consensus LogP provide information on molecular size and lipophilicity, respectively, whereas LogS (ESOL) estimates aqueous solubility. GI absorption predicts the potential for gastrointestinal absorption, while CYP450 inhibition indicates the predicted potential of a compound to inhibit cytochrome P450 enzymes involved in drug metabolism. Lipinski's Rule of Five assesses drug-likeness based on molecular weight, lipophilicity, and hydrogen-bonding properties. The bioavailability score provides an estimate of the likelihood of achieving adequate oral bioavailability, while synthetic accessibility estimates the relative ease of chemical synthesis. Predicted LD_{50} and toxicity class provide estimates of acute toxicity.
These compounds, with molecular weights ranging from 272.25 to 355.32 g/mol, were predicted to exhibit high gastrointestinal (GI) absorption, except for ferulic acid 4-O-glucoside (FAG), quercetin-3-O-rhamnoside (QOR), and gallic acid 4-O-glucoside (GAG), which were predicted to have low GI absorption. The predicted consensus log P values ranged from −0.89 to 1.84 for most compounds, whereas caffeic acid phenylethyl ester (CPE, log P 3.09) and caffeic acid cinnamyl ester (CCE, log P 3.21) exhibited higher predicted lipophilicity. The predicted aqueous solubility (log S) values ranged from −3.89 to −1.14, with the caffeic acid derivatives showing relatively lower predicted solubility.

Table 4.2. ADME-Tox Properties and Drug-Likeness of the Top Propolis Compounds Identified from Molecular Docking.

Compound	MW ($g \cdot mol^{-1}$)	Consensus LogP	LogS (ESOL)	GI Absorption	CYP450 Inhibition	Lipinski (✓/✗)	Bioavailability Score	Synthetic Accessibility	Predicted LD_{50} (mg/kg)	Predicted Toxicity class
CDG	272.25	1.84	−3.49	High	No	✓	0.55	3.01	2000	4
FAG	355.32	−0.89	−1.14	Low	No	✓	0.56	4.46	4000	5
QME	316.26	1.75	−3.89	High	Yes	✓	0.55	3.29	5000	5
PIN	285.27	1.63	−2.52	High	No	✓	0.55	3.34	2500	5
QOR	448.38	0.26	−3.33	Low	No	✗	0.17	5.28	5000	5
CPE	284.31	3.09	−4.24	High	No	✓	0.55	2.64	5000	5
GAG	331.25	−1.87	−0.76	Low	No	✓	0.11	4.13	3750	5
HES	304.29	1.61	−3.10	High	No	✓	0.55	4.02	435	4
CCE	296.32	3.21	−3.96	High	No	✓	0.55	2.62	1520	4
ML334 (reference)	459.51	2.52	−4.49	High	No	✓	0.56	4.10	210	3

*- **MW:** molecular weight; Consensus **LogP:** predicted lipophilicity based on multiple LogP prediction methods; **LogS (ESOL):** predicted aqueous solubility estimated using the ESOL model; **GI:** gastrointestinal; **CYP450:** cytochrome P450; **Lipinski:** compliance with Lipinski's Rule of Five; **Bioavailability Score:** predicted probability of achieving at least 10% oral bioavailability; **Synthetic Accessibility:** predicted relative ease of chemical synthesis; **LD_{50}:** predicted median lethal dose. Toxicity classes range from 1 (most toxic) to 6 (least toxic) according to ProTox-II (Banerjee et al., 2018; Banerjee & Ulker, 2022). **CDG:** 1,3-O-caffeoyl-dihydrocaffeoylglycerol; **FAG:** ferulic acid 4-O-glucoside; **QME:** quercetin-3-methyl-ether; **PIN:** pinobanksin; **QOR:** quercetin-3-O-rhamnoside; CPE: caffeic acid phenylethyl ester; **GAG:** gallic acid 4-O-glucoside; **HES:** hesperetin; **CCE**: caffeic acid cinnamyl ester.*

Crucially, none of the compounds were predicted to inhibit the major cytochrome P450 isoforms evaluated, with the exception of QME, suggesting a potentially lower risk of cytochrome P450-mediated drug–drug interactions. All compounds except quercetin 3-*O*-rhamnoside (QOR) comply with Lipinski's Rule of Five, indicating favorable drug-likeness (Lipinski, 2004). Oral bioavailability scores ranged from 0.55 to 0.56 for most compounds, falling within acceptable limits, while QOR (0.17) and GAG (0.11) showed lower scores. Synthetic accessibility scores range from 2.62 to 4.46, indicating that these compounds are synthetically feasible.

Regarding toxicity, the predicted LD_{50} values ranged from 435 to 5000 mg/kg, placing the samples within toxicity classes 4 to 5 (on a scale of 6, where class 6 is the safest) (Banerjee & Ulker, 2022). Hesperetin (HES) showed the lowest LD_{50} (435 mg/kg, class 4), while most compounds fell into class 5 (2500–5000 mg/kg), indicating relatively low acute toxicity. Notably, the reference ligand ML334 exhibited a lower LD_{50} (210 mg/kg, class 3), suggesting that the natural propolis compounds are safer than the synthetic reference inhibitor.

The combined docking and ADME-Tox analysis indicates that Algerian propolis contains multiple compounds with strong binding affinity toward the Keap1 binding site, exceeding that of the reference inhibitor ML334. Among them, CDG emerged as the most promising candidate, combining high binding affinity with favorable pharmacokinetic properties and low predicted toxicity. These findings provide a molecular rationale for the antioxidant activity of Algerian propolis observed in Chapter 2 and support its potential role modulating the Keap1-Nrf2 pathway.

2. Antibacterial Targets (DNA Gyrase and PBP2a): Docking and ADME-Tox of Propolis and Honey Compounds

While the Keap1–Nrf2 pathway addresses host-directed antioxidant defense, the antibacterial potential of propolis and honey also requires investigation at the level of direct bacterial targets. The following sections therefore examine the interactions of selected compounds with DNA gyrase and PBP2a through molecular docking and ADME-Tox analysis.

a. *In Silico* Molecular Docking

The docking protocol was validated by re-docking the co-crystallized ligands into the active sites of DNA gyrase (4URO) and PBP2a (3UDI). The resulting RMSD values were 0.788 Å and 0.421 Å, respectively, indicating good agreement between the docked and crystallographic conformations and confirming the reliability of the docking procedure.

In both cases, the native ligands aligned closely with their predicted binding poses, demonstrating that the selected docking parameters could accurately reproduce the experimental binding modes Figure 4.2.

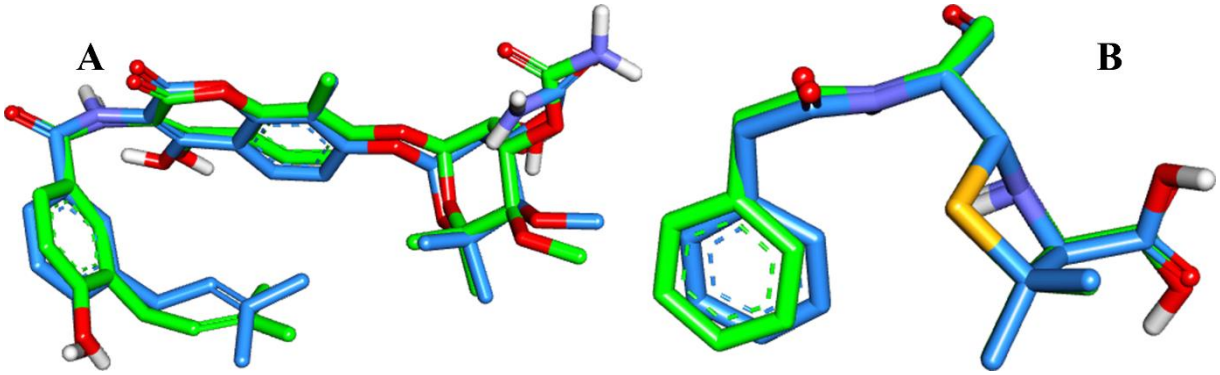


Figure 4.2. Superposition of Co-Crystallized Ligands (Green) and Re-Docked Poses (Blue) in the Active Sites of **(A)** DNA Gyrase and **(B)** PBP2a.

b. Binding Affinity

Binding energy analysis showed that the reference antibacterial drugs exhibited predicted interactions with the selected molecular targets. Nalidixic acid (NAL) exhibited binding energies of −5.53 kcal/mol with DNA gyrase and −5.10 kcal/mol with PBP2a, whereas penicillin (PEN) showed binding energies of −6.18 kcal/mol and −7.55 kcal/mol, respectively (Table 4.3). DNA gyrase was selected as a relevant target for Gram-negative pathogens, such as *E. coli*, because of its essential role in bacterial DNA replication and its established association with quinolone antibacterial activity, including that of nalidixic acid (Sugino *et al.*, 1977). PBP2a was selected as a relevant target for Gram-positive pathogens, particularly *S. aureus*, because of its role in peptidoglycan cross-linking and β-lactam resistance (Yocum *et al.*, 1980). The binding affinities of the reference drugs therefore provided comparative benchmarks for evaluating the predicted interactions of the propolis and honey compounds with these targets.

Among the propolis compounds, several molecules displayed strong predicted binding to DNA gyrase, with binding affinities more favorable than that of the native ligand (−7.11 kcal/mol). The highest binding affinities were observed for Pinobanksin-3-O-acetate (PNA, −8.57 kcal/mol), Pinobanksin-3-O-propionate (PNR, −8.40 kcal/mol), Isorhamnetin (ISO, −8.27 kcal/mol), Kaempferol (KAE, −8.01 kcal/mol), and Pinobanksin (PIN, −7.84 kcal/mol). For PBP2a, the most favorable interactions were recorded for Naringenin hexoside (NHX, −8.21 kcal/mol), ISO (−8.02 kcal/mol), QME (−7.74 kcal/mol), and PNA (−7.71 kcal/mol), all showing more favorable predicted binding affinities than the native ligand (−6.88 kcal/mol).

Similarly, several honey constituents demonstrated notable predicted binding affinities against both molecular targets. For DNA gyrase, Quercetin (QUE, −8.23 kcal/mol), Curcumin (−8.21 kcal/mol), Resveratrol (RES, −8.01 kcal/mol), and Riboflavin (RBF, −7.89 kcal/mol) showed the most favorable interactions. In the case of PBP2a, Oleanolic Acid (OLA, −10.05 kcal/mol), and β-carotene (CAR, −9.81 kcal/mol) displayed the strongest binding, followed by Curcumin (−8.63 kcal/mol), Rutin (RUT, −8.18 kcal/mol), and QUE (−7.62 kcal/mol).

Overall, several compounds from both propolis and honey displayed more favorable binding energies than the native ligands and the reference antibiotics (nalidixic acid and penicillin), indicating strong theoretical affinity with DNA gyrase and PBP2a.

Table 4.3. Binding Energies (ΔG, kcal/mol) of Reference Drugs and Propolis/Honey Compounds Against DNA Gyrase (4URO) and PBP2a (3UDI).

Ligand	4URO	3UDI	Ligand	4URO	3UDI
Native ligand	-7.11	-6.88	**MCE**	-7.26	-7.40
NAL	-5.53	-5.10	**NAR**	-7.60	-6.96
PEN	-6.18	-7.55	**NHX**	-7.74	-8.21
API	-7.54	-7.05	**OLA**	-7.68	-10.05
CAF	-5.53	-5.85	**OLP**	-6.01	-5.99
CAR	-7.32	-9.81	**PCA**	-5.29	-5.41
CCE	-7.52	-7.58	**PCH**	-5.82	-6.97
CDG	-6.79	-7.14	**PCI**	-7.42	-6.22
CHR	-7.47	-6.62	**PIN**	-7.84	-6.92
CIE	-7.01	-7.14	**PME**	-7.19	-7.30
CPE	-6.96	-7.12	**PNA**	-8.57	-7.71
CUR	-8.21	-8.63	**PNP**	-7.61	-7.43
EPI	-7.03	-7.26	**PNR**	-8.40	-7.45
FAG	-5.95	-7.62	**QDE**	-7.54	-7.78
GAG	-5.33	-6.17	**QME**	-7.05	-7.74
HES	-7.76	-7.29	**QOR**	-7.39	-7.97
ISO	-8.27	-8.02	**QUE**	-8.23	-7.62
KAE	-8.01	-7.25	**RBF**	-7.89	-6.75
KFD	-7.46	-6.96	**RES**	-8.01	-6.68
KOJ	-4.80	-4.31	**RUT**	-6.08	-8.18
LUT	-7.59	-7.42	**SIA**	-5.30	-5.71
MBA	-3.42	-5.36	**VNA**	-4.35	-4.97

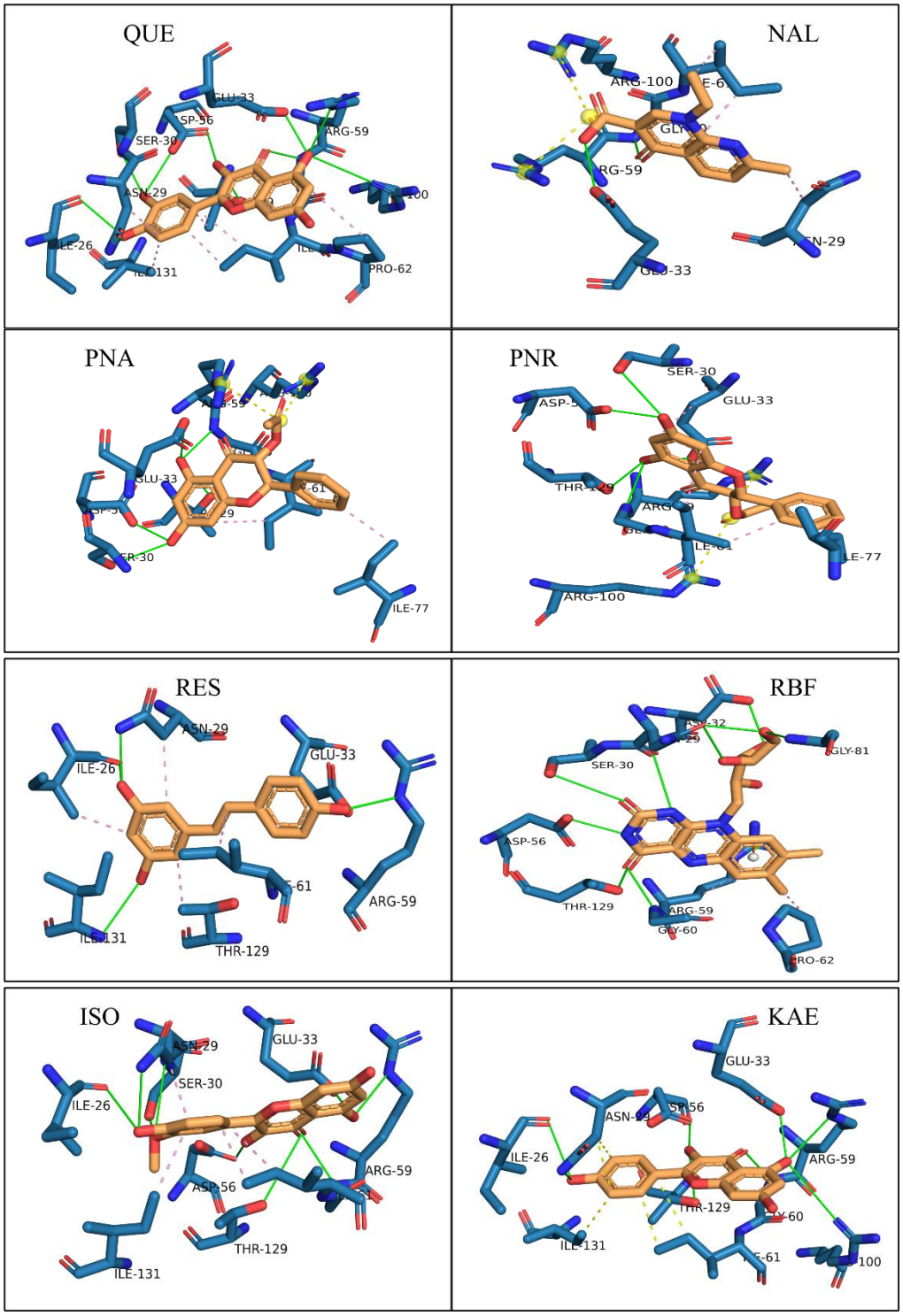
-NAL: *nalidixic acid;* ***PEN:*** *penicillin****; API:*** *apigenin****; CAF:*** *caffeic acid;* ***CAR:*** *β-carotene;* ***CCE:*** *caffeic acid cinnamyl ester;* ***CDG:*** *1,3-O-caffeoyl-dihydrocaffeoylglycerol;* ***CHR:*** *chrysin;* ***CIE:*** *caffeic acid isoprenyl ester;* ***CPE:*** *caffeic acid phenylethyl ester;* ***CUR:*** *curcumin;* ***EPI:*** *epicatechin;* ***FAG:*** *ferulic acid 4-O-glucoside;* ***GAG:*** *gallic acid 4-O-glucoside;* ***HES:*** *hesperetin;* ***ISO:*** *isorhamnetin;* ***KAE:*** *kaempferol;* ***KFD:*** *kaempferide;* ***KOJ:*** *kojic acid;* ***LUT:*** *luteolin;* ***MBA:*** *2-methoxybenzoic acid;* ***MCE:*** *p-methoxycinnamic acid cinnamyl ester;* ***NAR:*** *naringenin;* ***NHX:*** *naringenin hexoside;* ***OLA:*** *oleanolic acid;* ***OLP:*** *oleuropein;* ***PCA:*** *p-coumaric acid;* ***PCH:*** *p-coumaric acid hexose;* ***PCI:*** *p-coumaric acid isoprenyl ester;* ***PIN:*** *pinobanksin;* ***PME:*** *pinobanksin-5-methyl ether;* ***PNA:*** *pinobanksin-3-O-acetate;* ***PNP:*** *pinobanksin-3-O-pentanoate;* ***PNR:*** *pinobanksin-3-O-propionate;* ***QDE:*** *quercetin-dimethyl ether;* ***QME:*** *quercetin-3-methyl-ether;* ***QOR:*** *quercetin-3-O-rhamnoside;* ***QUE:*** *quercetin;* ***RBF:*** *riboflavin;* ***RES:*** *resveratrol;* ***RUT:*** *rutin;* ***SIA:*** *sinapic acid;* ***VNA:*** *vanillic acid.*

c. Interaction Analysis

Detailed analysis of ligand-protein interactions revealed that both reference drugs and natural compounds established multiple stabilizing interactions within the DNA gyrase binding site (Appendix E). NAL formed hydrogen bonds with GLU33 and GLY60 (1.85–1.92 Å), along with hydrophobic interactions involving ASN29 and ILE61. In addition, salt-bridge interactions with ARG59 and ARG100 were observed (3.94-4.73 Å), consistent with its moderate binding affinity (Figure 4.3).

Propolis flavonoids, including PNA, PNR, ISO, PIN, and KAE, exhibited extensive hydrogen bonding and hydrophobic interactions with key residues such as SER30, GLU33, ASP56, GLY60, ARG59, and THR129 (Figure 4.3). Notably, PNA and PNR also showed salt-bridge or π-cation interactions with ARG59 and ARG100 (4.07-4.61 Å), which may contribute to binding stabilization.

Similarly, honey constituents such as QUE, RES, and RBF formed multiple hydrogen bonds within the active site, involving residues ASN29, SER30, GLU33, ASP56, and GLY60 (Figure 4.3). RBF additionally displayed a π-cation interaction with ARG59 (4.26 Å). Overall, the interaction analysis suggests that multiple complementary interactions with key active-site residues, particularly GLU33, GLY60, ARG59, and ARG100, may contribute to the predicted binding affinities of the evaluated compounds. Among the identified molecules, propolis constituents (PNA, PNR, ISO, KAE, and PIN) together with selected honey compounds (QUE, RES, and RBF) emerged as promising candidates based on their favorable binding energies and interaction profiles. These findings provide a molecular basis for prioritizing these compounds for further investigation.


QUE
NAL
PNA
PNR
RES
RBF
ISO
KAE

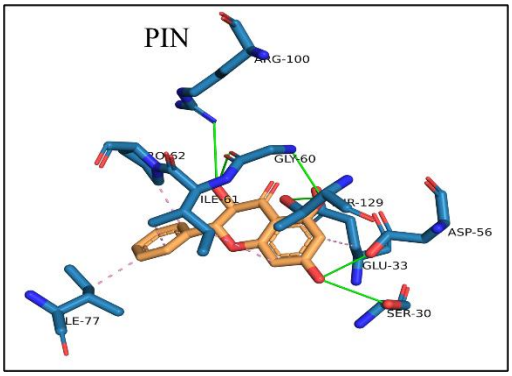


Figure 4.3. 3D Binding Interaction Diagrams of Nalidixic Acid (NAL), Quercetin (QUE), Pinobanksin-3-O-Acetate (PNA), Pinobanksin-3-O-Propionate (PNR), Resveratrol (RES), Riboflavin (RBF), Isorhamnetin (ISO), Kaempferol (KAE), And Pinobanksin (PIN) Docked into the DNA Gyrase Binding Site.

Docking analysis of PBP2a (Appendix F) demonstrated that penicillin (PEN) interacts through hydrogen bonds with residues SER348, THR535, and THR537, along with hydrophobic interaction at THR537 and a salt bridge with LYS534 (Figures 4.4).

Among honey constituents, OLA exhibited the most favorable binding affinity and formed hydrogen bonds with SER348, THR535, THR537, and ASN539, as well as hydrophobic interactions with THR537, ASN539, and TYR572, and a salt bridge with LYS534. In contrast, CAR interacted mainly through hydrophobic contacts with residues such as THR523, THR535, THR537, ARG569, and TYR572, without hydrogen bonding (Figure 4.4).

Propolis flavonoids (QUE, ISO, QME, PNA) formed multiple hydrogen bonds within the PBP2a binding pocket, primarily involving SER348, ASN403, ASN539, ASP540, and ARG569, along with hydrophobic interactions with THR537 and TYR572 (Figure 4.4). Notably, PNA also exhibited a π-stacking interaction with TYR399 (4.71 Å), which may enhance binding stability.

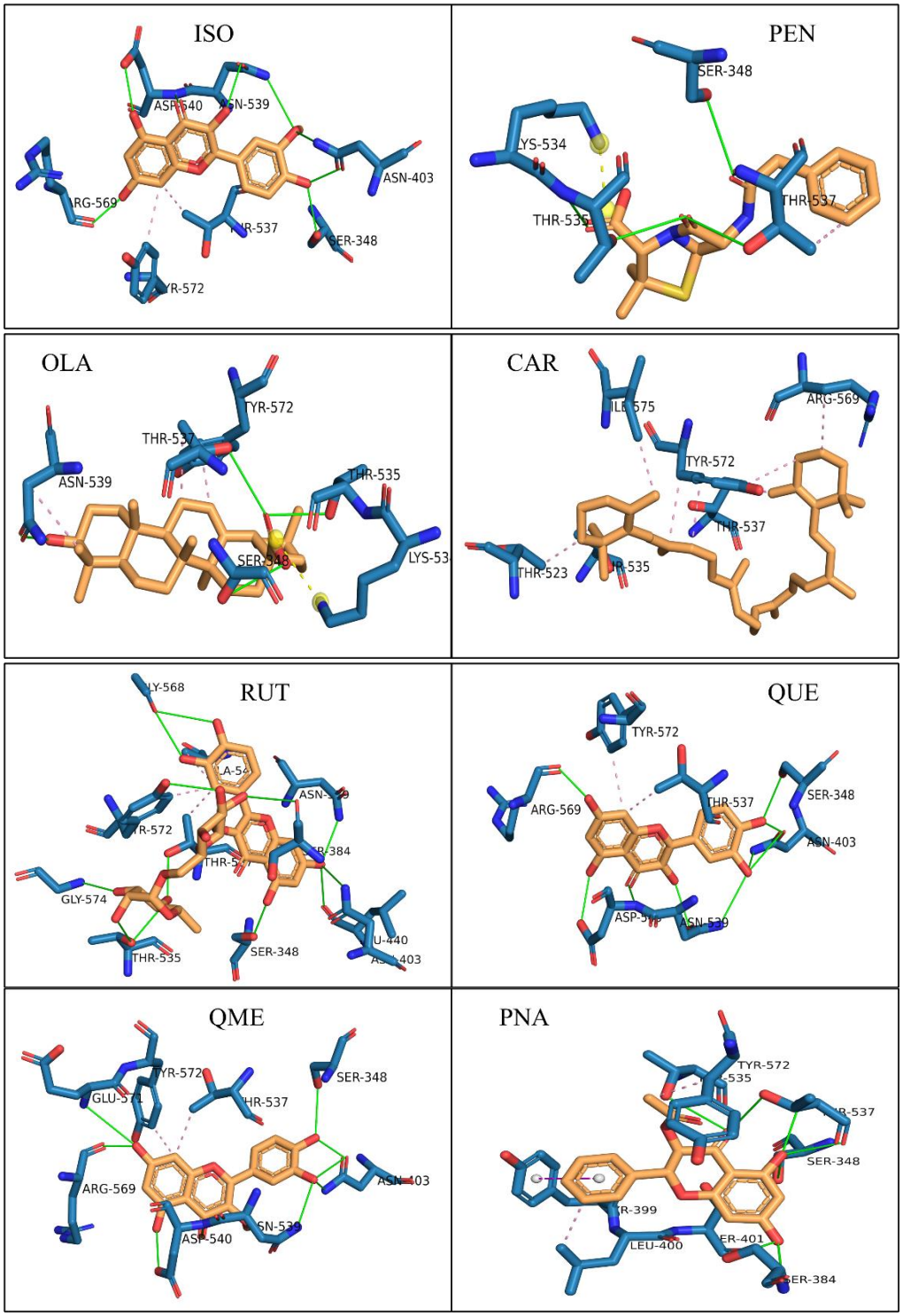


Figure 4.4. 3D Binding Interaction Diagrams of Penicillin (PEN), Isorhamnetin (ISO), Oleanolic Acid (OLA), B-Carotene (CAR) Rutin (RUT), Quercetin (QUE), Quercetin-3-Methyl-Ether (QME), and Pinobanksin-3-O-Acetate (PNA) Docked into the PBP2a Binding Site.

Overall, the docking results were consistent with those observed *in vitro* antibacterial activity. Among propolis samples, PE2 exhibited stronger activity against Gram-positive bacteria (*S. aureus, S. uberis, and S. dysgalactiae*; MIC 33.75-225 µg/mL) compared to PE1 (MIC 66.5–300 µg/mL). This trend correlates with LC-MS data showing higher concentrations of bioactive flavonoids and phenolics in PE2, including PNA (9730 vs 4530 µg/g), PNR (1121 vs 470 µg/g), ISO (2767 vs 907 µg/g), PIN (7102 vs 3140 µg/g), and QME (1398 vs 1263 µg/g). These compounds also demonstrated strong affinities to DNA gyrase (−7.84 to −8.57 kcal/mol) and PBP2a (−6.92 to −8.02 kcal/mol).

Similarly, among honey samples, H1 exhibited lower MIC values than H2 and H3, consistent with its higher content of key compounds such as QUE (0.62 µg/g), OLA (0.04 µg/g), RUT (0.15 µg/g), RES (1.95 µg/g), and NAR (0.22 µg/g). Although H2 and H3 contained lower concentrations of these compounds, their favorable docking affinities (DNA gyrase: −7.60 to −8.23 kcal/mol; PBP2a: −6.68 to −10.05 kcal/mol) suggest potential multi-target interactions, contributing to moderate antibacterial activity.

Overall, the *in vitro* antibacterial activity observed in Chapter 3 can be linked not only to compound abundance but also to the capacity of flavonoids and phenolic compounds to establish thermodynamically stable interactions with essential bacterial enzymes. These docking findings provide a molecular rationale for the observed antibacterial efficacy and set the stage for the ADMET profiling, assessing the pharmacokinetic viability of the most promising candidates, presented in the following section.

d. ADMET Analysis

i. Drug-likeness

Drug-likeness evaluation was performed using Lipinski, Ghose, Veber, Egan, and Muegge criteria to assess the suitability of the selected compounds for oral bioavailability (Table 4.4). Overall, most compounds exhibited favorable drug-like properties, with the majority fully complying with Lipinski's Rule of Five. All propolis compounds (ISO, PIN, PNA, and PNR) showed no violations, indicating appropriate molecular weight, polarity, and hydrogen bonding capacity.

Similarly, honey compounds such as NAR, QUE, and RES, as well as reference drugs (NAL and PEN), also satisfied Lipinski criteria. In contrast, QME and RUT showed multiple violations, mainly due to high polarity and excessive hydrogen bonding capacity (HBA/HBD), along with elevated TPSA values. Specifically, QME violated Lipinski (2

violations) and Veber/Egan rules due to TPSA > 140 $Å^2$, while RUT exhibited multiple violations across Lipinski, Ghose, Veber, and Muegge criteria., which indicate reduced compliance with the evaluated drug-likeness filters.

Regarding the Ghose filter, all compounds except OLA and RUT met the required criteria. Oleanolic Acid exceeded limits for lipophilicity and molar refractivity, while RUT showed multiple deviations related to molecular size and polarity. Similarly, OLA failed the Egan and Muegge criteria due to its high lipophilicity (LogP > 5), suggesting potential solubility and absorption limitations despite acceptable Lipinski compliance All compounds, except QME and RUT, complied with the Veber and Egan criteria, indicating favorable physicochemical properties according to these empirical drug-likeness filters.

Bioavailability scores (BAS) further supported these observations, with most compounds showing moderate values (~0.55), while OLA and NAL exhibited higher scores (0.85), suggesting better predicted oral availability. In contrast, QME and RUT displayed lower BAS values (0.17), consistent with multiple rule violations.

Overall, the drug-likeness evaluation indicated that most identified constituents complied with the evaluated empirical filters, whereas RUT, QME, and OLA exhibited the highest number of deviations from the recommended physicochemical criteria.

Table 4.4. Drug-likeness Properties and Physicochemical Descriptors of Selected Propolis and Honey Compounds.

Rule	**NAL**	**ISO**	**PIN**	**PNA**	**PNR**	**QME**
MW	232.24	316.26	272.25	314.29	328.32	478.40
ROTB	2	2	1	3	4	5
HBA	4	7	5	6	6	12
HBD	1	4	2	2	2	7
TPSA	72.19	120.36	86.99	93.06	93.06	199.51
MR	64	82.50	70.71	80.45	85.25	114.63
Cons Log P	1.38	1.65	1.39	1.83	2.11	0.07
Lipinski	Yes; 0V	Yes; 0V	Yes; 0V	Yes; 0V	Yes; 0V	No; 2V
Ghose	Yes	Yes	Yes	Yes	Yes	Yes
Veber	Yes	Yes	Yes	Yes	Yes	No; 1V
Egan	Yes	Yes	Yes	Yes	Yes	No; 1V
Muegge	Yes	Yes	Yes	Yes	Yes	No; 3V
BAS	0.85	0.55	0.55	0.55	0.55	0.17
Rule	**PEN**	**NAR**	**OLA**	**QUE**	**RES**	**RUT**
MW	334.39	272.25	476.70	302.24	228.24	610.50
ROTB	5	1	1	1	2	6
HBA	4	5	3	7	3	16
HBD	2	3	2	5	3	10
TPSA	112.01	86.99	57.53	131.36	60.69	269.43
MR	89.86	71.57	136.65	78.03	67.88	141.38
Cons Log P	1.29	1.84	6.07	1.23	2.48	-1.51
Lipinski	Yes; 0V	Yes; 0V	Yes; 1V	Yes; 0V	Yes; 0V	No; 3V
Ghose	Yes	Yes	No; 3V	Yes	Yes	No; 4V
Veber	Yes	Yes	Yes	Yes	Yes	No; 1V
Egan	Yes	Yes	No; 1V	Yes	Yes	No; 1V
Muegge	Yes	Yes	No; 1V	Yes	Yes	No; 4V
BAS	0.56	0.55	0.85	0.55	0.55	0.17

*-**NAL:** nalidixic acid; **PEN:** penicillin; **ISO:** isorhamnetin; **PIN:** pinobanksin; **PNA:** pinobanksin-3-O-acetate; **PNR:** pinobanksin-3-O-propionate; **QME:** quercetin-3-methyl-ether; NAR: naringenin; **OLA:** oleanolic Acid; **QUE:** quercetin; **RES:** resveratrol; **RUT:** Rutin. **MW:** molecular weight (g·mol⁻¹); **ROTB:** number of rotatable bonds; **HBA/ HBD:** number of. Hydrogen-bond acceptors/ donors; **TPSA:** topological polar surface area (Å²); **MR:** molar refractivity; **Cons:** consensus octanol/water partition coefficient (lipophilicity); **BAS:** bioavailability score, representing the predicted probability of achieving at least 10% oral bioavailability in rats/ humans according to the SwissADME model; **V:** number of rule violations.*

ii. Absorption

Absorption is a key pharmacokinetic process describing the movement of a compound from its site of administration to the site of action or systemic circulation. It is influenced by several factors, including the route of administration, the physicochemical properties of the compound (e.g., solubility and membrane permeability), and species-specific physiological characteristics (Bucao and Solidum 2022). In the present study, gastrointestinal (GI) absorption predictions generated by SwissADME were used as standardized descriptors of the absorption-related properties of the identified compounds. These predictions provide

comparative information on membrane permeability but should not be interpreted as direct predictors of absorption following intramammary administration.

As shown in Table 4.5, most compounds exhibited high predicted gastrointestinal (GI) absorption, whereas QME and RUT were classified as having low GI absorption. Water solubility (LogSwat) values ranged from −2.47 to −4.20 log mol/L, indicating acceptable predicted aqueous solubility for all compounds. In the context of natural extracts and local formulations, adequate aqueous solubility is an important physicochemical property that influences dissolution, homogeneous dispersion in aqueous media (e.g., milk and biological fluids), and the availability of the bioactive constituents at the site of administration (Aulton & Taylor, 2013). Among the evaluated compounds, PNA (−3.94) and PNR (−4.20) exhibited comparatively lower predicted solubility than the other constituents, whereas NAL (−2.75) and QUE (−2.92) showed relatively higher predicted solubility.

Caco-2 (intestinal epithelial cell) permeability analysis revealed variability among the compounds. High permeability (> 0.90×10^{-6} cm/s) was observed for PIN (1.07), NAR (1.02), OLA (1.16), and RES (1.17), suggesting efficient intestinal transport (Pires *et al.*, 2015). In contrast, ISO (−0.003), QME (0.053), PNA (0.136), and PNR (0.146) showed low permeability, which may limit absorption despite favorable GI predictions. RUT (−0.949) and QUE (−0.229) exhibited particularly poor permeability, consistent with their reduced absorption profiles.

Skin permeability (log Kp) values ranged from −3.77 to −10.26 cm/s, indicating generally low transdermal permeability. OLA (−3.77) and RES (−5.47) showed relatively higher skin permeability compared to other compounds, while RUT (−10.26) exhibited the lowest, suggesting limited suitability for transdermal delivery (Daina *et al.*, 2017).

Regarding transport mechanisms, most compounds were predicted to be P-glycoprotein (P-gp) substrates, except NAL, PEN, OLA, and QUE. As P-gp functions as an efflux transporter, substrate recognition may reduce intracellular accumulation, a feature generally considered less favorable because it may decrease the effective intracellular concentration of the compounds (Daina *et al.*, 2017).

Overall, the predicted absorption profile indicates that compounds such as PIN, NAR, OLA, and RES exhibited favorable absorption-related properties, combining high predicted GI absorption and good Caco-2 permeability. In contrast, QME and RUT were characterized by low predicted GI absorption and poor Caco-2 permeability, whereas PNA and PNR exhibited comparatively lower Caco-2 permeability despite acceptable predicted aqueous solubility.

Table 4.5. Predicted Absorption Properties of Selected Propolis and Honey Compounds.

Rule	**NAL**	**ISO**	**PIN**	**PNA**	**PNR**	**QME**
GI absorption	High	High	High	High	High	Low
$logS_{wat}$ (mol/L)	-2.756	-3	-3.003	-3.94	-4.198	-2.984
Caco2 perm	1.278	-0.003	1.077	0.136	0.146	0.053
log Kp (cm/s)	-6.72	-6.90	-6.78	-6.63	-6.38	-8.73
P-gp substrate	No	Yes	Yes	Yes	Yes	Yes
Rule	**PEN**	**NAR**	**OLA**	**QUE**	**RES**	**RUT**
GI absorption	High	High	Low	High	High	Low
$logS_{wat}$ (mol/L)	-2.47	-3.224	-3.261	-2.925	-3.178	-2.892
Caco2 perm	0.114	1.029	1.168	-0.229	1.170	-0.949
log Kp (cm/s)	-7.04	-6.17	-3.77	-7.05	-5.47	-10.26
P-gp substrate	No	Yes	No	No	Yes	Yes

*- **GI absorption:** predicted gastrointestinal absorption; **LogS** (mol/L): predicted aqueous solubility expressed as log molar solubility; **Caco-2 permeability:** predicted permeability across Caco-2 human intestinal epithelial cells; **Log Kp** (cm/s): predicted skin permeability coefficient (log cm/s); **P-gp substrate:** prediction of whether a compound is a substrate of P-glycoprotein (P-gp), an efflux transporter; **NAL:** nalidixic acid; **PEN**: penicillin; **ISO**: Isorhamnetin; **PIN:** Pinobanksin; **PNA:** Pinobanksin-3-O-acetate; **PNR:** Pinobanksin-3-O-propionate; **QME**: Quercetin-3-methyl-ether; **NAR:** Naringenin; **OLA:** Oleanolic Acid; **QUE:** Quercetin; **RES:** Resveratrol; **RUT:** Rutin.*

iii. Distribution

Distribution properties were evaluated using the predicted steady-state volume of distribution (VDss), blood-brain barrier (BBB) permeability, and central nervous system (CNS) permeability (Table 4.6). The evaluated compounds showed variable predicted tissue distribution, with ISO, PIN, QME, QUE, and RUT exhibiting higher VDss values than the remaining constituents. Predicted BBB and CNS permeability were generally low, indicating limited central nervous system exposure for most compounds. Although these parameters contribute to a comprehensive in silico ADMET profile, their direct relevance to the present study is limited, as the primary objective is to evaluate honey and propolis for the treatment of bovine mastitis rather than for central nervous system delivery.

Table 4.6. Predicted Distribution Properties of Selected Propolis and Honey Compounds.

Rule	**NAL**	**ISO**	**PIN**	**PNA**	**PNR**	**QME**
VDss (log L/kg)	-0.885	1.123	0.862	-0.611	-0.547	0.845
BBB perm (log BB)	-0.324	-1.135	-0.418	0.096	0.086	-1.837
CNS perm (log PS)	-2.899	-3.188	-2.28	-3.223	-3.204	-4.34
Rule	**PEN**	**NAR**	**OLA**	**QUE**	**RES**	**RUT**
VDss (log L/kg)	-1.905	-0.015	-1.009	1.559	0.296	1.663
BBB perm (log BB)	-0.864	-0.578	-0.143	-0.048	1.559	-1.899
CNS perm (log PS)	-2.943	-2.215	-1.176	-2.067	-3.065	-5.178

*- **VDss (log L/kg):** predicted steady-state volume of distribution**; BBB perm (logBB):** predicted blood-brain barrier permeability**; CNS perm (logPS):** predicted central nervous system permeability; **NAL:** nalidixic acid; **PEN:** penicillin; **ISO:** Isorhamnetin; **PIN:** Pinobanksin; **PNA:** Pinobanksin-3-O-acetate; **PNR**: Pinobanksin-3-O-propionate; **QME:** Quercetin-3-methyl-ether; **NAR**: Naringenin; **OLA:** Oleanolic Acid; **QUE:** Quercetin; **RES**: Resveratrol; **RUT:** Rutin.*

iv. Metabolism

Cytochrome P450 (CYP450) enzymes play a central role in drug metabolism, particularly isoforms CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, which are responsible for the majority of drug biotransformation (Zanger and Schwab 2013).

As shown in Table 4.7, most compounds were not predicted to be CYP3A4 substrates, except OLA and RES, suggesting that their metabolism may partially depend on this enzyme, while other compounds may follow alternative metabolic pathways (Deodhar *et al.*, 2020).

Regarding enzyme inhibition, the majority of compounds exhibited low inhibitory potential toward CYP isoforms, indicating a reduced likelihood of drug–drug interactions (Kokkonen *et al.*, 2021). However, some exceptions were observed. ISO showed inhibitory activity toward CYP1A2, CYP2D6, and CYP3A4, while QUE and RES were predicted to inhibit multiple enzymes, including CYP1A2 and CYP3A4, with RES also inhibiting CYP2C9. Additionally, NAR showed inhibition of CYP1A2. These findings suggest that compounds with multi-CYP inhibition profiles (e.g., ISO, QUE, and RES) may present a higher risk of drug–drug interactions by affecting the metabolism of co-administered drugs.

Overall, the metabolism profile indicates that most compounds exhibit a favorable metabolic profile with limited CYP inhibition, although compounds such as ISO, QUE, and RES may require further evaluation due to potential metabolic interactions.

Table 4.7. Predicted Metabolism and Excretion Profiles of Selected Propolis and Honey Compounds.

Rule	**NAL**	**ISO**	**PIN**	**PNA**	**PNR**	**QME**
CYP3A4 substrate	No	No	No	No	No	No
CYP1A2 inhibitor	No	Yes	No	No	No	No
CYP2C19 inhibitor	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	No	No
CYP2D6 inhibitor	No	Yes	No	No	No	No
CYP3A4 inhibitor	No	Yes	No	No	No	No
CL_{tot}	0.825	0.508	0.053	0.236	0.262	0.473
Renal OCT2	No	No	No	No	No	No
Rule	**PEN**	**NAR**	**OLA**	**QUE**	**RES**	**RUT**
CYP3A4 substrate	No	No	Yes	No	Yes	No
CYP1A2 inhibitor	No	Yes	No	Yes	Yes	No
CYP2C19 inhibitor	No	No	No	No	No	No
CYP2C9 inhibitor	No	No	No	No	Yes	No
CYP2D6 inhibitor	No	No	No	Yes	No	No
CYP3A4 inhibitor	No	No	No	Yes	Yes	No
CL_{tot}	0.197	0.06	-0.081	0.407	0.076	-0.369
Renal OCT2	No	No	No	No	No	No

-CYP: *cytochrome P450 enzyme;* ***CYP3A4 substrate:*** *predicted substrate of CYP3A4;* ***CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4 inhibitor:*** *predicted inhibition of the respective cytochrome P450 isoenzyme;* ***CLtot:*** *predicted total clearance (log mL/min/kg);* ***Renal OCT2:*** *renal organic cation transporter 2;* ***NAL:*** *nalidixic acid;* ***PEN:*** *penicillin;* ***ISO:*** *Isorhamnetin;* ***PIN:*** *Pinobanksin;* ***PNA:*** *Pinobanksin-3-O-acetate;* ***PNR:*** *Pinobanksin-3-O-propionate;* ***QME:*** *Quercetin-3-methyl ether;* ***NAR:*** *Naringenin;* ***OLA:*** *Oleanolic acid;* ***QUE:*** *Quercetin;* ***RES:*** *Resveratrol;* ***RUT:*** *Rutin.*

v. Excretion

Excretion properties were evaluated based on total clearance (CL_{tot}) and interaction with the renal organic cation transporter 2 (OCT2) (Table 4.7).

Total clearance values varied among compounds, indicating differences in elimination rates. Higher clearance was observed for NAL (0.825), ISO (0.508), and QME (0.473), suggesting faster elimination. In contrast, PIN (0.053), RES (0.076), and NAR (0.06) showed lower clearance values, indicating slower elimination and potentially longer systemic retention. Negative values observed for OLA (−0.081) and RUT (−0.369) suggest very low predicted clearance, which may result in prolonged exposure and possible accumulation.

None of the compounds were predicted to be renal OCT2 substrates, indicating a low likelihood of transporter-mediated renal drug–drug interactions (Hacker *et al.*, 2015).

Overall, the predicted excretion profile revealed variability in the elimination characteristics of the identified constituents, with most compounds exhibiting moderate to low predicted clearance and none predicted to be renal OCT2 substrates. These findings provide

complementary information on the predicted pharmacokinetic behavior of the individual constituents.

vi. Toxicity

Toxicity prediction is a critical step in drug development to identify potential safety risks at an early stage (Bucao and Solidum 2022). In this study, mutagenicity (AMES test), cardiotoxicity (hERG I inhibition), hepatotoxicity, and acute toxicity (LD_{50}) were evaluated (Table 4.8).

The Ames test results indicated that most compounds were non-mutagenic, suggesting a low risk of genotoxicity. However, RES showed a positive AMES prediction, indicating a potential mutagenic risk that may require further experimental validation.

Cardiotoxicity assessment revealed that none of the compounds were predicted to inhibit hERG I, suggesting a low risk of cardiac toxicity. Hepatotoxicity analysis showed that the majority of compounds were predicted to be non-hepatotoxic, except PEN and OLA, which exhibited potential hepatotoxicity.

Acute toxicity, ranged from 1.716 to 2.736 mol/kg, indicating moderate toxicity levels. PEN (1.716 mol/kg) showed the lowest LD_{50} value, suggesting higher toxicity compared to most tested compounds. Similarly, PNA (1.814 mol/kg) and PNR (1.820 mol/kg) exhibited relatively higher toxicity, although still comparable to PEN. In contrast, NAL (2.480 mol/kg) showed lower toxicity than several compounds, while QME (2.736 mol/kg), RES (2.529 mol/kg), and RUT (2.491 mol/kg) demonstrated the highest LD_{50} values, indicating lower toxicity.

Overall, the predicted toxicity profile suggests that most evaluated constituents possess a favorable predicted safety profile, with low predicted risks of mutagenicity and cardiotoxicity. However, the predicted alerts for RES (mutagenicity) and OLA (hepatotoxicity) warrant further experimental validation through in vitro and in vivo studies before confirming their safety profiles.

Table 4.8. Predicted toxicity of selected propolis and honey compounds.

Rule	**NAL**	**ISO**	**PIN**	**PNA**	**PNR**	**QME**
AMES toxicity	No	No	No	No	No	No
hERG I	No	No	No	No	No	No
Hepatotoxicity	No	No	No	No	No	No
LD_{50} (mol/Kg)	2.480	2.407	2.142	1.814	1.820	2.736
Rule	**PEN**	**NAR**	**OLA**	**QUE**	**RES**	**RUT**
AMES toxicity	No	No	No	No	Yes	No
hERG I	No	No	No	No	No	No
Hepatotoxicity	Yes	No	Yes	No	No	No
LD_{50} (mol/Kg)	1.716	1.791	2.196	2.471	2.529	2.491

*- **AMES toxicity:** predicted mutagenicity according to the Ames test; **hERG I:** cardiotoxicity; **Hepatotoxicity:** predicted potential to induce liver toxicity; **LD_{50} (mol/kg):** predicted median lethal dose following acute oral exposure. **NAL:** nalidixic acid; **PEN:** penicillin; **ISO:** isorhamnetin; **PIN:** pinobanksin; **PNA:** pinobanksin-3-O-acetate; **PNR:** pinobanksin-3-O-propionate; **QME:** quercetin-3-methyl-ether; **NAR:** naringenin; **OLA:** oleanolic acid; **QUE:** quercetin; **RES:** resveratrol; **RUT:** rutin.*

IV. Conclusion: From *In Silico* Affinity to Therapeutic Potential

The *in silico* investigation conducted in this chapter provides a mechanistic extension of the experimental findings by elucidating the molecular interactions underlying the bioactivity of Algerian propolis and honey. Molecular docking analysis demonstrated that a wide range of identified compounds exhibit strong binding affinity toward key bacterial targets, namely DNA gyrase and PBP2a, as well as the Keap1 protein involved in antioxidant defense. Binding affinities reached values as low as approximately −10 kcal/mol for certain honey-derived compounds, while several propolis flavonoids exhibited energies around −8 to −8.5 kcal/mol, consistently surpassing those of native ligands and reference antibiotics. These results indicate a strong theoretical potential to interfere with both bacterial survival mechanisms and host oxidative stress pathways.

Importantly, the selection of DNA gyrase and PBP2a as molecular targets provides direct relevance to antimicrobial resistance mechanisms. DNA gyrase is associated with fluoroquinolone resistance, while PBP2a mediates β-lactam resistance in staphylococci. The favorable predicted binding affinities observed for multiple propolis and honey-derived compounds toward these targets suggest their potential to interact with proteins involved in antimicrobial resistance mechanisms, thereby providing a molecular rationale for their observed antibacterial activity. These observations are consistent with the *in vitro* findings presented in Chapter 2, where multidrug-resistant isolates remained susceptible to the tested bee products. However, the precise mechanisms underlying these predicted interactions and their contribution to the observed antibacterial activity require further experimental validation.

Beyond direct antibacterial action, the predicted interaction of propolis compounds with the Keap1–Nrf2 pathway suggests a potential complementary mechanism related to antioxidant regulation. By potentially modulating cellular antioxidant defenses, these compounds could contribute to reducing oxidative stress associated with mastitis. Together with their predicted antibacterial activity, these findings suggest complementary molecular effects. However, the biological relevance of these predicted interactions, including their dependence on the route of administration and their influence on host responses, remains to be established through experimental studies.

Complementary ADME-Tox analysis further characterized the identified constituents by predicting their pharmacokinetic and safety properties. Several compounds, particularly pinobanksin (PIN), naringenin (NAR), quercetin (QUE), and resveratrol (RES), exhibited favorable drug-likeness, absorption, and toxicity profiles. Although certain compounds,

including rutin (RUT), quercetin methyl ether (QME), and oleanolic acid (OLA), presented predicted limitations related to bioavailability or toxicity, the overall ADME-Tox analysis indicates that most identified constituents exhibit generally favorable predicted properties, thereby strengthening the molecular interpretation of the antibacterial potential of the experimental findings.

Overall, the *in silico* findings are consistent with and reinforce the biological activity observed *in vitro*, supporting the interpretation that the antibacterial potential of Algerian bee products may be explained not only by their phytochemical composition but also by the predicted ability of specific bioactive constituents to establish stable interactions with key molecular targets. By integrating molecular docking with ADME-Tox predictions, this study provides a molecular framework for interpreting the antibacterial properties of Algerian propolis and honey and establishes a foundation for future mechanistic investigations, formulation development, and *in vivo* validation for bovine mastitis management.

General Discussion

Bovine mastitis remains one of the most economically damaging diseases of dairy herds worldwide, while the intensive use of antimicrobials for its control contributes to the broader antimicrobial resistance (AMR) challenge (Halasa *et al.*, 2007; Okeke *et al.,* 2024). In Algeria, this challenge is expressed by the high prevalence of mastitis, the gap between domestic milk production and national demand, and the documented occurrence of resistant bacterial strains in dairy settings (Seddar-Yagoub *et al.,* 2023; Bounoua and Souna, 2025; Akkou *et al.,* 2024). Within a One Health framework, these challenges highlight the need to investigate sustainable approaches that may complement conventional antimicrobial strategies (Eloit, 2021). The present thesis addressed this context through an integrated physicochemical, biological, and computational investigation of Algerian honey and propolis, linking their chemical composition to antioxidant and antibacterial activities and subsequently exploring selected bioactive constituents through *in silico* approaches.

The chemical characterization demonstrated substantial differences in the composition of the investigated bee products according to geographical and botanical origin. Batna propolis (PE1), despite displaying higher total phenolic and flavonoid contents than Biskra propolis (PE2), did not exhibit the strongest antioxidant activity (Kouadri *et al.,* 2021; Teggar *et al.,* 2023). Conversely, PE2, with lower TPC and TFC values, showed greater activity in several antioxidant assays. A similar dissociation between bulk phenolic content and biological activity was observed for the honey samples. These findings suggest that antioxidant and antibacterial performance is more closely associated with qualitative phytochemical composition and specific molecular signatures than with total phenolic abundance alone. The multivariate analysis further supported the distinct chemical profiles of the two propolis samples, with the first two principal components accounting for 87.15% of the total variance. Differences in environmental conditions may contribute to these compositional patterns, although the limited number of samples in our study prevents establishing a direct causal relationship between environmental conditions and metabolite accumulation (Sorucu and Oruç, 2019; do Nascimento *et al.,* 2019).

The chemical profiling also highlighted the diversity and novelty of the investigated matrices. The identification of *p*-methoxycinnamic acid cinnamyl ester and 1,3-*O*-caffeoyl dihydrocaffeoylglycerol represents a significant addition to the chemical profile of Algerian propolis, with the latter constituent representing a novel finding for propolis within the available literature. The honey samples, although sharing a conserved metabolic core, also displayed distinct regional and botanical signatures. In particular, kojic acid characterized H1, while H2 exhibited a distinct *Ziziphus lotus* profile, and differences in phenolic composition

were observed among the samples. The integration of melissopalynological and physicochemical characteristics further demonstrated the value of combining compositional, botanical, and physicochemical criteria when characterizing honey.

The antibacterial evaluation provided the main evidence for the biological activity of the investigated products. Honey and propolis inhibited the five bacterial groups represented in the clinical isolate panel, including multidrug-resistant isolates. Notably, H1 showed the lowest MIC values among the honey samples, including a MIC of 0.44% against one *S. aureus* isolate, despite its comparatively lower TPC and TFC values. PE2 also generally exhibited greater antibacterial activity than PE1. These findings reinforce the observation that antibacterial performance cannot be reliably inferred from total phenolic content alone and may instead reflect differences in the specific compounds and chemical profiles present in each product. The observed activity is consistent with previous reports describing antibacterial effects of honey and propolis against mastitis-associated pathogens (Aamer *et al.*, 2014; Fiordalisi *et al.*, 2016; Klhar *et al.*, 2019; Wang, K. *et al.*, 2016). Importantly, the antibiotic-resistance phenotypes represented by the multidrug-resistant *E. coli* and linezolid-resistant *S. aureus* isolates did not coincide with an apparent loss of activity toward the tested bee products. However, the present findings do not establish the mechanisms responsible for this observation.

The combination experiments provided a more nuanced perspective on the antibacterial potential of these products. The results showed that combining honey and propolis did not systematically enhance antibacterial activity relative to the individual products. Instead, the combinations produced isolate- and pathogen-dependent interaction profiles, with additive, favorable, or less favorable responses observed according to the specific product pair and bacterial isolate (Postali *et al.*, 2022; Sánchez-Martín *et al.*, 2025). In the inhibitory combinations, the propolis component generally remained at concentrations comparable to or higher than its individual MIC, whereas lower concentrations of honey were required in several combinations relative to H1 alone. Thus, the combination approach should not be interpreted as inherently superior to the use of individual products. Rather, individual products such as PE2 and H1 remain important on the basis of their intrinsic antibacterial activity, while PE1H1 and PE2H1 may provide complementary options in specific pathogen–product contexts where favorable interactions were observed. Overall, these findings support a pathogen-dependent interpretation of bee-product activity rather than a single universally optimal formulation.

The *in silico* investigation extended the experimental findings by providing a molecular perspective on selected bioactive constituents. Docking analyses against DNA gyrase and PBP2a identified several compounds with favorable predicted interactions within these bacterial targets, including constituents with docking scores comparable to or stronger than those of the reference compounds (Spencer and Panda, 2023; Shalaby *et al.*, 2020; Rajakumari *et al.*, 2024). These results provide hypotheses regarding possible molecular interactions that may contribute to the antibacterial activity observed experimentally, but they do not constitute experimental confirmation of target engagement or mechanism. Similarly, nine of the 28 compounds identified by LC-MS showed favorable predicted interactions within the Keap1 binding site, supporting the hypothesis that selected constituents may interact with the Keap1–Nrf2 pathway and potentially contribute to the antioxidant properties (Diniyah *et al.*, 2023; Culletta *et al.*, 2024; Crisman *et al.*, 2023). This antioxidant dimension is particularly relevant to mastitis, in which oxidative stress is involved in inflammatory and tissue-damaging processes (Turk, 2017).

ADME-Tox profiling provided an additional preliminary assessment of the predicted pharmacokinetic and toxicity characteristics of the identified constituents. Most compounds displayed favorable predicted profiles, while specific limitations were identified for compounds such as rutin, quercetin methyl ether, and oleanolic acid (Daina *et al.*, 2017; Bucao and Solidum, 2022). These predictions can assist in prioritizing compounds for subsequent investigation but cannot establish pharmacokinetic behavior or safety *in vivo*. Thus, the ADME-Tox results should be regarded as a prioritization tool rather than evidence of therapeutic suitability.

Taken together, the three analytical levels of this thesis-chemical characterization, *in vitro* biological evaluation, and *in silico* molecular investigation provide complementary perspectives on the biological potential of Algerian bee products. The chemical analyses established substantial compositional diversity and identified distinctive molecular signatures, the microbiological experiments demonstrated antibacterial activity against clinically relevant bovine mastitis isolates, and the computational analyses provided hypotheses regarding molecular interactions that may contribute to the observed activities. The integration of these approaches is particularly relevant for complex natural products, for which biological effects are unlikely to be explained adequately by a single global chemical parameter or isolated mechanism.

The present findings should be interpreted within several methodological and logistical limitations. First, the study was restricted to two propolis extracts and three honey samples collected during limited sampling periods and therefore cannot capture the full geographical, botanical, and seasonal diversity of Algerian bee products. Moreover, honey and propolis were collected independently according to their availability and accessibility rather than through a paired sampling design. This limited sampling scope also restricts broader conclusions regarding product standardization.

Second, melissopalynological profiling was constrained by the limited availability of comprehensive reference resources for Algerian pollen identification. Some pollen grains could therefore remain unidentified or be assigned only to higher taxonomic levels, limiting the precision of botanical attribution and the interpretation of specific floral contributions to honey composition and biological activity, particularly where medicinal or pharmacologically relevant plant species may contribute to the bioactive profile of the honey.

Third, antimicrobial assessment also presented some methodological constraints. CLSI breakpoints were unavailable for several antimicrobial-organism combinations, limiting direct clinical interpretation. For streptococcal isolates, the opacity of blood-supplemented media complicated MIC determination, the use of Todd-Hewitt broth provided a suitable alternative and allowed the assays to be completed, although it required an additional incubation period and increased experimental time.

Finally, some analyses were performed in different collaborating laboratories using different analytical platforms and procedures, limiting complete methodological harmonization across the study. The COVID-19 pandemic and limited financial resources also constrained laboratory access, sampling opportunities, and the number of complementary analyses that could be undertaken.

Overall, this thesis provides an integrated characterization of selected Algerian honey and propolis samples, and demonstrates their measurable antioxidant capacity, and antibacterial activity against clinical bovine mastitis isolates. The results do not establish a ready-to-use therapeutic formulation, nor do they demonstrate specific molecular mechanisms *in vivo*. Rather, they identify chemically distinctive bee products with reproducible experimental activity, reveal that product composition is an important determinant of biological performance, and show that honey-propolis combinations may produce context-dependent rather than universally enhanced effects. The molecular docking and ADME-Tox analyses further provide testable hypotheses and a basis for prioritizing selected constituents for future experimental investigation. In this context, the work establishes a foundation for subsequent

studies addressing formulation, standardization, pharmacokinetic and pharmacodynamic behavior, and ultimately *in vivo* efficacy and safety within a One Health framework.

General Conclusion

General Conclusion and Perspectives

Antimicrobial resistance (AMR) represents a major global health challenge, driven by the extensive use of antimicrobials in both human and animal sectors. Within the One Health framework, there is an increasing need for sustainable alternatives to conventional antimicrobial approaches. In this context, the present work provides an integrated evaluation of Algerian honey and propolis as potential natural approaches for the management of bovine mastitis, a disease that remains a major constraint to dairy productivity and an important contributor to antimicrobial use and resistance.

The study revealed substantial chemical and biological diversity among the investigated Algerian bee products. Distinct phytochemical profiles were identified according to product origin, while differences in antibacterial and antioxidant activities highlighted the importance of product-specific composition. Notably, the most active products were not consistently those with the highest total phenolic or flavonoid contents, emphasizing the relevance of specific bioactive constituents within these complex matrices.

The *in vitro* antibacterial investigation demonstrated that the tested honey and propolis samples inhibited the major bovine mastitis pathogens represented in the clinical isolate panel, including multidrug-resistant *E. coli* and linezolid-resistant *S. aureus*. The combination experiments did not demonstrate a universal advantage over individual products, supporting a pathogen-tailored strategy rather than a single optimal formulation.

The *in silico* analyses provided a mechanistic explanation for these observations, demonstrating strong binding affinities of multiple compounds toward DNA gyrase and PBP2a. Several compounds exhibited binding energies surpassing those of reference antibiotics, suggesting their ability to interfere with resistance-associated pathways. In parallel, the interaction of propolis-derived compounds with the Keap1–Nrf2 pathway highlights a host-directed mechanism, whereby modulation of oxidative stress may enhance the animal's resilience to infection. This dual antibacterial and antioxidant mode of action represents a significant therapeutic advantage in complex diseases such as mastitis.

ADME-Tox analysis further supports the translational potential of these compounds, with most exhibiting favorable safety profiles and acceptable pharmacokinetic properties. While certain molecules present limitations related to bioavailability or lipophilicity, these constraints are less critical for localized applications such as intramammary therapy, where systemic exposure is limited. This reinforces the feasibility of developing bee product-based formulations for veterinary use.

Future research should focus on *in vivo* validation and formulation development, including evaluation of efficacy, local tolerance, pharmacokinetic and pharmacodynamic behavior, and safety under relevant intramammary conditions. Expanding sampling across different regions, botanical origins, and seasons would improve the characterization of the chemical and biological diversity of Algerian honey and propolis. Further work should also address standardized, quality-controlled formulations and improve the clinical interpretation of antimicrobial susceptibility data where standardized breakpoints remain unavailable. Experimental investigation of selected compounds and molecular targets will be necessary to test the hypotheses generated by the docking and ADME-Tox analyses. Ultimately, controlled clinical studies in dairy cattle will be required to determine whether the antibacterial potential observed *in vitro* can translate into effective and safe veterinary applications.

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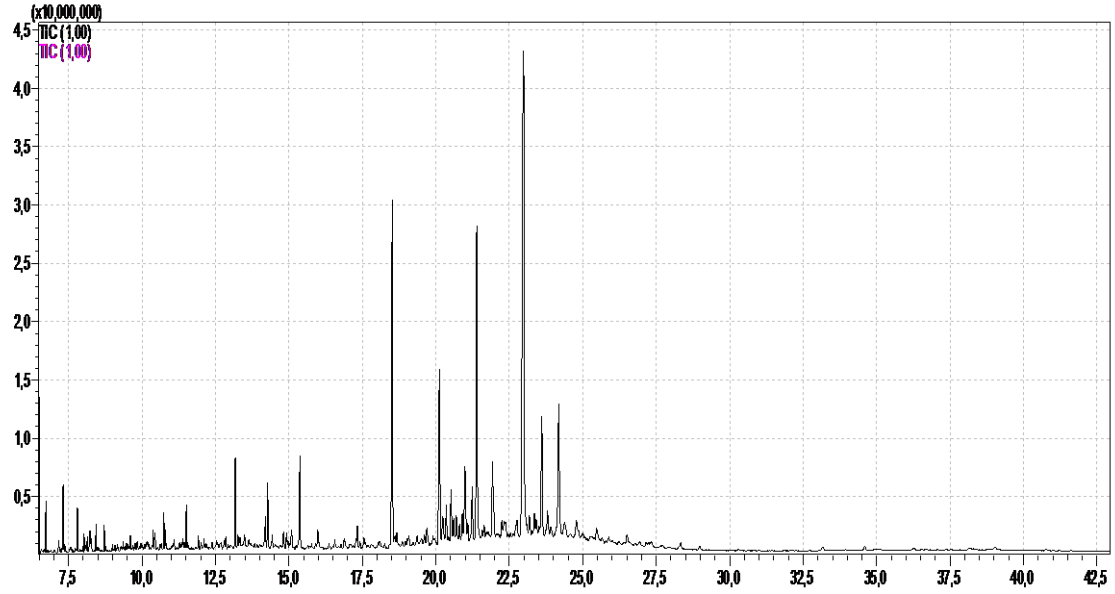
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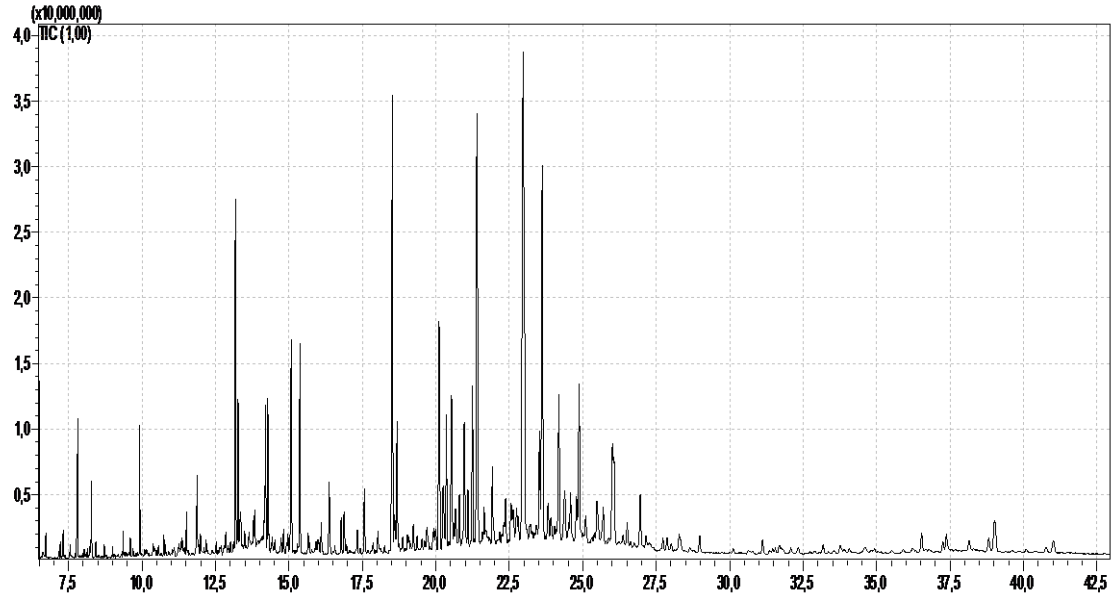
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Appendices

A.

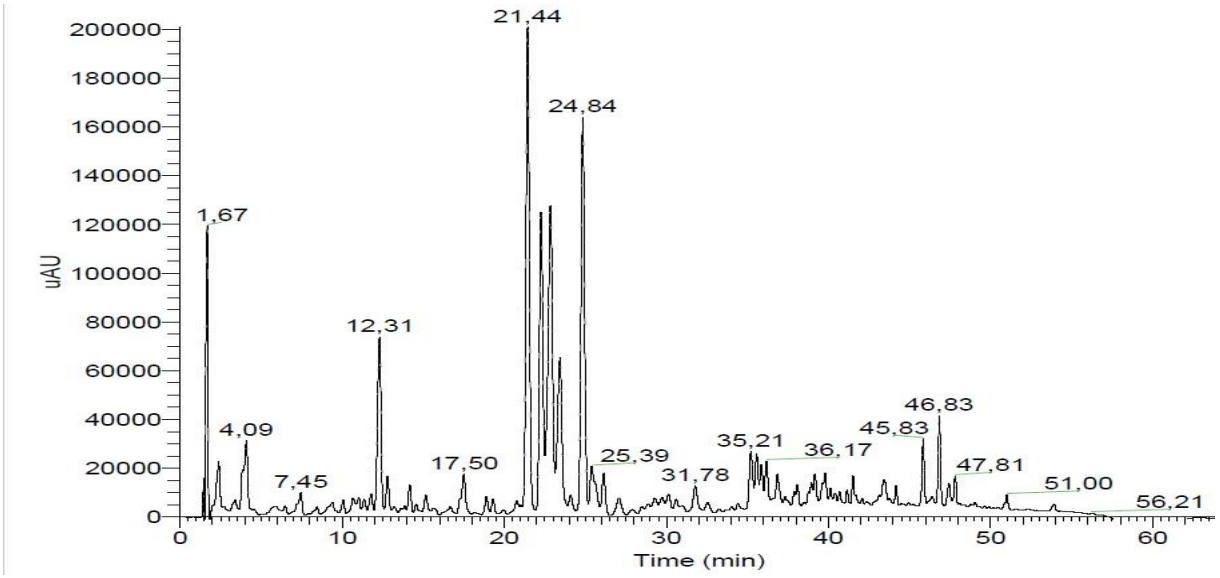


B.

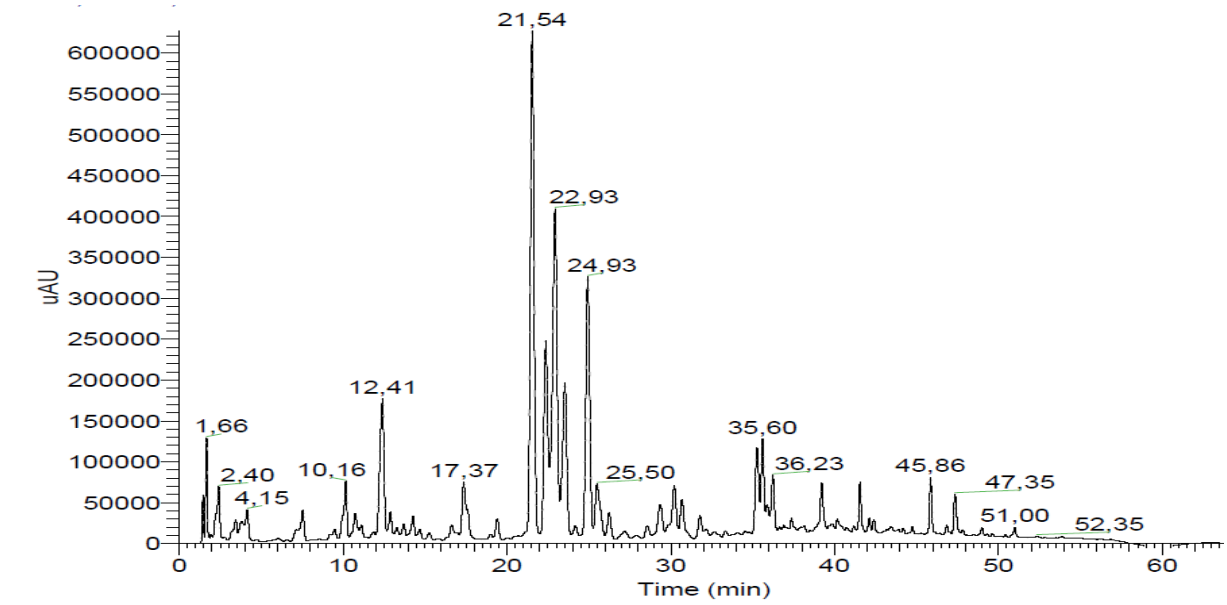


Appendix A. Total ion chromatograms (TIC) of Algerian propolis extracts: (A) PE1 and (B) PE2 (TIC vs. retention time, min).

C.

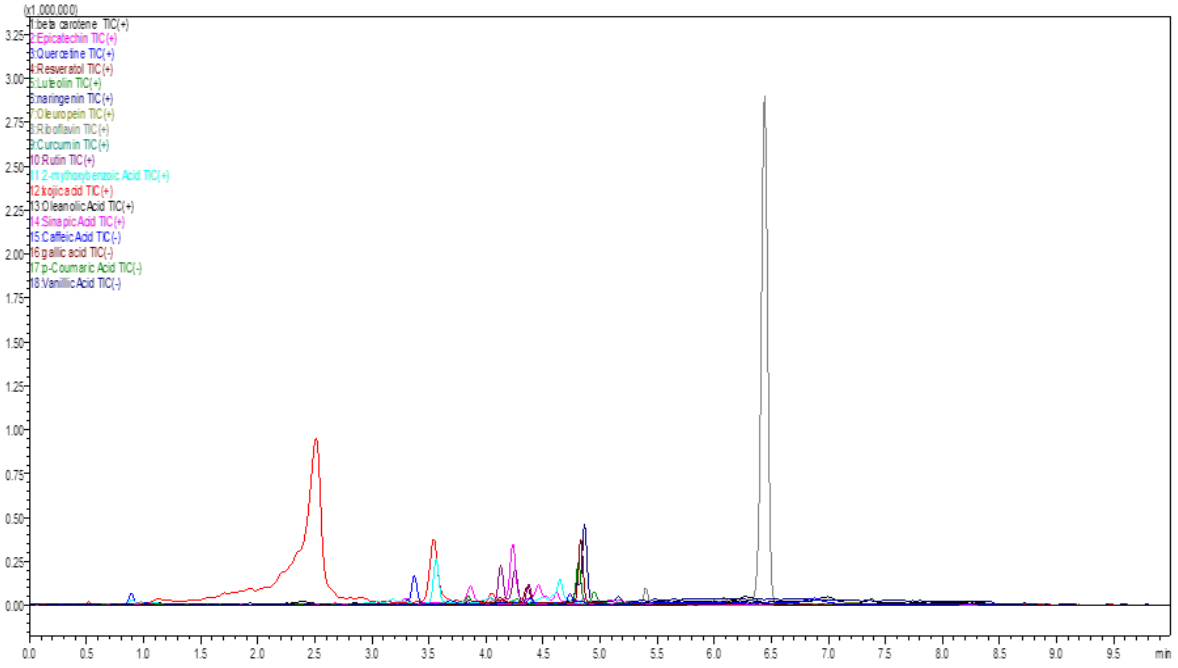


D.

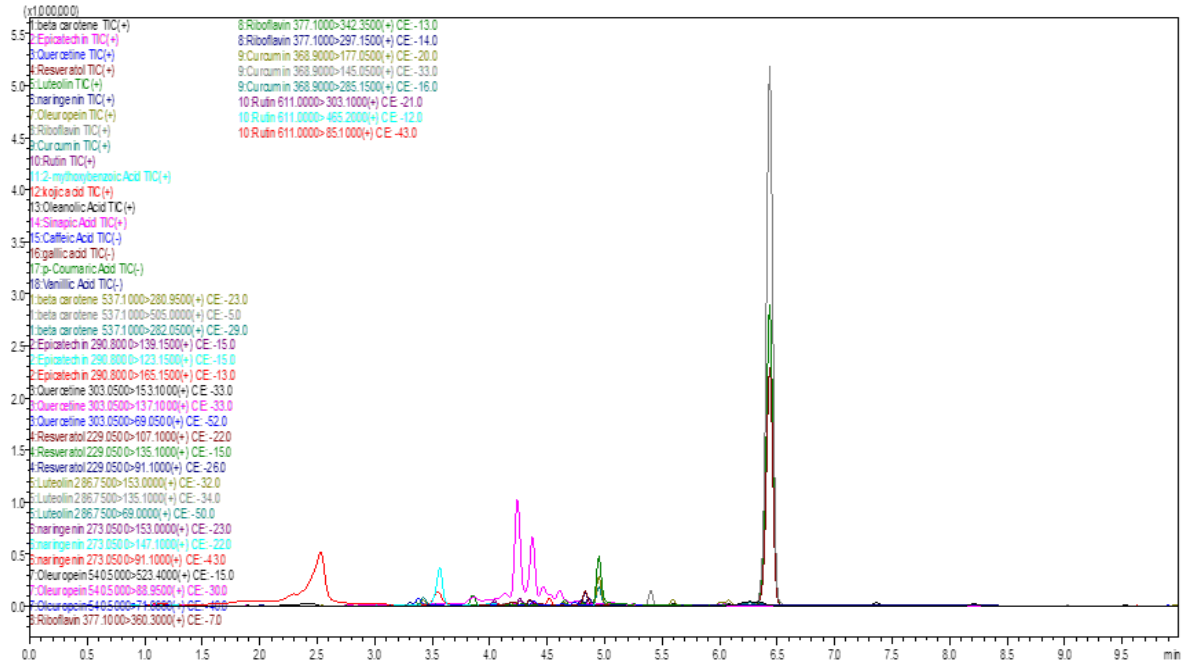


Appendix B. UHPLC-DAD-ESI/MS chromatograms of Algerian propolis extracts: (C) PE1 and (D) PE2.

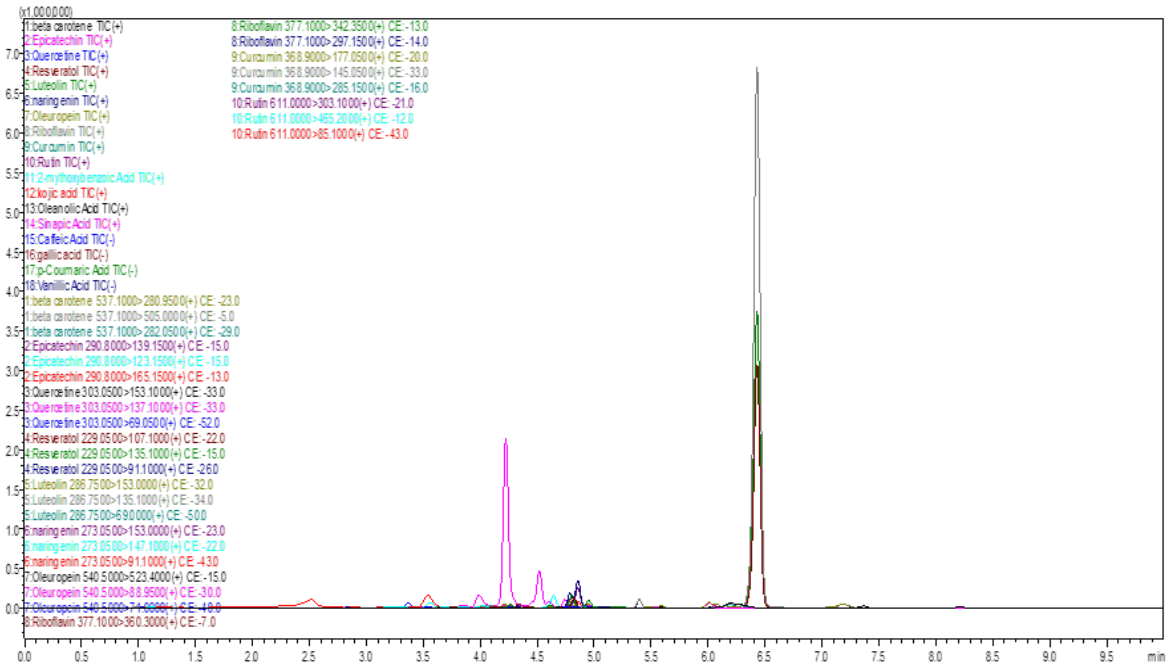
H1.


(x1,000,000)
1:beta carotene TIC(+)
2:Epicatechin TIC(+)
3:Quercetine TIC(+)
4:Resveratol TIC(+)
5:Luteolin TIC(+)
6:naringenin TIC(+)
7:Oleuropein TIC(+)
8:Riboflavin TIC(+)
9:Curcumin TIC(+)
10:Rutin TIC(+)
11:2-mythoxybenzoic Acid TIC(+)
12:kojic acid TIC(+)
13:Oleanolic Acid TIC(+)
14:Sinapic Acid TIC(+)
15:Caffeic Acid TIC(-)
16:gallic acid TIC(-)
17:p-Coumaric Acid TIC(-)
18:Vanillic Acid TIC(-)
min

H2.

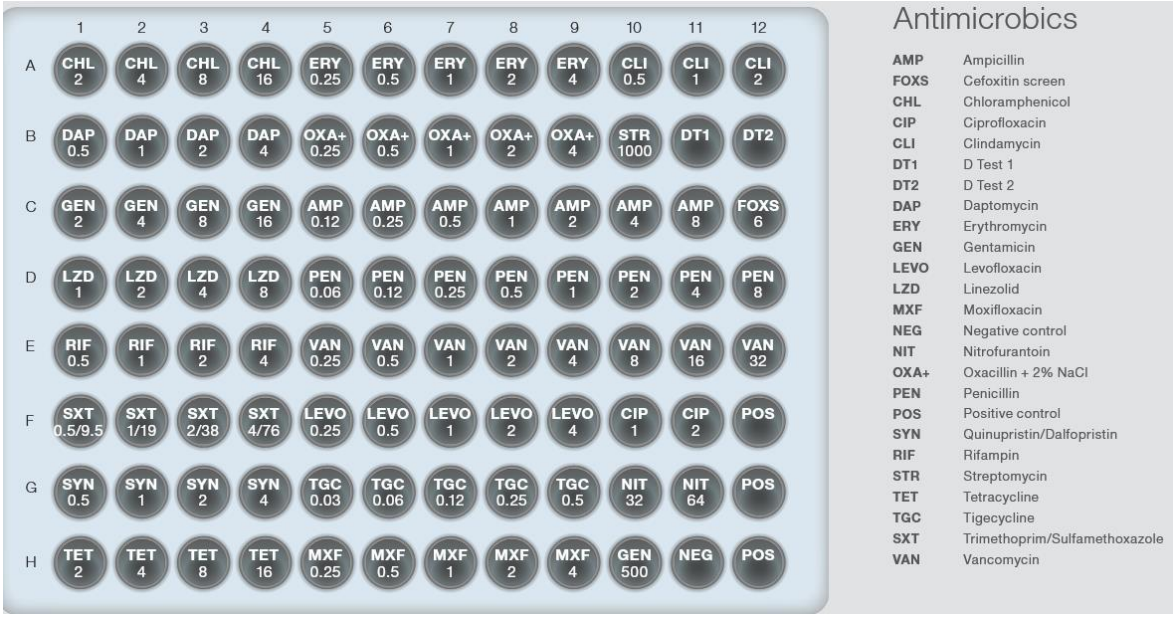

(x1,000,000)
1:beta carotene TIC(+)
2:Epicatechin TIC(+)
3:Quercetine TIC(+)
4:Resveratol TIC(+)
5:Luteolin TIC(+)
6:naringenin TIC(+)
7:Oleuropein TIC(+)
8:Riboflavin TIC(+)
9:Curcumin TIC(+)
10:Rutin TIC(+)
11:2-mythoxybenzoic Acid TIC(+)
12:kojic acid TIC(+)
13:Oleanolic Acid TIC(+)
14:Sinapic Acid TIC(+)
15:Caffeic Acid TIC(-)
16:gallic acid TIC(-)
17:p-Coumaric Acid TIC(-)
18:Vanillic Acid TIC(-)
8:Riboflavin 377.1000>342.3500(+) CE:-13.0
8:Riboflavin 377.1000>297.1500(+) CE:-14.0
9:Curcumin 368.9000>177.0500(+) CE:-20.0
9:Curcumin 368.9000>145.0500(+) CE:-33.0
9:Curcumin 368.9000>285.1500(+) CE:-16.0
10:Rutin 611.0000>303.1000(+) CE:-21.0
10:Rutin 611.0000>465.2000(+) CE:-12.0
10:Rutin 611.0000>85.1000(+) CE:-43.0
1:beta carotene 537.1000>280.9500(+) CE:-23.0
1:beta carotene 537.1000>505.0000(+) CE:-5.0
1:beta carotene 537.1000>282.0500(+) CE:-29.0
2:Epicatechin 290.8000>139.1500(+) CE:-15.0
2:Epicatechin 290.8000>123.1500(+) CE:-15.0
2:Epicatechin 290.8000>165.1500(+) CE:-13.0
3:Quercetine 303.0500>153.1000(+) CE:-33.0
3:Quercetine 303.0500>137.1000(+) CE:-33.0
3:Quercetine 303.0500>69.0500(+) CE:-52.0
4:Resveratol 229.0500>107.1000(+) CE:-22.0
4:Resveratol 229.0500>135.1000(+) CE:-15.0
4:Resveratol 229.0500>91.1000(+) CE:-26.0
5:Luteolin 286.7500>153.0000(+) CE:-32.0
5:Luteolin 286.7500>135.1000(+) CE:-34.0
5:Luteolin 286.7500>69.0000(+) CE:-50.0
6:naringenin 273.0500>153.0000(+) CE:-23.0
6:naringenin 273.0500>147.1000(+) CE:-22.0
6:naringenin 273.0500>91.1000(+) CE:-43.0
7:Oleuropein 540.5000>523.4000(+) CE:-15.0
7:Oleuropein 540.5000>88.9500(+) CE:-30.0
7:Oleuropein 540.5000>71.8000(+) CE:-46.0
8:Riboflavin 377.1000>360.3000(+) CE:-7.0
min

H3.

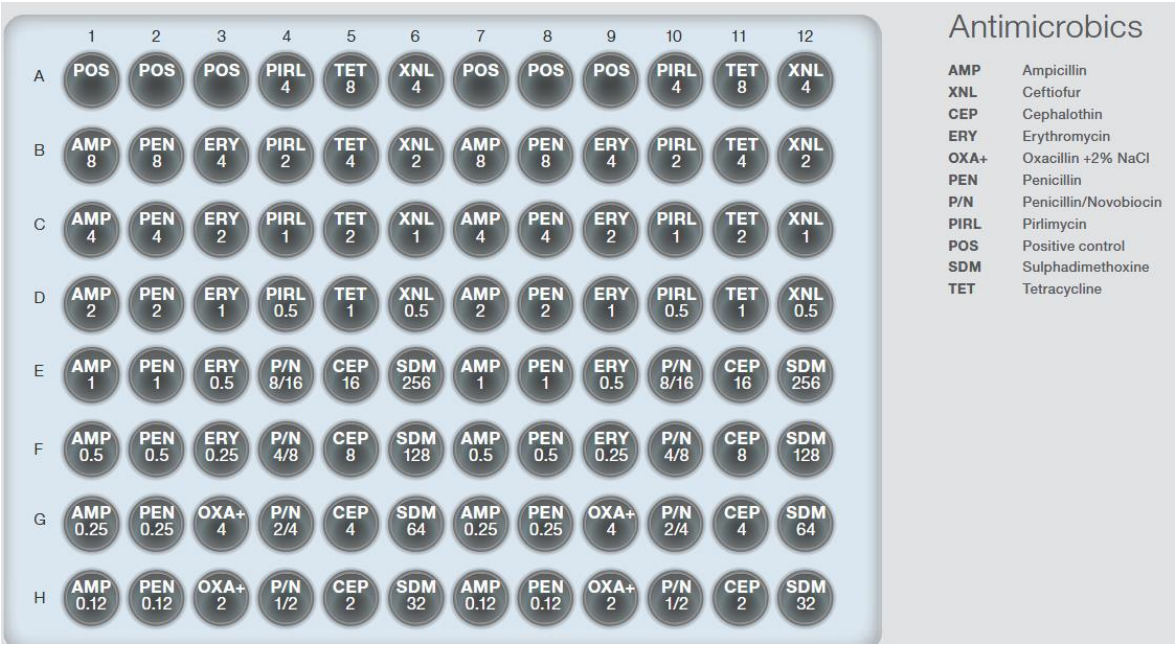


Appendix C. Total ion chromatograms (TIC) of Algerian honey samples (H1, H2, H3) obtained in negative and positive ionization modes by UPLC-ESI-MS/MS.

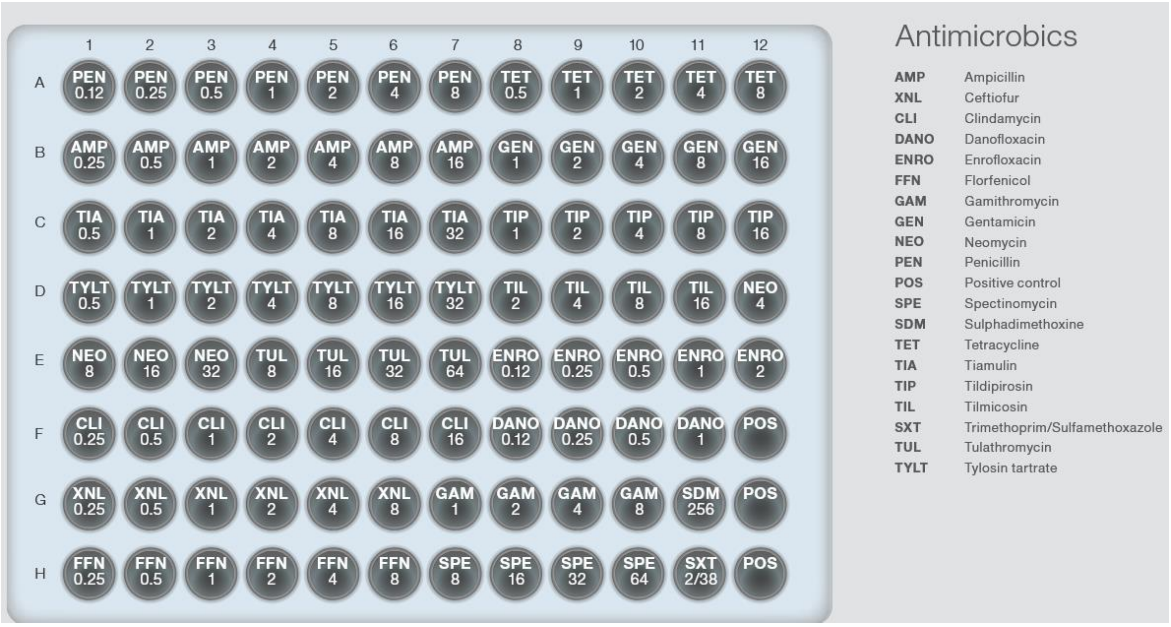
(A)



(B)



(C)



Appendix D. Sensititre™ plate layouts and antimicrobial concentration ranges: **(A)** GPALL1F (Gram-positive plate), **(B)** CMV1AMAF (Veterinary mastitis plate), and **(C)** BOPO7F (Bovine plate).

Appendix E. Binding interaction overview of selected ligands docked into the DNA gyrase binding site.

Ligand	Interaction	Residue	Distance (Å)
NAL	Hydrogen Bond	GLU33, GLY60	1.85–1.92
	Hydrophobic	ASN29, ILE61	3.19–3.76
	Salt bridges	ARG59, ARG100	3.94–4.73
QUE	Hydrogen Bond	ILE26, ASN29, SER30, GLU33, ASP56, ARG59, GLY60, ARG100, THR129	1.71–3.15
	Hydrophobic	ASN29, ILE61, PRO62, THR129, ILE131	3.46–3.98
RES	Hydrogen Bond	ILE26, ASN29, GLU33, ARG59, ILE131	1.85–2.28
	Hydrophobic	ILE26, ASN29, ILE61, THR129	3.05–3.63
RBF	Hydrogen Bond	ASN29, SER30, ASP32, ASP56, GLY60, GLY81, THR129	1.81–2.87
	Hydrophobic	ARG59, PRO62	3.35–3.69
	π-Cation	ARG59	4.26
PNA	Hydrogen Bond	SER30, GLU33, ASP56, GLY60, THR129	1.77–3.1
	Hydrophobic	ILE61, ILE77	3.17–3.92
	Salt Bridges	ARG59, ARG100	4.07–4.61
PNR	Hydrogen Bond	SER30, GLU33, ASP56, GLY60, THR129	1.78–2.78
	Hydrophobic	GLU33, ILE61, ILE77	3.20–3.97
	π-Cation	ARG59, ARG100	4.30–4.42
ISO	Hydrogen Bond	ILE26, ASN29, SER30, GLU33, ASP56, ARG59, GLY60, THR129	1.77–3.08
	Hydrophobic	ASN29, ILE61, ILE131	3.22–3.58
KAE	Hydrogen Bond	ILE26, ASN29, GLU33, ASP56, ARG59, GLY60, ARG100, THR129	1.74–3.17
	Hydrophobic	ASN29, ILE61, ILE131	3.22–3.58
PIN	Hydrogen Bond	SER30, GLU33, ASP56, GLY60, ARG100, THR129	1.81–2.98
	Hydrophobic	GLU33, ILE61, PRO62, ILE77	3.33–3.99

NAL: *nalidixic acid;* ***QUE:*** *quercetin;* ***RES:*** *resveratrol;* ***RBF:*** *riboflavin;* ***PNA:*** *pinobanksin-3-O-acetate;* ***PNR:*** *pinobanksin-3-O-propionate;* ***ISO:*** *isorhamnetin;* ***KAE:*** *kaempferol;* ***PIN:*** *pinobanksin.*

Appendix F. Binding interaction overview of selected ligands docked into the PBP2a binding site.

Ligand	Interaction	Residue	Distance (Å)
PEN	Hydrogen Bond	SER348, THR535, THR537	1.97–3.00
	Hydrophobic	THR537	3.63
	Salt Bridges	LYS534	2.65
OLA	Hydrogen Bond	SER348, THR535, THR537, ASN539	1.95–3.24
	Hydrophobic	THR537, ASN539, TYR572	3.34–3.91
	Salt Bridges	LYS534	3.63
CAR	Hydrophobic	THR523, THR535, THR537, ARG569, TYR572, ILE575	2.96–3.93
RUT	Hydrogen Bond	SER348, SER384, ASN403, LEU440, THR535, THR537, ASN539, GLY568, TYR572, GLY574	1.74–3.11
	Hydrophobic	THR537, ALA541, TYR572	3.35–3.84
QUE	Hydrogen Bond	SER348, ASN403, ASN539, ASP540, ARG569	1.68–3.09
	Hydrophobic	THR537, TYR572	3.55
ISO	Hydrogen Bond	SER348, ASN403, ASN539, ASP540, ARG569	1.71–2.33
	Hydrophobic	THR537, TYR572	3.49–3.53
QME	Hydrogen Bond	SER348, ASN403, ASN539, ASP540, ARG569, GLU571	1.67–3.01
	Hydrophobic	THR537, TYR572	3.27–3.41
PNA	Hydrogen Bond	SER348, SER384, SER401, THR535, THR537	1.79–2.46
	Hydrophobic	LEU400, TYR572	3.49–3.54
	π-Stacking	TYR399	4.71

PEN: *penicillin;* ***OLA:*** *oleanolic acid;* ***CAR:*** *β-carotene;* ***RUT:*** *rutin;* ***QUE:*** *quercetin;* ***ISO:*** *isorhamnetin;* ***QME:*** *quercetin-3-methyl-ether;* ***PNA:*** *pinobanksin-3-O-acetat*

Research Output

Research Article

Comparative Molecular Profiling and Bioactivity Analysis of Algerian Propolis: Antioxidant, Antibacterial Activities, and *In Silico* NRF2-KEAP1 Pathway Modulation. Current Issues in Molecular Biology, 2025, https://doi.org/10.3390/cimb47090761

Oral Communications

2nd National Seminar on Ethnobotany and Valorization of Natural Substances.

1st Virtual National Conference on Animal Production.

1st National Seminar on Bees and Bee Products.