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Evaluation of the effectiveness of Algerian propolis in the treatment of urinary tract infections

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“In loving memories spent during this work”

DEDICATIONS

This valuable work is dedicated

To my **loving parents**; whose unending love was priceless. When I read Charles Dickens' words, "*Reflect upon your present blessings,*" you immediately come across my mind.

To you **Mom**, for your support, your advice, your faith, and your love.

To you **Dad**, for your tenderness, your sacrifices, your encouragement, and your generosity.

To you, **Boubakeur**, you have been wonderfully thoughtful, supportive, caring, and unconditionally loving

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To my brother-in-law, **Badr-Eddine**

To my adorable nieces and nephews: **Jawed**, **Douaa, Baraa, Aissa, Moustafa, Assinat, Touwaiba, Rahil, and Adem**

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To the strongest person I know, **me**

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$A_{0.5}$: Concentration in which the absorbance is 0.50
ABTS: 2,2′-azino-bis (3- ethylbenzothiazoline-6-sulfonate)
$AlCl_3$: Aluminum chloride
ATCC: American type culture collection
BHA: Butylated hydroxyanisole
BHT: Butylated Hydroxytoluene
C_6HSL: N-hexanoyl-L-Homoserine lactone
CFU: Colony-forming unit
CUPRAC: Cupric Reducing Antioxidant Capacity.
***CV12472*:** *Chromobacterium violaceum (*CV12472*)*
CV026: Chromobacterium violaceum (CV026)
DMSO: Dimethylsulfoxide.
DPPH: 1,1-Diphenyl-2-PicrylHydrazyl.
EUCAST: The European Committee on Antimicrobial Susceptibility Testing
FDA: Food and Drug Administration
FCR: The Folin-Ciocalteu Reagent
GAE: Equivalent of Gallic acid
HBP: High Biofilm Producer
HPLC-DAD: High-performance liquid chromatography- Diode Array Detector
IC_{50}: Inhibition concentration of 50%
MARI: Multiple-antibiotic Resistance Index
MDR: Multi-drug Resistant
MHB: Muller-Hinton Broth
MIC: Minimum Inhibitor Concentration
MBC: Minimum Bactericidal Concentration
NCCLS: National Committee for Clinical Laboratory Standard
OD: Optical Density
OS: Oxidative Stress
QE: Equivalent of quercetin
QS: Quorum-Sensing
R: Resistant.
Rt: Retention time
rUTI: Recurrent urinary Tract Infections
S: Susceptible
UTI: Urinary Tract Infections
UV-Vis: Ultraviolet/Visible

ABSTRACT

This study sought to investigate the pharmaceutical efficiency of propolis collected from different Algerian eastern localities against multi-drug resistant (MDR) uropathogens isolated from womens suffering from recurrent urinary tract infections (rUTI). In this regard, propolis was examined for its capacity to inhibit several uro-virulence factors of MDR uropathogens such as bacterial resistance, urease, biofilm, and quorum sensing (QS). Furthermore, antioxidant properties were discovered to investigate the possible limitation of free radicals' harmful effects, as well as chemical profile investigations to determine the primary components responsible for the latter activities. The results of the chemical content of propolis extracts revealed variable polyphenolic content, the quantification of total phenolic content ranged from 111.54±4.49 to 1117.23 ± 2.03 µg GAE/mg E, while total flavonoid content was within the range of 96.45±1.76 to 321.31±4.86 (µg QE/mg E). The phenolic profile using HPLC-DAD analyses revealed the presence of 19 different compounds among the different studied extracts caffeic acid was the highly detected compound in PTBL extract with a value of 20mg/ml. Interestingly, cynarin was detected in all extracts, this compound was not previously identified in Algerian propolis. Furthermore, all the tested extracts displayed potential anti-radical activity in the different antioxidant assays, the strongest antioxidant activity was recorded for the PTBL extract showing the highest scavenging within all the employed approaches. The antibacterial activity of propolis against MDR uropathogens showed varying inhibition potency and the highest activity was recorded against Gram-positive bacteria specifically MDR *S. aureus* strains. According to the micro-dilution results, the sensitivity of bacteria to propolis varied among the tested strains and the propolis extracts used, the MIC values ranged between 0.625 to 20 mg/ml. Whereas the MBC values ranged from 2.5 to 20 mg/ml. The screening of the antibiofilm activity against high biofilm producers showed a positive capacity in eradicating biofilm formation of different HBP. The highest activity was recorded by PSH extract against *S. aureus* $_{(MDR3)}$, and biofilm eradication was up to 70%. On the other hand, the anti-QS activity showed that all propolis extracts demonstrated a substantial decrease in violacein production using the model strain *Chromobacteriem violaceum 12475*. PSH, PTBL, PTAK, and PACH extracts showed the highest activity with complete violacein inhibition at MIC (100%). Furthermore, the inhibition of QS using CV026 showed moderate to no capacity to eliminate QS activity. Moreover, propolis extracts were studied for their enzyme inhibitory effect against urease. All extracts were found not active except for PACH and PTBL extracts. The highest activity was recorded within the PACH extract with an IC_{50} of 1.08±0.37µg/ml which was more strongly active compared to the employed standards Thiourea (11.57±0.68µg/ml). The finding of the anti-inflammatory activity showed that different extracts exerted a potent preventive effect on thermally-albumin denaturation in a dose-dependent manner. The cytotoxic effect against brine shrimp larvae demonstrated that propolis extracts showed distinctive toxicity (moderate to high toxicity).

Overall, these findings indicate that propolis is a suitable agent that is capable of countering MDR uropathogens. Therefore, it can be used as a promising therapeutic agent to prevent recurrent UTIs. With that being said, we believe that the supplementation of propolis as a therapeutical agent is mandatory in rUTI patients.

***Keywords*:** Propolis, HPLC-DAD, Multi-drug resistant, Urinary tract infection, Antibiofilm, Anti-quorum sensing, Antioxidant.

RESUME

Cette étude visait à étudier l'efficacité pharmaceutique de la propolis algérienne collectée à partir de différentes localités de l'Est algérienne contre des uropathogènes multi-résistants isolés des femmes atteintes d'infections urinaires récidivantes. À cet égard, la propolis a été examinée pour sa capacité à inhiber plusieurs facteurs d'uro-virulence adoptés par des uropathogènes multi-résistants tels que la résistance bactérienne, l'uréase, formation de biofilm et le quorum sensing (QS). En outre, les propriétés antioxydants de la propolis étudiés ont été réalisés pour but d'éleminer l'effets negative des radicaux libres, en plus le profile chimique a été analysé pour déterminer les principaux composants active responsables d'activité précédentes. Les résultats de la teneur chimique des extraits de propolis ont révélé une teneur polyphénolique variable, la quantification de la teneur phénolique totale variait de 111.54 ± 4.49 à 1117.23 ± 2.03 µg GAE/mg E, tandis que la teneur totale en flavonoïdes était comprise entre 96.45 ± 1.76-321.31 ± 4.86 (µg QE/mg E). L'analyse chromatographique par HPLC-DAD a permis d'identifier 19 composés dans les différents extraits étudiés. L'acide caféique était le composé phénolique hautement détecté dans l'extrait de PTBL avec une valeur de 20 mg/ml. Le cynarin a été detecté dans tous les extraits, ce composé a été détecté pour la premier fois dans la propolis algerienne. De plus, tous les extraits testés ont présenté une activité antiradicalaire potentielle dans tous les tests antioxydants employés, cependant, l'activité antioxydante la plus forte a été révélée pour l'extrait PTBL présentent une activité chélatrice la plus élevée dans toutes les approches utilisées. L'étude de l'activité antimicrobienne vis-à-vis des bactérie urinaires multi-résistantes a montrés que les extraits de propolis possèdent un pouvoir d'inhibition variable avec une forte capacité d'inhibition contre les Gram-positive, en particulier *S. aureus* multi-résistantes. Les résultats de microdilution ont montré que la sensibilité des bactéries à la propolis variait selon la propolis et les souches testées. Les valeurs de CMI variaient généralement entre 0.625 et 20 mg/ml. Alors que les valeurs de MBC variaient de 2.5 à 20 mg/ml. De plus, l'étude de l'activité antibiofilm a montré que la propolis possède une capacité inhibitrice importante sur la prévention de formation de biofilm chez les uropathogens multi-résistantes fortement productrice de biofilm. L'extrait PSH présentait le pourcentage d'inhibition le plus élevé sur *S. aureus* $_{(MDR3)}$, et l'éradication du biofilm a atteint 70 %. D'autre part, l'activité anti-QS a montré que tous les extraits de propolis démontraient une diminution substantielle de la production de violacéine chez *Chromobacteriem violaceum 12475*. Les extraits PSH, PTBL, PTAK, et PACH présentaient l'activité la plus élevée avec une inhibition complète de la violacéine (100 %). De plus, l'inhibition de la communication microbienne (QS) à l'aide du CV026 a montré une capacité modérée, voire nulle. Tous les extraits se sont révélés inactifs contre l'urease, à l'exception des extraits PACH et PTBL. L'activité la plus élevée a été détecté par l'extrait de PACH avec une IC_{50} de 1.08 ± 0.37 µg/ml qui était plus fortement active par rapport aux standards utilisés Thiouré (11.57 ± 0.68 µg/ml). La détermination de l'activité anti-inflammatoire a montré que les différents extraits exerçaient un puissant effet préventif sur la dénaturation thermique de l'albumine. De plus, l'effet cytotoxique contre les larves d'artémias a démontré que les extraits de propolis présentaient une toxicité distinctive. Ces résultats indiquent que la propolis est un agent approprié, capable de lutter contre les uropathogènes multi-résistantes. Par conséquent, il peut être utilisé comme agent thérapeutique prometteur pour prévenir les infections urinaires récidivantes. En plus, il peut être une alternative dans le traitement de nombreuses maladies provoqué par les radicaux libres.

***Mots clés*:** Propolis, HPLC-DAD, Multi-resistantes, Infections urinaires, Antibiofilm, Anti-quorum sensing, Antioxidant.

الملخص

تهدف هذه الدراسة إلى التحقق من الفعالية الفرماكولوجية للعكبر الذي تم جمعه من مختلف المناطق الشرقية الجزائرية ضد البكتيريا المقاومة للعديد من المضادات الحيوية، المسببة لالتهاب المسالك البولية و المعزولة من النساء المصابات بالتهابات المسالك البولية المتكررة. في هذا الصدد، تم فحص فعالية العكبر على تثبيط العديد من عوامل الفوعة للبكتيريا المقاومة للعديد من المضادات الحيوية والمسببة لالتهاب المسالك البولية مثل: المقاومة، اليوريز، الأغشية الحيوية ، واستشعار النصاب. علاوة على ذلك، تم اكتشاف الخصائص المضادة للاكسدة للعكبر التي من المحتمل انها تساهم في الحد من التأثيرات الضارة للجذور الحرة. بالاضافة الى ذلك تم تحديد التركيب الكيميائي لمستخلصات العكبر بهدف تحديد المكونات الأساسية المسؤولة عن الأنشطة الأخيرة. كشفت نتائج المستويات الفينولية لمستخلصات العكبر تباين في المحتوى الكلي للفينولات، حيث تراوحت كمية المحتوى الفينولي الإجمالي من 111.54 ± 4.49 إلى 1117.23± 2.03 ميكروغرام مكافئ الغاليك/ملغ من المستخلص ، بينما كان إجمالي محتوى الفلافونويد ضمن النطاق 96.45 ± 1.76-321.31 ±2.86 ميكروغرام مكافئ الكرسيتين/ملغ من المستخلص. بينت نتائج تحليل المحتوى الفينولي باستخدام الكروماتوغرافيا السائلة ذات المردود العالي وجود 19 مركبًا مختلفًا بين العينات المدروسة حيث كان حمض الكافيين هو المركب الفينولي الرئيسي في عينة PTBL. بشكل مثير للانتباه تم اكتشاف مركب السينارين قي كل العينات تعتبر هذه الدراسة الاولى التي تكشف عن السينارين كاحد مكونات العكبر الجزائري. علاوة على ذلك، أظهرت جميع المستخلصات المختبرة نشاطًا قويا مضادًا للاكسدة في مختلف فحوصات مضادات الأكسدة. اظهر مستخلص PTBL أقوى نشاط مضاد للأكسدة في جميع أساليب فحوصات مضادات الأكسدة المستخدمة. أظهر النشاط المضاد للبكتيريا المقاومة للعديد من المضادات الحيوية و المسببة لالتهاب المسالك البولية قوة تثبيط متفاوتة وتم تسجيل أعلى نشاط ضد البكتيريا إيجابية الجرام وتحديدًا سلالات المكورات العنقودية الذهبية المقاومة للعديد من للأدوية. وفقا لنتائج التخفيف الجزئي، تباينت حساسية البكتيريا للعكبر بين السلالات المختبرة ومستخلصات العكبر المستخدمة،حيث تراوحت قيم MIC بين 0.625 الي 20 مغ/م, بينما تراوحت قيم MBC

من 2.5 إلى 20 ملغم/مل. أظهر فحص النشاط المضاد لتكوين البيوفيلم (الأغشية الحيوية) قدرة إيجابية في القضاء على التكوين العالي للأغشية الحيوي. تم تسجيل أعلى نشاط لمستخلص PSH ضد بكتيريا *S.aureus* (MDR3) ، وكانت نسبة استئصال الأغشية الحيوية تتعدى 70%. من ناحية أخرى، أظهر النشاط المضاد لـ QS أن جميع مستخلصات العكبر أظهرت انخفاضًا كبيرًا في إنتاج الفيولاسين عند *Chromobacteriem violaceum 12475*. *اضهرت مستخلصات العكبرPACH،PTAK ، PSH* و *PACH* قدرة عالية على التثبيط الكلي لانتاج الفيولاسين (100%). بالاضافة, كشف تثبيط استشعار النصاب باستخدام CV026 قدرة متوسطة الى معدومة. كذلك تم دراسة قدرة العكبر على تثبيط انزيم اليوريازحيث بينت النتائج ان كل العينات غير فعالة باستثناء العينتين PTBL و PACH حيث اظهرت العينة PACH اعلى نشاط بتركيز مثبط IC_{50}= 1.08±0.37 ميكروجرام / مل والذي اظهر فعالية اقوى من المعيار المستخدم الثيوريا (11.57 ± 0.68 ميكروجرام/مل). أظهر النشاط المضاد للالتهابات أن المستخلصات المختلفة لها تأثير وقائي قوي على تمسخ الألبومين حرارياً بطريقة تعتمد على التراكيز المختلفة للعكبر. علاوة على ذلك، أظهر التأثير السام للخلايا ضد يرقات الأرتيميا أن مستخلصات العكبر أظهرت سمية مختلفة (متوسطة إلى عالية السمية) . بشكل عام، تشير هذه النتائج إلى أن البروبوليس هو عامل مناسب قادر على مكافحة مسببات التهابات المسالك البولية المقاومة للأدوية المتعددة. ولذلك، يمكن استخدامه كعامل علاجي واعد لمنع عدوى المسالك البولية المتكررة. ومع ذلك، نعتقد أن مكملات البروبوليس كعامل علاجي إلزامية لدى مرضى التهاب المسالك البولية.

الكلمات المفتاحية: العكبر، HPLC-DAD، مقاومة للعديد من المضادات الحيوية ، التهابات المسالك البولية، مضاد للاغشية الحيوية، مضاد اشعارالنصاب، مضاد للاكسدة.

Introduction

From time immemorial, man has been curious and fascinated by the natural world around him, and has harnessed all of these forces to benefit from the environment, focusing on the search for natural resources that can serve as therapeutic agents.

The pharmaceutical industry has always considered natural products as a valuable source of bioactive molecules since many of the drugs available for the treatment of human diseases are extracted from plants, animals, bacteria, and fungi. About 75% of anti-infective substances are extracted from natural products (Kolayli & Keskin, 2020). Between 1981 and 2021, over 1310 drugs have been approved by the FDA for the treatment of several diseases (Rodriguez-Monguio *et al.*, 2023). Despite the fact that many countries have approved synthetic antimicrobial drugs, many researchers are interested in the use of natural products obtained from various creatures such as microbes, plants, and animals, these latter remain significant sources of innovation (Qadri *et al*., 2022).

The relationship between humans and bees dates back to prehistoric times and predates the industrial revolution (Abutaha, 2020). Over time, humans have successfully exploited the products of the beehive, proving that these natural products can contribute to the enhancement of the quality of human life, hence the importance of beehive products in the development of natural medicine (Cortés *et al*., 2011). Some products of this trend are used frequently in everyday life, especially in food such as honey, in medicine, and in pharmaceuticals (royal jelly, propolis, pollen, beeswax, and bee venom), which help to supplement the conventional treatment of certain pathologies (Alvarez-Suarez *et al*., 2010; Sadhana *et al*., 2017). Among these products, propolis is a natural remedy that has been used since ancient times. The Egyptians benefited from its anti-putrefactive properties to embalm their dead, and its usefulness as an antiseptic and healing agent was also recognized by Greek and Roman physicians (Rojczyk *et al*., 2020). Furthermore, interest in this product has increased significantly due to its chemical composition and several pharmacological effects: antibacterial, anti-inflammatory, anticancer, and antiviral (Karagecili *et al*., 2023).

Infectious diseases are undoubtedly the world's greatest health threat. After respiratory infections, the second most common infectious disease is urinary tract infections. It is more prevalent in women than in men, with almost 40–50% of women experiencing at least one episode of UTI in their lives (Lila *et al*., 2023). Generally, UTI occurs when bacteria attack and persist in a part of the urinary tract, causing vascular damage to the urinary bladder (Al-Badr & Al-Shaikh, 2013). Furthermore, among the concerning factors that intensify the

severity of UTIs and contribute to recurrence and drug failure is the emergence of MDR uropathogens (Khoshnood *et al*., 2017). Moreover, the ability of bacteria to both establish and sustain infections is directly related to several virulence factors such as biofilm formation, quorum sensing, urease …etc. On the other hand, oxidative stress (OS) is another situation found to be involved in the etiology of UTI. Several lines of evidence point out that free radicals increase lipid peroxidation, hence contributing to the persistence of UTI (Grant & Hung, 2013; Kurutas *et al*., 2005; M. D. Liu *et al*., 2022).

Besides that, the inflammation response is one of the body's defense strategies against external and internal attacks, and it results in the onset of several pathological diseases. Given that, one of the hallmarks of inflammation is protein denaturation. Most denaturized proteins lose their biological roles, resulting in the generation of autoantigens and the induction of many autoimmune dysfunctions. Indeed, tissue protein denaturation is a well-known cause of inflammation and several illnesses (Mouffouk *et al*., 2018).

In recent years, the emergence of multi-drug resistant uropathogens has increased significantly, endangering the potency of available treatments. It is undeniable that antibiotics have been a successful therapy option in modern medicine. They offer protection against a variety of infections by eliminating bacteria and preventing their growth. However, there are numerous cases where antibiotics show many side effects (Kaushik *et al*., 2018; Shekhar & Petersen, 2020). Antibiotic misuse and overuse increase the prevalence of antibiotic resistance in the community resulting in an increasing proportion of multidrug-resistant bacteria that are untreatable and eventually resulting in a blunting of our antimicrobial arsenal. Moreover, the indiscriminate use of antibiotics might potentially harm the body instead of healing it (Serwecińska, 2020). Therefore, many world scientists search for solutions to mitigate these issues by focusing on the discovery of new bioactive molecules through the use of natural products such as propolis, which has already well-known pharmaceutical properties.

The first part of this manuscript focuses on two chapters that present bibliographical knowledge. In the first chapter, we focus on a global and common problem which is urinary tract infection in women. Our research is centered on the epidemiological and microbiological characteristics of this disease and the emergence of antibiotic resistance of bacteria responsible for urinary tract infections, and more precisely the related virulence factors including bacterial resistance, biofilm, quorum sensing, urease…etc.

In the second chapter, we discuss initially a general overview of the golden beehive product “Propolis”, then a more detailed study reveals its chemical composition, specifically the physicochemical aspect in connection with its botanical origin and its biological activities. The second part describes the methodological approaches by addressing the quantitative and qualitative analyses of the extracts of our propolis samples and the evaluation of their biological activities. In the last part, we report and discuss our results. We concluded this study with perspectives for future research.

Chapter I

Urinary tract infections

1 The urinary system in women

1.1 Description

The urinary system, also known as the urinary tract is one of the excretory biological systems, with the basic role of protecting the vital state of the human body. The urinary system's crucial functions include toxic waste elimination, blood volume and pressure regulation, electrolyte and metabolite control, and blood pH regulation. This natural body's filtration system ensures the collection, transport, storage, and expel urine periodically and in a highly coordinated fashion. It is comprised of multiple anatomical structures (Robertson, 2001; Naber & Purohit, 2021).

The urinary tract is divided into two parts: the upper and lower urinary tracts. The upper urinary tract includes the kidneys and ureters, while the lower urinary tract consists of the urinary bladder and urethra (Naber & Purohit, 2021).

1.1.1 The upper urinary-collecting system

1.1.1.1 Kidney

The kidneys are considered the first gross structure of the upper urinary collecting system. This organ takes a bean-shaped structure located on the back of the abdominal wall behind the peritoneum, and it is held in place by a connective tissue, called renal fascia. the human adult kidney is approximately 3 cm thick and about 12 cm in length. The kidney structure includes the peripheral cortex and the central medulla. This functional organ performs crucial and mandatory functions for the human body, by removing wastes such as urea, salts, and excess water, and excreting them in the form of urine. Generally, the filtration process is achieved through tiny filtering units called nephrons. This unit consists mainly of small blood capillaries (glomerulus) and a small tube called a renal tubule (Moinuddin & Dhanda, 2015).

1.1.2 The lower urinary-collecting system

1.1.2.1 Bladder

The bladder is an active, hollow vital organ, triangle-shaped, responsible for temporary urine storage that is conveyed to it continuously by the ureters. In females, the bladder is situated directly in front of the genital tract (vagina) and below the uterus. To store urine, the bladder's walls relax and expand, and then contract and flatten for urination through the urethra (de Groat & Yoshimura, 2015).

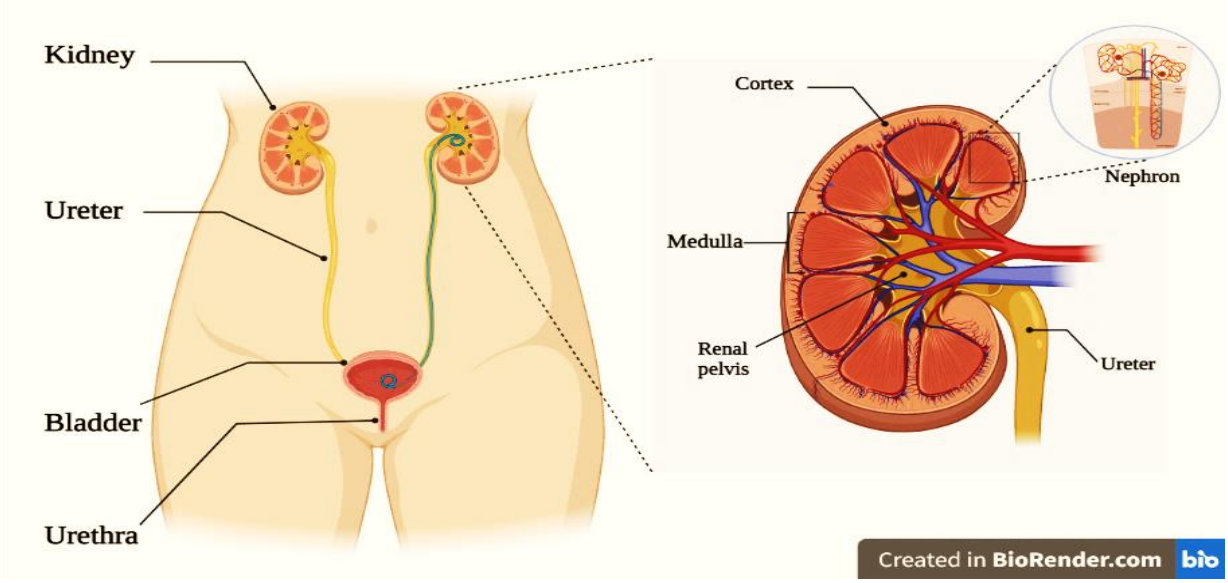


Figure 1. Normal anatomy of the urinary tract and kidney in the female Human Body. (Created in BioRender.com, with permission of the publisher).

1.1.2.2 The female urethra

The Urethra is a thin-walled tube that transports urine from the bladder to the outside, it is considered as the final passageway for urine flow. In females, the urethra is narrow and about 3-4 cm long. It extends from the bladder neck to the external urethral orifice in the vaginal vestibule (Mahadevan, 2019).

2 Urinary tract infections (UTI)

2.1 Epidemiology among women

Nowadays, health problems are considered one of the most existential threats that can affect humanity. Among these global health issues, we may coin the epidemiology of urinary tract infection (UTI), which can be regarded as the most common and widely spread infectious disease. Furthermore, it is one of the most formidable challenges in clinical practice given its high prevalence, frequent recurrence, myriad associated morbidities, and rapidly evolving antimicrobial resistance. Women are extremely targeted by such diseases. Consequently, a third of women are diagnosed with a UTI before the age of 24 years and half develop at least one episode by 35 years of age. Up to 70% of women experience a UTI at least once during their lifetime, and of those, 30% will have recurrent UTIs (rUTIs) (Tan & Chlebicki, 2016; Medina & Castillo-Pino, 2019).

2.2 Accounts on UTI

UTI is an inflammatory response at the level of the urothelium to fight a bacterial infection. It is almost always associated with bacteriuria and pyuria. Generally, UTI occurs

when bacteria attack and persist in a part of the urinary tract, causing vascular dysfunction and damaging the urinary system (Al-Badr & Al-Shaikh, 2013). Considering that women are highly susceptible to UTI because of their genital anatomy, which allows easier access of the bacteria to the urethra (Minardi *et al*., 2011).

The diagnostic symptoms for UTI differ according to the urine tract infection site, the symptoms generated from the lower urinary tract include; urgency, frequency, urethral tenderness, and dysuria. Besides the latter symptoms, systemic symptoms such as fevers, nausea, and flank pain, are signals of upper urinary tract infection. Furthermore, a positive urine culture and a consistent clinical picture are mandatory for UTI diagnosis (Chu & Lowder, 2018).

2.3 Urinary tract infection categories

The classification of UTIs is mandatory for appropriate diagnostic and clinical decisions. UTI are generally classified based on different systems guidelines (CDC, IDSA, ESCMID, FDA). The concept of classification adopted by the latter guidelines is limited to merely two categories, uncomplicated and complicated UTI. Consequently, this classification does not sufficiently reflect the complexity of the clinical spectrum. Therefore, a recent classification was proposed to improve the diagnostics and management of urologic diseases worldwide, known as the ORENUC classification system, it employs several basic including, the clinical presentation of the UTI, the anatomical level of the UTI, the grade of severity of the infection, the categorization of risk factors and availability of appropriate antimicrobial therapy (Smelov *et al*., 2016; Haddad *et al*., 2020;).

2.3.1 Asymptomatic bacteriuria (ASB)

Asymptomatic bacteriuria is very common in clinical practice; it refers to the presence of one or more species of bacteria (potentially uropathogenic) growing in the urine at specified quantitative counts ($\geq 10^5$colony-forming units [CFU]/mL) in two consecutive samples (standard cultures) in women. Specifically, it is recognized by the absence of signs or symptoms attributable to UTI. Most patients with ASB have no adverse consequences. Thus, antibiotics intake is unnecessary (Salvatore *et al*., 2011; Tan & Chlebicki, 2016).

2.3.2 Acute uncomplicated UTI

Uncomplicated urinary tract infections (uUTIs) are one of the most common problems that affect women worldwide. Generally, it occurs in healthy women with no urological abnormalities or comorbidities, it includes, lower tract infections (Cystitis and urothertits) and also regroup cases of upper tract infections (pyelonephritis with no complications) (Smelov *et al*., 2016). Uncomplicated UTIs can be classified as:

a) Acute uncomplicated cystitis

Acute Uncomplicated cystitis (AUC) is a superficial bacterial infection in the bladder mucosa, it is a common type of UTI especially in healthy women (Baerheim, 2012). A colony count of $\geq 10^3$ CFU/ml of uropathogens is diagnostic in women who present with symptoms of acute uncomplicated cystitis (Smelov *et al*., 2016). The classical symptoms include urinary frequency, dysuria, urgency, and in several cases haematuria. AUC is usually with no serious complication, and in the majority of cases, narrow-spectrum oral antibiotics with a low potential are frequently recommended for clinical treatment (Di Vico *et al*., 2020).

b) Acute uncomplicated pyelonephritis

Acute uncomplicated pyelonephritis (AUP) is a bacterial infection of the upper urinary tract (renal parenchyma and renal pelvis) in patients with normal urinary tract anatomy and renal function (Ramakrishnan & Scheid, 2005). This type of infection is typically the outcome of an ascending bacterial invasion that originates in the lower urinary tract. In acute pyelonephritis, colony counts $\geq 10^4$ CFU/ml of uropathogens are considered to be clinically relevant bacteriuria (Smelov *et al*., 2016). Besides the clinical symptoms of cystitis mentioned above, flank pain, fever, nausea, and vomiting may also occur in typical cases. AUP is usually treated promptly with oral antibiotics to avoid chronic complications (Colgan *et al*., 2011).

2.3.3 Complicated UTI

Complicated UTI (CUTI) is an infection that occurs in patients with predisposing factors. This kind of infection carries a greater risk of treatment failure and frequently requires longer exposure to antibiotic treatment. Furthermore, it has a higher morbidity and mortality rate than the former UTI. The predisposing factors include urinary tract obstruction, diabetes, pregnancy, the presence of an indwelling urinary catheter, structural and functional abnormalities in the urinary tracts, renal transplantation, and immunosuppression (Wagenlehner *et al*., 2020). Significant bacteriuria in complicated UTI is defined as counts of 10^5 CFU/mL in the urine specimen of women. In CUTIs, the bacterial spectrum is much broader than in uncomplicated UTIs (Smelov *et al*., 2016).

2.3.4 Recurrent urinary tract infection (rUTI)

Recurrent UTI (rUTI) is extremely common in young healthy women and is estimated to affect 25% of women with a history of UTI.

rUTI are classified into two categories “relapses” where infection is caused by the same uropathogen or “reinfection” where a new uropathogens is the main causative of the infection (Salvatore *et al*., 2011; Sihra *et al*., 2018). Generally, this type of infection is the outcome of a long-multiple antibiotic treatment exposure, which most of the time leads to the

development of multi-drug resistant uropathogens that render antibiotic selection difficult. In this case, it will be more suitable to develop more approaches such as non-pharmacological methods to reduce recurrences (Rané, 2013). For women with rUTIs, a colony count of 10^3 CFU/mL of uropathogens is considered to be diagnostic. The majority of recurrences occur within the first three months of the primary infection, and there can often be a clustering of infections (Epp *et al*., 2010).

2.4 Etiology

2.4.1 Causative uropathogens and physiopathology

Most UTIs result from bacteria invasion of the urinary system. The current paradigm of urinary tract infection (UTI) pathogenesis takes into account the contamination of the periurethral space by specific pathogens residing in the gut, vagina, and urinary bladder jointly implicated in the pathogenesis of UTI (Meštrović *et al*., 2020). The uropathogenic spectrum of UTI is broad and includes several species of gram-negative pathogens such as *Escherichia coli, Proteus sp, Pseudomonas aeruginosa, Klebsiella sp, Citrobacter sp, Serratia sp,* and *Providencia sp*. On the other hand, gram-positive pathogens are also involved and include *Staphylococcus sp, Enterococcus sp,* and group B *Streptococci*. The predominant causative uropathogens agent is *E.coli* which accounts for >75% of UTI cases, while 5-10% of cases are caused by *S. saprophyticus, K. pneumoniae, P. mirabilis, and Enterococci spp* (Ahmed *et al*., 2019; Haddad *et al*., 2020).

Women are highly targeted by uropathpgens invasion, due to anatomical structure. In this regard, the shortness of the urethra, and its proximity to the anus are the leading cause that comforts easier access of the uropathogens through the urinary tract. Accordingly, most UTI cases are caused by the ascent of uropathogens via the urethra to the bladder. It may also reach up to the kidneys via the ureters, causing pyelonephritis. However, some uropathogens can reach the urinary tract by hematogenous or lymphatic spread (Salvatore *et al*., 2011; Zare *et al*., 2022).

On the other hand, women are extremely vulnerable to rUTIs, and several theories regarding the physiopathology of rUTI have emerged over the past decades. Indeed, this hypothesis differs according to multiple factors such as the species of uropathogen, and the patient's recurrence history. One of the most long-standing scenarios for rUTI is when the initially infecting bacteria persist within the gastro-intestinal flora after elimination from the urinary tract and keep recolonizing the periurethral surface, subsequently retrograde ascension. Also, alteration of the urinary microbiota through the introduction of native microbes from the vagina such as *Gardnerella vaginalis*, which may potentiate the recurrence

of UTI (Murray *et al*., 2021). Many host factors increase the recurrence of infection among healthy young women. These include local pH alteration, greater adherence of uropathogenic bacteria to the uroepithelium; and possibly pelvic anatomic differences, such as shorter urethra-to-anus distance (Kodner & Gupton, 2010).

2.4.2 Urobiome dysbiosis and its correlation to UTI

Traditionally, the human urinary microbiome known as the urobiome was considered sterile. However, the implication of advanced high-throughput sequencing technologies such as 16S rRNA sequencing, and expanded quantitative urine culture, revealed the existence of a wide range of bacterial species in urine, even in healthy, asymptomatic individuals (Jayalath & Magana-Arachchi, 2021).

The studies related to urobiome are limited and still unclear, thus more investigations are needed to explore their role in maintaining urinary tract homeostasis. In this regard, emerging evidence suggests that the urinary microbiome may be involved in the pathogenesis of UTIs. In other words, the perturbations or the dysbiosis of the urinary microbiome lead to a subsequent development of the microbiome pathogenicity, thus prompting the emergence of UTI (Meštrović *et al*., 2020). This was confirmed by a study conducted by Bossa *et al*. (2017) who noticed that urinary microbiome changes preceded the development of a UTI and that the urinary microbiome essentially normalized after treatment, implying its relative stability over time.

2.4.3 Urovirulence factors

Virulence factors are defined as bacterial-associated molecules that are mandatory for bacterial pathogenesis to successfully evade their host defenses and cause disease that can often be fatal. A wide range of factors has been identified among different uropathogens such as pili, adhesins, exopolysaccharides, glyco and lipoproteins, toxins, ureases, proteases, and iron-scavenging siderophores. Accordingly, these factors confer a long-lasting invasion capability to uropathogens by helping them to colonize, adhere, and cause tissue damage, as well as to evade host defenses, ultimately increasing their persistence in the urinary tract (Epler Barbercheck *et al*., 2018; B. O. Murray *et al*., 2021).

2.4.3.1 Adhesion pili

Bacterial adherence is a critical step that precedes every process in UTI pathogenesis. It is usually initiated by adhesion pili, also called ‘fimbriae’, expressed on the surface of pathogenic bacteria and confers virulence, colonization, biofilm formation, invasion, and sustained adherence of bacteria to target cells (Govindarajan *et al*., 2020). Multiple uropathogens adhesins recognize receptors in the urinary tract, notably in the bladder and

kidney uroepithelium using stereochemical specificity, consequently invading the uropithelium. Pili of both gram-positive and gram-negative uropathogenic bacteria are covered by multi-sub-unit pili proteins that are assembled via two distinct pili biogenesis pathways. One is the chaperone/ usher pathway (CUP) pili of gram-negative bacteria, and the other is the sortase assembled (SA) pili of gram-positive bacteria (Govindarajan & Kandaswamy, 2022).

Uropathogens produce a wide variety of adhesive pili that facilitate bacterial colonization of the urinary tract. For instance, uropathogenic *E. coli* (UPEC) require Type 1 fimbriae for effective colonization of the bladder epithelium, these fimbriae adhere to the mannose-sensitive receptor on the host bladder cells, thus preventing the elimination of bacteria during urination. Similarly, uropathogenic *K. pneumoniae* uses type 1 fimbriae to form biofilm and bladder colonization (Dhakal *et al.*, 2009).

On the other hand, *S. saprophyticus* differs in pathogenesis because of its virulence proteins. The virulence factors include several cell-wall virulence proteins such as Autolysin/adhesin that have autolytic, and adhesive properties, and subsequently exhibit an affinity towards fibronectin and human ureters. Moreover, Sdrl is another cell wall-associated protein that binds to collagen found in a minority of *S. saprophyticus* strains and plays a major role in acute UTI and persistent in the kidney (Kline *et al*., 2010).

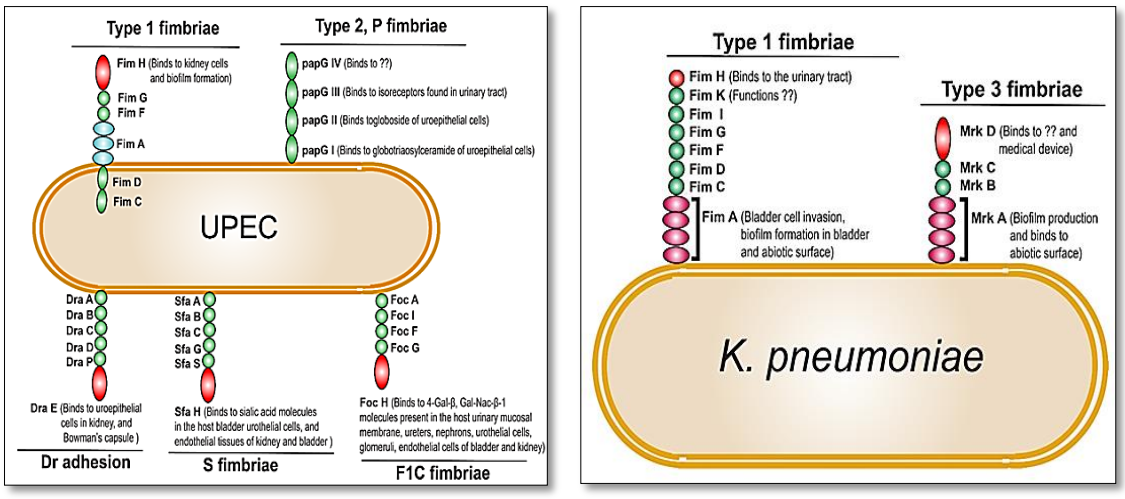


Figure 2. Adhesin-pili types of different uropathogens in host cell surface receptors (Govindarajan & Kandaswamy, 2022).

2.4.3.2 Prominent toxins of uropathogenic

Toxins are one of the major virulence factors identified in uropathogens, they are extremely involved in the intensity of the UTI. The majority of uropathogens employ toxins for offensive reasons (Popoff, 2020). Generally, toxins are classified as

either exotoxins or endotoxins, usually, exotoxins are proteins with enzymatic activity that interfere with host cells triggering the symptoms associated with the disease (Barbieri, 2009), while endotoxins refer to the part of the outer plasma membrane of uropathogens such as lipopolysaccharide (LPS) or lipooligosaccharide (LOS) (Kim *et al*., 2012).

The bladder environment is limited in nutrients; thus to survive and grow within the urinary tract, uropathogens produce toxins that damage the host tissue to release nutrients, while also providing a niche for bacterial invasion and dissemination, for instance, UPEC produces three main types of toxin include, hemolysin, cytotoxic necrotizing factor 1 (CnF1), and secreted autotransporter toxins. Hemolysin integrates into the host cell membranes, including, uropepithium, and leads to pore formation, thus resulting in cell lysis. Furthermore, this toxin stimulates exfoliation, leading to deeper uropithelium colonization, thus increasing epithelial damage and bladder hemorrhage. As is the case for hemolysin, CnF1 causes apoptosis of bladder cells, induces neutrophil dysfunction, and increases epithelial-cell invasion (Nielubowicz & Mobley, 2010). Besides hemolysin (alpha, beta, gamma, and delta) the uropathogenic Staphyloccocys can produce a variety of toxins such as the shock syndrome toxin 1 (TSST-1), exfoliative toxins, staphylococcal enterotoxins (SE), hemolysins, and leukocidins (Abril *et al*., 2020). Also, *P. aeruginosa* produces toxins that are implicated in UTI such as elastase, exoenzyme S (ExoS), and hemolytic phospholipase C, responsible for the establishment of *P. aeruginosa* during UTI (Mittal *et al*., 2006; Gupta *et al*., 2013).

2.4.3.3 Urease

A wealth of clinical evidence has classified uropathogenic urease as a virulence factor. In fact, ureolytic activity has a key role in the colonization and the persistence of different uropathogens such as *P. mirabilis*, *Klebsiella pneumonia*, *Staphylococcus saprophyticus*, *Providencia stuartii*, *Proteus vulgaris* and *Morganella morganii* (Mora & Arioli, 2014).

Urease catalysis the breakdown of urea to ammonia. The latter compound resulting from urease activity causes an increase in urine pH, turning it alkaline which creates an adequate environment for bacterial growth. Furthermore, inorganic ions contained within it, such as magnesium and calcium phosphate crystals, precipitate. Subsequently, these aggregates accumulate in the urine and provide an optimal home for bacterial growth and biofilm formation, thus promoting uropathogenic persistence in the urinary tract. Alarmingly, the accumulation of ammonia becomes toxic for the uroepithelial cells, inducing direct tissue damage (Flores-Mireles *et al*., 2015; Armbruster *et al*., 2017). For instance, *P.mirabilis* urease is expressed continuously due to urea induction, leading to uroepithelium irritation, exacerbating inflammation, and providing additional surfaces for biofilm formation (Duran

Ramirez *et al*., 2022). Considering the high activity of this enzyme, crystals are rapidly formed and trapped within biofilm polysaccharides, leading to the formation of crystalline biofilms on catheters. Moreover, this structure blocks urine drainage from the ureters, potentially resulting in reflux and promoting progression to pyelonephritis, septicemia, and shock. Additionally, uropathogenic *S. saprophyticus* exhibits urolithic activity that contributes to the invasion of bladder tissue (Gatermann *et al*., 1989). Furthermore, over 90% of *S. aureus* strains are urease-producing and play a crucial role in *S.aureus* pathogenesis, since it facilitate pH homeostasis under weak acid stress, and enhances bacterial persistence (Zhou *et al*., 2019).

Many strains of *K. pneumoniae* produce the extracellular enzyme urease, which is involved in virulence and pathogenesis mediation. Compared to Proteus, this uropathogens has lower urolytic activity, resulting in less salt precipitation on the abiotic surfaces in vitro, and thus lower encrustations forming efficiency (Clegg & Murphy, 2017).

2.4.3.4 Siderophores

Iron is mandatory for living uropathogens as it is considered an important cofactor for many bacterial processes. Considering that, siderophores have important functions for bacteria in iron acquisition and as virulence factors, these natural iron chelators possess a high affinity for ferric iron and they are usually produced under iron deficiency. In pathogenic microorganisms, siderophores are involved in the stealing of iron from the host proteins (Albelda-Berenguer *et al*., 2019).

In the case of UTI, the bladder environment is iron-limited (Flores-Mireles *et al*., 2015). Therefore, to colonize the human urine, uropathogens employes siderephroses for iron (Fe3+) scavenging. For instance, iron-chelating siderophores, are known virulence factors for UPEC, these strains produce four types of siderophores, including, enterobactin, salmochelin, yersiniabactin, and aerobactin. Among these siderophores, it was found that aerobactin and yersiniabactin are essential for the colonization of the urinary tract (Cavas & Kirkiz, 2022). Likewise, *P. aeruginosa* produces two types of siderophores, pyochelin, and pyoverdin, which support iron acquisition from host iron-binding proteins such as lactoferrin and transferrin. In this regard, recent studies explored the impact of *P.aeruginosa* siderophore in UTI, since this uropathogens was more virulent in the iron-deplete medium; showing increased renal bacterial load and tissue pathology, compared to iron-replete grown bacteria (Mittal *et al*., 2008). Besides, numerous iron-scavenging siderophore systems are utilized by other uropathogens: *K. pneumoniae* produces enterobactin and aerobactin. *P. mirabilis* uses proteobactin and yersiniabactin.

3 Antimicrobial resistance

3.1 The burden of antibiotic resistance

The sharp increase and the widespread of antimicrobial resistance (AMR) is considered an ominous threat to public health**,** as infection with AMR leads to serious illnesses, and most of the time, treatment failures. According to the 2022 GRAM Global Burden Report, bacterial AMR is associated with 4.95 million deaths in 2019, placing AMR as a leading cause of death worldwide (Murray *et al*., 2022).

AMR occurs when bacteria become able to adapt and persist within the presence of an antibacterial agent that once targeted them (Dadgostar, 2019). This feature is usually associated with a genetic mutation that significantly alters an aspect of its anatomy or physiology, allowing this function. Consequently, through time these resistant bacteria spread and become more prevalent, making antimicrobial therapy ineffective.

3.2 Origin basis of bacterial resistance

AMR can be divided as either intrinsic or acquired, in the case of intrinsic resistance, the type of resistance is chromosomally-encoded, and typically found within the genome of a group of bacteria or within a bacterial species. For instance, gram-negative bacilli are naturally resistant to vancomycin, gram-positive cocci are resistant to aztreonam. Also, *P. aeruginos*a provides another example of intrinsic resistance, which is naturally resistant to a wide range of antibiotics including, ampicillin, cephalosporins (^{1} and ^{2}generation) sulfonamides, tetracycline, chloramphenicol (Reygaert, 2018; Morrison & Zembower, 2020).

Worryingly, acquired resistance poses a severe threat to human health, since encountering these bacteria is challenging. This type of bacteria is initially susceptible to a common medication, subsequently, changing its status to resistance due to genetic material modifications. Generally, acquired resistance involves bacterial gene modifications, either by bacterial gene mutations or the acquisition of horizontal resistance gene transfer (transduction, conjugation, and transformation), resulting in resistance gene integration and dissemination. The most recognized example of acquired resistance is the mobilization of the chromosomal β-lactamase gene ampC to a plasmid (Peterson & Kaur, 2018).

3.3 Mechanisms of antibiotic resistance

Antibiotics are widely used worldwide for the purpose of bacterial eradication, employing specific physiological blocking actions. On the other side, bacteria became resistant to different therapeutical agents using multiple mechanisms. Generally speaking, bacteria employ four main mechanisms, but considering the differences in the structures of both Gram-negative and Gram-positive the mechanisms used may differ, for instance, the

Gram-positive bacteria cell wall is composed of a thick peptidoglycan layer which has a large permeability threshold, conversely, Gram-negative cell wall is more complex and contains a thin peptidoglycan layer that is surrounded by a thick plasma membrane composed of a double layer of phospholipids linked with the inner membrane by lipopolysaccharides (LPS) which contribute to reducing membrane fluidity (Cox & Wright, 2013).

3.3.1 Drug uptake limitation

Considering the differences in structure mentioned previously, the drug uptake limitation differs due to the outer cell wall rich in LPS in Gram-negative bacteria, the latter provides a barrier to a wide range of antimicrobial agents, thus giving innate resistance, moreover, porins contribute to the resistance of many bacteria using two main ways: retarding the influx of antibiotic using several mechanisms (limitations, hydrophobicity, charge repulsion) or mutations that change porins selectivity. Fortunately, this mechanism is not available in $Gram^+$ bacteria, because of the lack of cell walls (lack of LPS outer membrane) (Uddin *et al*., 2021).

3.3.2 Drug inactivation

Antibiotics can be rendered ineffective, either by complete destruction or altering their structure. The chemical modification of the antibiotic is ensured by bacteria-released enzymes, which have the ability to add various chemical groups to the drugs, preventing antibiotic binding to the target site in the bacterial cell. Transfer of phosphoryl, acetyl, and adenyl groups to the antibiotic structure is the most common method of drug inactivation by chemical group transfer. Whereas, drug destruction involves enzymes that cause antibiotic degradation, such as hydrolases (Uddin *et al*., 2021; Harikumar & Krishanan, 2022)

3.3.3 Modification of drug target sites

In general, each antibiotic recognizes its specific target, thus any structural modification in those targets prevents drug binding, resulting in drug inefficiency. Remodeling antibiotic's target site is extremely adopted by bacteria, it is usually ensured through spontaneous mutations in gene coding sites. For example, Staphylococcus grants resistance by employing genetic changes impacting the active site of penicillin-binding proteins (PBPs) which provide resistance to methicillin and oxacillin (Jubcair *et al*., 2021).

3.3.4 Drug efflux

To achieve antibacterial efficiency, antibiotics must get access to the bacterial cell at specific amounts and stay for a considerable time to perform their action (Jubcair *et al*., 2021). This availability across the bacterial cell is inhibited by a proteinaceous transporters located in the cytoplasmic membrane called efflux pumps, which exclude conventional

antibiotics, leading to a decrease in their concentration, thus increasing their minimum inhibitory concentration within the bacterial cell, in some cases, the loss of their antimicrobial activity. This resistance mechanism involves antibiotics that exert their antibacterial activity by inhibiting bacterial protein and DNA synthesis, especially tetracyclines, macrolides, and quinolones (Annunziato, 2019).

3.4 Multi-drug resistant bacteria

3.4.1 Overview

Multidrug-resistant (MDR) bacteria are a complex phenotype whose main characteristic is resistance to a broad range of antimicrobial drugs. MDR bacteria are described as bacterial resistant to at least 1 antibiotic in 3 or more antibiotic classes (Alemayehu, 2021). These types of antibiotic-resistant bacterial strains use several mechanisms to avoid the harmful impact of antibiotics such as the accumulation of multiple resistance genes within a single cell, excessive expression of multidrug efflux pump genes which pump out a diverse range of drugs, secretion of several degrading enzymes (Bharadwaj *et al*., 2022).

3.4.2 The emerging threat of multidrug-resistant uropathogens among UTI

The sharp increase and widespread of multidrug-resistant (MDR) among uropathogens is considered an emergent global health problem especially in UTI patients, as the outcome associated with MDR-uropathogens is worse compared with patients infected with none MDR-uropathogens. Furthermore, treatment failure is highly expected due to the scarcity of novel antibiotics and the inefficiency of the available antibacterial agents (Zowawi *et al*., 2015; Jubair *et al*., 2021).

MDR uropathogens include a wide range of potential urinary tract pathogens, such as MDR-*E. coli*, MDR-*P. aeruginosa*, MDR-*K. pneumonia*, and MRSA-*S. aureus*. The most prevalent MDR-uropathogenic is MDR-UPEC which is considered as the major causative factor of UTI. Devastatingly, causing 700,000 deaths worldwide (Rozwadowski & Gawel, 2022). Besides the possession of an arsenal of virulence factors mentioned above, MDR-UPEC strains are extremely resistant due to the existence of the worrisome phenomenon "broad-spectrum β-lactamases", which confer resistance to all β-lactams, including carbapenems (Madrazo *et al*., 2021). In this regard, many other uropathogenic belonging to the Enterobacteriaceae family possess this spectrum due to the possible transmission of ESBL genes between bacteria. One important aspect of acquiring ESBL or carbapenemase genes is that they are frequently co-located on plasmids which makes their spread easier, thus, rendering strains MDR. Another well-known example is methicillin-resistant *S.aureus* (MRSA), which is resistant not only to methicillin but to a wide range of antibiotics

including, tetracycline, aminoglycosides, macrolides, and chloramphenicol (Nikaido, 2009; Zowawi *et al*., 2015).

MDR-uropathogens may colonize the urinary tract and avoid host-killing mechanisms such as antimicrobial peptides and cytokines, neutrophil influx, inflammation, and apoptosis. Furthermore, MDR-uropathogens can evade host defenses by forming biofilm as a protected intracellular niche within epithelial cells during the early acute phases of UTI, as well as by surviving in quiescent intracellular reservoirs, which may act as a reservoir for recurrent infection (Zowawi *et al*., 2015).

3.4.3 Relation between MDR-uropathogens and rUTI

rUTIs are common among young women, even when no underlying anatomical or physiological abnormalities can be identified (Zare *et al*., 2022). As previously stated, the excessive use of antibiotics leads to the emergence of MDR-uropathogens among UTI. In fact, MDR uropathogens exhibit a high rate of persistence within the urinary bladder following antibiotic therapy, which extremely contributes to the pathophysiology of rUTIs.

Even though research on the possible link between MDR uropathogens and rUTI are quite limited, several elementary studies hypothesized that the possible reason behind the recurrences of UTI infections is the high capacity of MDR pathogen to form a peptidoglycan layer within the uropethelium, called biofilm. The latter is characterized by a great ability to resist and thus it is considered as the possible source of rUTI (Chakrabarty *et al*., 2022). In this regard, community-onset UTIs due to Enterobacteriaceae demonstrating extended-spectrum cephalosporin-resistance are associated with a significantly increased hazard of rUTIs compared to extended-spectrum cephalosporin susceptible Enterobacteriaceae (Moussa *et al*., 2020). On the other hand, the study conducted by Ahn *et al*. (2019), evidenced that MDR is involved in rUTI, as a large proportion of the initial UTIs caused by ESBL-producing *E. coli* had ESBL-positive *E. coli* on subsequent recurrence episodes of UTIs (Ahn *et al*., 2019).

3.4.4 Factors contributing to the emergence of MDR

MDR pathogens are rising day by day endangering human health worldwide. Earlier, these types of antibiotic-resistant bacterial strains were rare, but nowadays, they have become very common due to several inappropriate human activities. Indeed, many uropathogens became resistant to a wide range of antibiotic drugs because of several factors including, abuse and overuse, non-prescribed use, uncompleted dosages, inappropriate prescription, and antimicrobial drug accessibility (Begum & Shamsuzzaman, 2015; Bharadwaj *et al*., 2022). Alarmingly, resistance has emerged to almost all antibiotics used to treat urinary tract

infections, for instance, resistance to ampicillin and amoxicillin, these two antibiotics are commonly prescribed for UTI, unfortunately, they are no longer recommended for UTI treatment because of the emergence of resistance to these antibiotics among uropathogens. In another case, the widespread use of cephalosporins and quinolones engendered the ability to acquire MDR among uropathogens (Zhanel *et al*., 2006; Gupta *et al*., 2011). Furthermore, the overuse of carbapenems for the treatment of UTI patients caused by drug-resistant uropathogens has led to the emergence and spread of carbapenem-resistant Enterobacteriaceae (Moussa *et al*., 2020).

It is not negligible that the current viral COVID-19 pandemic may further contribute to the sharp spread of MDR-uropathogens, due to the lack of an effective therapeutic protocol, and the inappropriate use of antibiotics. Eventually, the scarcity of new antibiotics is one of the devastating purposes that contributed to the incapacity to control the spread of MDR-uropathogens (Catalano *et al*., 2022).

4 Biofilms and its role in the pathogenesis of UTI

4.1 Overview

As a part of their survival processes, bacteria form biofilms in response to a variety of stressful conditions such as nutrient limitation, osmolality, and immune responses. Biofilms are described as bacterial clusters that are irreversibly adhered to a biotic or abiotic surface and embedded in a self-produced extracellular polymeric matrix which accounts for 90% of the biomass. The matrix consists of several compounds including proteins, polysaccharides, and eDNA as well as different components from the surrounding environment, e.g. host´s components. Biofilm communities are regulated by a complex microbial cells' communication called quorum sensing (QS) regulation system (Delcaru *et al*., 2016; Sharma *et al*., 2019; Holá *et al*., 2021;).

Bacterial biofilms are a serious global health concern since these unique niches protect bacteria not only from the former harsh conditions but also from toxic components such as antibiotics entering the bacterial biofilm community. As a result, the biofilm matrix increases bacteria's resistance to antibiotics, resulting in the establishment of MDR bacteria (Sharma *et al*., 2019).

4.2 Stages of biofilm development in the urinary tract

Recently, most chronic rUTI have been associated with biofilm formation within the urinary system. To ensure biofilm development, uropathogens need to transit from the planktonic form to the sessile form which is ensured by various physiological and structural changes. The biofilm development is classified into five stages as follows:

4.2.1 Reversible attachment of planktonic bacteria to surfaces

Uropathogens possess various virulence factors that grant adhesion to the uroepithelium including fibrinogen and fibronectin-binding proteins. In the early stages of biofilm formation, planktonic uropathogens swim to the host surface using physical forces or bacterial appendages such as flagella and pili. Afterwards, bacteria can easily adhere to the uroepithelium through hydrophobic and electrostatic interaction that offers reversible adherence (Jagannathan & Viswanathan, 2018; Fu *et al*., 2021).

4.2.2 Irreversible attachment to surfaces

At this stage, many reversibly adsorbed bacteria remain irreversibly attached and stay immobilized due to the secretion of extracellular polymeric substances (EPS) that complex with surface host cells and/or receptor-specific ligands located on pili, and fimbriae. Consequently, enhancing rigid and irreversible adsorption between bacterial community and host cell surface allows firm attachment (Abebe, 2020).

4.2.3 Microcolony formation

Uropathogens embedded in the EPS proliferate in a coordinated community, resulting in the establishment of micro-colonies which are the basic units of biofilm. Accordingly, this process is usually the outcome of bacterial and EPS accumulation leading to the formation of a layer film compartmentalized by channels with different distinct micro-environments. After these sequential events, micro-colonies further develop into colonies (Abebe, 2020).

4.2.4 Biofilm maturation

In the mature stage, biofilm is characterized by a well-arranged three-dimensional structure. Under ideal conditions, the biofilm develops and matures, thickens to the point it becomes macroscopic. In this complex structure, each microenvironment is interspersed with fluid-filled channels through vital substances such as oxygen, and nutrient circulate. The coordinated functions of biofilm are assured by microbial cells communicating via QS using signal molecules such as acyl-homoserin lactones (AHL) which are used to communicate and orchestrate group behaviors, including virulence factor secretion and biofilm formation (Soto, 2014).

4.2.5 Biofilm dispersal

Many unfavorable conditions contribute to the detachment of microbial cells including abrasion, fluid shear, nutrient starvation, and insufficient oxygen. In this stage, biofilms rupture, and the bacterial cells start to disperse from the biofilm matrix as planktonic forms to colonize new cell surfaces, thus causing more infection (Samrot *et al*., 2021).

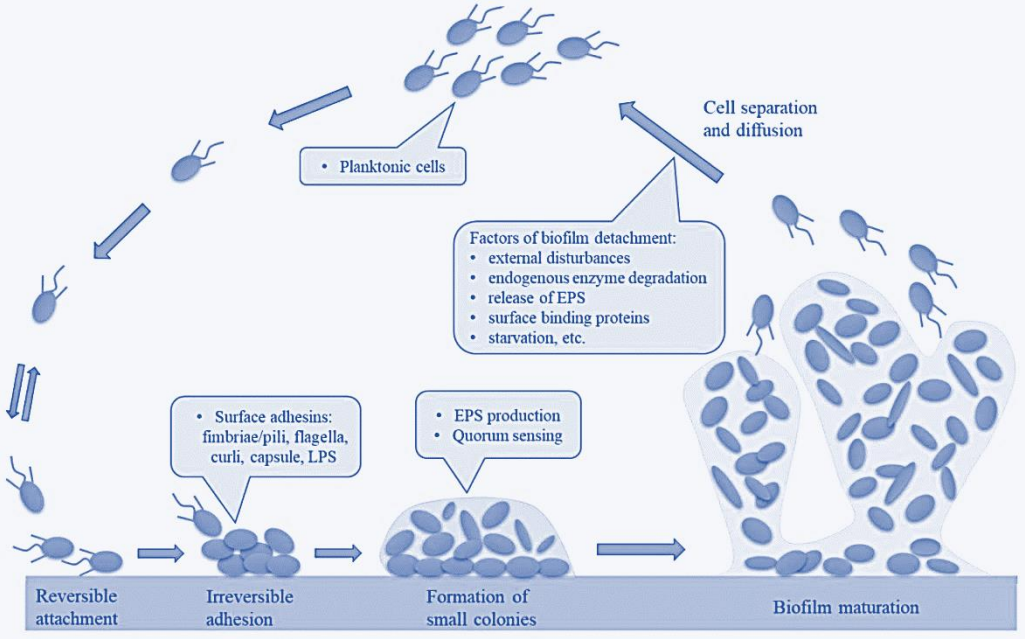


Figure 3. The five main phases leading to the development and formation of biofilm: (1) reversible attachment; (2) irreversible adhesion; (3) early development of biofilm structure (formation of small colonies); (4) biofilm maturation; (5) cell separation and diffusion (X. Liu *et al*., 2023).

4.3 Uropathogens and biofilms

Biofilm-forming bacteria were implicated in up to 80% of all infections, with urology being one of the key sectors where biofilm may become a severe problem. One of the most important factors contributing to infection severity and therapy failures is the capability of bacteria to form biofilms within the urinary tract. Bacterial biofilms play an important role in UTIs, responsible for persistent infections leading to recurrences and relapses. In severe UTI cases, uropathogens can adhere to the uropethelium and form biofilm thus invading the urinary tract and causing extreme damage. Biofilm can be detected in the urothelium, prostate stones, and foreign substances implanted (Soto, 2014).

Uropathogens are distinct in terms of the pathogenic processes and pathogenicity which allow them to invade and form biofilms within the urinary system. For instance, Uropathogenic *E. coli* (UPEC) is involved in the persistence of infection in the bladder due to its capacity to invade host urothelial cells and establish biofilm-like intracellular bacterial communities (IBCs). This structure confers bacterial immune evasion and protection from antibiotics. Furthermore, IBC has been observed by electron microscopy in shed epithelial cells in urine from women presenting with UTI (Vestby *et al*., 2020).

Another example is the ability of uropathogens such as *Providencia rettgeri* and *Proteus spp*. to generate urease. These uropathogens may hydrolyze urea, generating ammonia and carbon dioxide as a result. The latter components elevate urine pH and promote the formation of urinary salts, which can lead to kidney or bladder stones. Consequently, calcium crystals and magnesium ammonium phosphate precipitate and are typically incorporated into polysaccharide microbial capsules, resulting in the production of crystalline biofilms (Flores-Mireles *et al*., 2015; Delcaru *et al*., 2016).

4.4 Regulation systems in biofilms formation

To ensure the success of pathogenicity and persistent infections, bacteria use a very complex communication system called Quorum sensing. This system involves diffusible chemical signaling molecules called autoinducers (AIs), which coordinate growth and virulence. The increase in the bacterial density is compounded by the accumulation of signaling molecules, this accumulation reaches a threshold and results in its recognition and its binding to the cognate receptor that eventually activates and expresses the genes associated with biofilm formation (Zhou *et al*., 2020).

The phenomenon of QS is divided based on the types of AIs, the well-recognized system in Gram-negative bacteria is the AHL system, which employs the auto-inducers molecules such as N-acyl homoserine lactones (AHLs). The second one is the AIP system found only in Gram-positive bacteria, this system employs autoinducing peptides (AIPs). Among the several benefits provided by QS to bacterial pathogens is the capacity to colonize and/or infiltrate the host, as well as create biofilms on natural tissues (skin, mucosa, and uroepithelium) or medical devices (urinary catheters and other implants) (Lazar *et al*., 2021).

4.5 Biofilms and antimicrobial resistance

Biofilm-producing bacteria exhibit exceptional drug resistance, resulting in increased mortality and morbidity. This is due to the fact that biofilm development within a complex matrix can increase resistance to antimicrobials, making these pathogens difficult to eliminate and manage. When compared to planktonic bacteria, pathogens within a biofilm are more resistant to antibiotics. Biofilms, for example, may resist antimicrobial drugs 10-1000 times the concentration required to inactivate similar planktonic bacteria (Abebe, 2020).

Antibiotic resistance in biofilm is influenced by a variety of variables, including physical, physiological, and genetic factors. In this regard, bacteria within biofilm develop several mechanisms to protect their communities from unfavorable conditions such as the limitation of antibiotic diffusion through the biofilm matrix which retard and lowers their activities, transmission of resistance genes within the highly dense bacterial population,

production of persistent cells that are metabolically inert, drug efflux and inactivation of antibiotics (Stewart, 2002). Regarding the multifactorial nature of biofilm production and drug resistance, traditional antimicrobials face significant problems. To sum up, bacterial biofilm plays an important role in the emergence of MDR bacteria (Assefa & Amare, 2022).

4.6 Antibiotic treatment of UTI

Antimicrobial therapy for UTI involves multiple prescripted antibiotics chosen appropriately according to the susceptibility of the infecting bacteria, the concentrations of uropathogens in the urine, the severity of the infection, and the urinary complaint. Recently, the most recommended antibiotics for UTI include nitrofurantoin, trimethoprim sulfamethoxazole, ciprofloxacin, and ampicillin (Flores-Mireles *et al*., 2015). However, the widespread of antimicrobial resistance and the increase of rUTI rates are extremely challenging because of the scarcity of treatment options. Antibiotic recommendations by the Infectious Diseases Society of America (IDSA) and the European Association of Urology and their doses are summarized in Table 1.

Table 1. Antibiotic Recommendations According to Type of UTIs (Lee, 2018).

Antibiotics	Dose	Therapy Duration
Acute uncomplicated cystitis		
Recommended Agents		
Nitrofurantoin	100mg PO BID	5 days
Trimethoprim /sulfamethoxazole	160/800mg PO BID	3 days
Fosfomycin	3g PO once	once
Alternative agents		
Amoxicillin/clavulanate	500/125mg PO q8hr	5-7 days
Cephalexin	500mg PO BID	5-7 days
Ciprofloxacin	250mg PO BID	3 days
Levofloxacin	250-500mg PO daily	3 days
Acute uncomplicated pyelonephritis		
Recommended Agents		
Ciprofloxacin	500mg PO BID	7 days
Levofloxacin	750mg PO daily	5 days
Alternative		
trimethoprim /sulfamethoxazole	160/800mg PO BID	14 days

Amoxicillin/clavulanate	500mg PO TID	10-14 days
Acute complicated Cystitis		
Ciprofloxacin	500mg PO BID	5-7 days
Levofloxacin	750mg PO daily	5-7 days
Ampicillin/sulbactam	1.5-3g 6hr IV q6hr	/
Gentamicin/tobramycin	3-5mg/kg IV once	once
Acute complicated pyelonephritis		
Ceftriaxone	1g IV q24hr	/
Cevtazidime	1-2g IV q8hr	/
Piperacillin/tazobactam	3.375-4.5g IV q6hr	/
Aztreonam	1-2g IV q8hr	/
Meropenem	1g IV q8hr	/
Ertapenem	1g IVq24hr	/
Prevention of recurrent UTI		
Nitrofurantoin	50mg POqhs	/
Trimethoprim /sulfamethoxazole	40/200 mg PO daily	/

BID = twice daily; IV = intravenous(ly); PO = orally; q = every; QD = once daily; qhs = at night; TID = three times daily.

4.7 Alternative techniques for controlling UTI caused by MDR uropathogens

The prolonged use of antibiotics is one of the leading causes of the emergence of MDR pathogens. Furthermore, antibiotics in most cases present several side effects like disturbing microbiota, nausea, and vomiting. Many none antimicrobial alternative methods have been reported as promising options to manage UTI caused by MDR. Among theme, are the use of probiotics, D-mannose, phage therapy, phytotherapy (cranberry), and nanoparticles. In addition, recent research focuses on the use of therapeutic agents that target several bacterial virulence factors such as QS, biofilm, and urease. These agents don't exert significant selection pressure on bacteria thus decreasing the development of resistance mechanisms (Loubet *et al.*, 2020; Rodriguez-Mañas, 2020).

Considering the lack of progress in the discovery of novel therapeutic medicines, particularly those effective against drug-resistant strains, the research of additional alternative methods rather than antibiotics to treat UTI, and most particularly rUTI may be the greatest solution to solve this issue.

Chapter II
Propolis

1 Propolis

1.1 Overview

Bees are a tiny, venerable species that has been around for 100 million years, a truly balanced civilization that has always fascinated humanity, which has gradually learned to raise, maintain, and care for them (Ballot-Flurin, 2011). The bee is an insect belonging to the hymenopteran order and is also a member of the *Apoidea* superfamily, living in a colony in a hive or any other cavity (Clement *et al.*, 2006). By its anatomy, the bee is adapted to the collection of pollen and nectar and its adaptability to the plant world is vast. The bee is not bound to any particular species or even a limited botanical group. In contrast, its organs of harvest enable it to gainfully visit the most diverse plants. Flowering plants and bees have an interdependent relationship: one cannot exist without the other. Bees collect their food by foraging on flowers and their pollinating activity ensures the perpetuation of future generations of both plants and humans (Louveaux *et al*., 1966; Bradbear, 2005).

Bee products are among the most incredible natural resources because they provide a fascinating research topic and have numerous health, agronomic, and ecological advantages. The products of the hive are all derived from flowers or plants and transformed by bees. They include honey, propolis, royal jelly, pollen, and wax (Chahbar *et al*., 2011). Typically, honey comes to mind when we talk about bee products. However, there are other types of classified bee products, such as propolis, that benefit everyone in the world by bringing pleasure, health, and happiness.

1.2 Definition

Propolis is a natural product that belongs to the larger family of beekeeping products. The word propolis is a complex term derived from two Greek words propolis – pro – , for or in defense, and polis, the city (Kasiotis *et al.*, 2017). Propolis is an aromatic-resinous substance produced by honey bees it is a mixture of wax and botanical material such as buds, and plant resins, which the bees collect, and modify by adding some of their salivary secretions and wax (Wagh, 2013). Generally, Bees use propolis on their hives as protection against predators and microorganisms, to repair damages, as a thermal isolator, and to build aseptic locals to prevent microbial infection (Fokt *et al.*, 2010).

In terms of chemical content, propolis varies according to several factors including geographical and seasonal variation. The composition of propolis extremely depends on the local vegetation. It is generally composed of 50% resins, 30% waxes, and fatty acids, 10% essential oils, 5% pollen, and 5% mixed organic and minerals and organic compounds like phenolic acids and flavonoids (Irigoiti *et al.*, 2021).

2 Propolis qualitative properties

The qualitative proprieties of propolis vary from sample to sample and this variation is mainly due to several factors. In this regard, the vegetative flora and wax content are the main factors that contribute to the different physical characteristics of propolis, including color, flavor, texture, and consistency (Anjum *et al*., 2019).

2.1 Physical properties

2.1.1 Macroscopic analysis:

- **Consistency**

Propolis has a variable consistency depending on thermal changes, it is soft, springy, and very sticky when the temperature is between 25 and 45 °C. In contrast, propolis becomes rigid and brittle in lower temperatures (below 15 °C) (Blackiston, 2009; Boulechfar & Zellagui, 2023).

- **Color**

Propolis is a complex coniferous bee product whose appearance varies considerably, depending on many factors. Depending on the plant source and age, the color might be cream, yellow, green, light, or dark brown (Silva-Carvalho *et al*., 2015).

The propolis that comes from countries with a warm climate has a more or less pronounced brown color. Brown or European propolis originates mostly from poplar trees.

Figure 4. Honey bees with propolis in leg sack.

The majority of red propolis originates from the coastal areas of North East Brazil. In this regard, Li *et al*. (2007) discovered that bees reared in this location collected reddish exudates found on the surface of *Dalbergia ecastophyllum*, and their research revealed that the latter is the botanical source of red propolis.

Figure 5. Collection of propolis from reddish resinous exudates of *D. ecastophyllum* by africanized *Apis mellifera* (Daugsch *et al*., 2008).

Among the types of propolis found in Brazil, green propolis comes in small, rigid, and brittle blocks of a few cm. The main botanical source of 'green' propolis in Brazil is *Baccharis dracunculifolia*, followed by *Corymbia citriodora,* and *Araucaria angustifolia* (Nunes & Guerreiro, 2012).

Figure 6. Bee recolting green propolis from *Baccharis dracunculifolia* (Berretta et al., 2017; Toreti *et al*., 2013)

- **Smell and taste**

Propolis has a distinctively sweet, aromatic, and balsamic odor (Tosi *et al*., 2006), as well as an acrid, pungent flavor that varies depending on its origin (Balica *et al*., 2021).

2.1.2 Microscopic analysis

The majority of propolis particles appear in isolated form but with a propensity to form huge agglomerates. Accordingly, Tsibranska & Tylkowski, (2016) described the microscopic structure of propolis using electron microscopy, where the results of propolis samples from Bulgaria revealed rough surfaces coated with layers of wax and extracts, as well as the presence of plant constituents. On the other hand, Machado *et al*. (2015) reported that similarities in the microscopic appearance of samples of the same type were found in their profiles. For instance, in all the green propolis samples, vegetative constituents were found originating from the vegetative apexes of *Baccharis dracunculifolia*. Furthermore, various components from the flora visited by the bees, such as the Copaifera species, were found in the brown propolis samples.

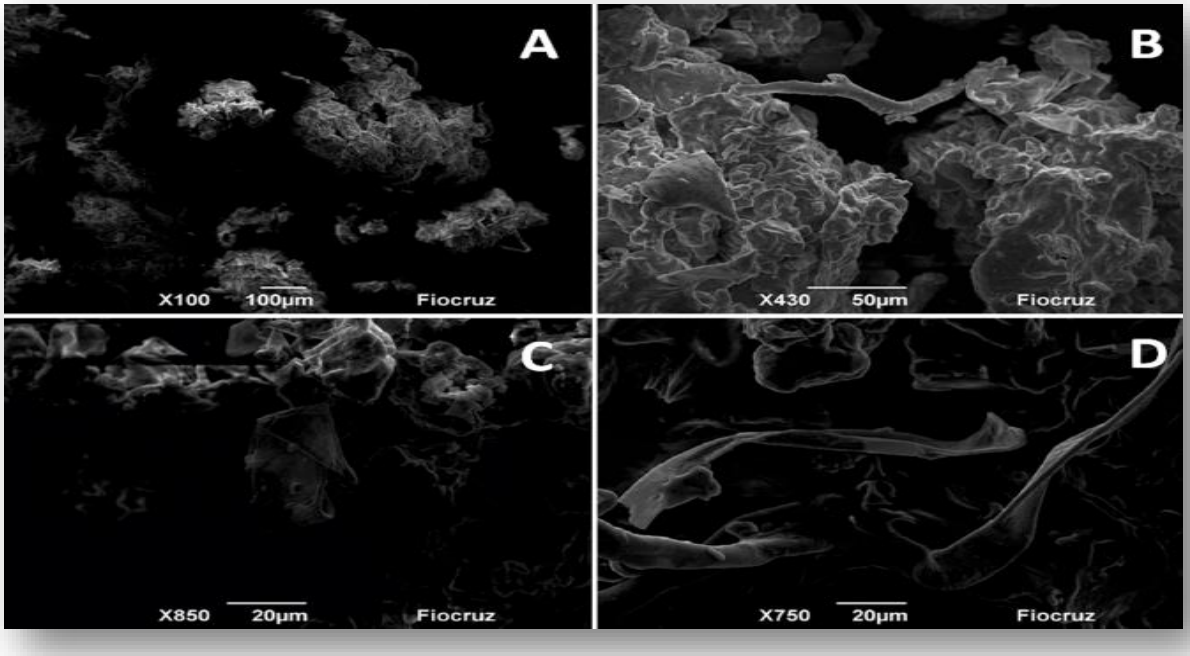


Figure 7. Images obtained by Scanning electron microscopy (SEM)for a sample of green propolis (Machado *et al*., 2015).

2.2 Chemical properties

- **Density and solubility**

Propolis has a density of 1.18, slightly heavier than water. However, it does not sink in water due to its high content in wax. It is easily soluble in petroleum ether, alcohol, gasoline, chloroform, turpentine, acetone, petroleum jelly, vegetable and animal fats, liquid ammonia, and acetic acid. Propolis is almost insoluble in water and its water-soluble fraction is 13%

more effective when heated. The choice of the right solvent is very important if the product is to be used for human consumption (Wagh, 2013; Potier, 2014).

3 Analytical composition: a wealth of active substances

In recent decades, propolis chemical composition has been widely investigated. More than 800 components were identified in different propolis samples (Kasote *et al*., 2022). Many analytical approaches have been employed to separate and identify propolis ingredients, in particular, propolis contains; polyphenols, flavonoids, chalcones, terpenoids, aliphatic and aromatic acids, ketones, aldehydes, sugars, amino acids, vitamins, minerals, waxes, and fatty acids … etc (Irigoiti *et al*., 2021). Several studies have been carried out on propolis from different geographic origins and revealed that the chemical content of propolis is highly variable because it depends on several factors such as botanical origin, season, bee species, harvesting time, and geographical area (Uzel *et al*., 2005).

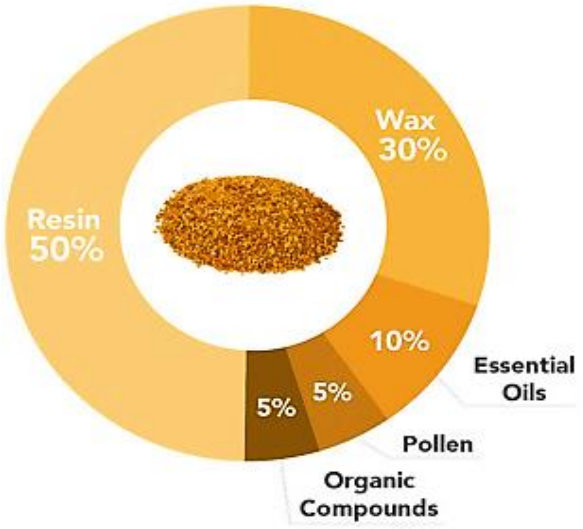


Figure 8. The composition of propolis: resin, wax, essential oils, pollen, and organic compounds (Easton-Calabria *et al*., 2019).

In general, when propolis comes from Europe or China, the main plant metabolites are flavonoids and phenolic acids, while metabolites from Southeast Brazil contain, in addition to phenolic compounds, large amounts of terpenoids and acid derivatives (Berretta *et al*., 2017). This variation in the content of propolis can determine a wide spectrum of different biological activities and underscores the effectiveness of the requirement for chemical standardization, necessary to link a particular chemical class of propolis to a specific type of biological activity (Piccinelli *et al*., 2013).

According to the above-mentioned information we can classify the components of propolis into the following groups:

- ✓ Polyphenols (phenolic acids and their esters, and flavonoids.)
- ✓ Terpenoids
- ✓ Essentiel oïl
- ✓ Aliphatic acids
- ✓ Proteins
- ✓ Lipids
- ✓ Sugars
- ✓ Others

3.1 Polyphenols

In terms of quantity and quality, phenolic compounds are the most dominant group of propolis components. They comprise phenolic acids and their esters, and flavonoids. Polish researchers have estimated the content of polyphenolic compounds in propolis to be about 58% (Kurek-Górecka *et al*., 2013; Kasote *et al*., 2022).

3.2 Flavonoids

Flavonoids are widely distributed in the plant kingdom, thus explaining their abundance in propolis. Flavonoids are a class of nearly 6,000 natural compounds that are almost ubiquitous in vascular plants. They consist of pigments responsible for the yellow, orange, and red coloring in various plant organs. It is well known that these chemical compounds are responsible for the main different biological activities of propolis. Hence, the determination of flavonoid content in propolis is considered a very important parameter to evaluate its quality (Ghedira, 2005). At present, more than 100 kinds of flavonoids have been separated and identified from different varieties of propolis, including myricetin, quercetin, chrysin, kaempferol, galangin, and so on, so it is considered to be the main biologically active component of propolis (Cui *et al*., 2022).

According to the chemical structure, flavonoids presented in propolis are classified into 11 sub-class: flavones, flavonols, flavanones, flavanonols, chalcones, dihydrochalcones, isoflavones, isodihydr, oflavones, flavans, isoflavans and neoflavonoids (Stojanović *et al*., 2020). The The flavonoid group in propolis includes mainly the following substances: apigenin, kaempferol, pinobanksin, chrysin, tektochrisin, pinocembrin, galangin, quercetin, myricetin, rutin, rhamnetin, isorhamnetin, luteolin, naringenin, acacetin, baicalein, hesperitin, sakuranetin, formononetin, liquiritigenin, isalpinin, daidzein, genistein, eupatorin, hispidulin, propolins, prokinawan, isosativan, medicarpin, vestitol, nymphaeol, isonymphaeol (Balica *et al*., 2021).

3.3 Phenolic acids

phenolic acids occupy a very interesting place in the raw composition of propolis such as benzoic, coumaric, caffeic,ferulic, and salicylic acids and especially their esters (Balica *et al*., 2021). In propolis other phenolic compounds (e.g., artepillin C) have been found as well as stilbenes and stilbene derivative resveratrol (Stojanović *et al*., 2020). On the other hand, aromatic acid esters are of great importance, caffeic acid ester (caffeic acid phenethyl ester or CAPE) is one of the most important medicinal components in propolis as it possesses diverse and important biological activities (Chang *et al*., 2017).

3.4 Volatile compounds (Terpenoids)

Essential oils are volatile and aromatic substances of plant origin (Ozturk, 2015). Based on results obtained between 1908 and 1948, propolis contains up to 10% of the essential oil. Recently published studies show a much lower proportion, generally up to 1% and rarely 2-3% (Bankova *et al*., 2014).

Despite their low concentrations, volatile compounds in propolis are extremely important. They comprise mainly terpenoids as well as aromatic and aliphatic compounds. Terpenoids are the second biggest and most important group of compounds and also the most abundant volatile components of propolis (Šturm & Ulrih, 2020). Among the identified terpoinds so far: geraniol, nerol, bisabolol, guaiol, farnesol, linalool, limonene, eudesmol, terpineol, camphor, squalene, copaene, calarene, calamenene, caryophyllene, patchoulene, elemene, ferruginol, junicedric acid, pimaric acid, abietic acid, isocupressic acid, acetylisocupressic acid, communic acid, imbricatoloic acid, totarol, amyrin, amyrone, lupeol, lupenone, moretenol, ferutinin, teferin, germanicol, agarospirol, lanosterol, erythrodiol, cycloartenol, ambonic acid, mangiferonic acid, ambolic acid (Balica *et al*., 2021).

In general, the chemical composition of the essential oils analyzed in various propolis samples revealed qualitative and quantitative variations, which are primarily attributed to the geographical local conditions and seasonal harvest times (Meurer *et al*., 2009). Furthermore, due to their aroma and biological activity, essential oils play a vital function in the characterization of propolis and might potentially increase its employment. In addition, volatile content might provide valuable data on the plant origins of propolis (Falcão *et al*., 2016).

In contrast to propolis constituents (such as phenolics, flavonoids, etc.), propolis essential oils appeared to have higher variability (Bankova *et al*., 2014). The mono- and sesquiterpenes were identified as the main components of propolis, despite the diversity and

high level of volatile components in propolis. For example, sesquiterpenes were predominant in most of the European propolis samples analyzed (Falcão *et al*., 2016).

3.5 Amino acids

While many propolis components have been identified and their biological properties analyzed, only a few reports have described the amino acid content of propolis. the protein content of propolis is generally in the minority. In low concentrations, aspartic acid, glutamic acid, alanine, arginine, cystine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine were detected in propolis extracts (Eroglu *et al*., 2016).

3.6 Carbohydrates

Propolis contains only a very small amount of carbohydrates. The reported isolated sugar from propolis includes xylose, galactose, mannose, glucuronic acid, lactose, maltose, melibiose, and erytritol. Xylitol and inositol also have been isolated from the Bee Propolis (Wali *et al*., 2017)

The origin of the sugars in propolis has not yet been determined. It is believed that nectar and honey are the sources of glucose, fructose, and sucrose. Others suggest that they come from the hydrolyzed flavonoid glycosides in propolis. In addition, mucilages containing a wide range of sugars, including alcoholic sugars, have been listed as a potential source of propolis sugars. However, numerous sugars, alcoholic sugars, and uronic acids have been identified in propolis from the Canary Islands and Malta, confirming the claim that plant mucilages are the source of these compounds (Huang *et al*., 2014).

3.7 Vitamins

In general, propolis has a low level of vitamin content, it contains some important vitamins such as B complex vitamins (B1, B2, B3, B6, B9), vitamins C and E (Eroglu *et al*., 2016).

3.8 Minerals

Iron (Fe), copper (Cu) and manganese (Mn) are the most reported minerals detected in propolis but there are also aluminum (Al), silver (Ar), barium (Ba), boron(Bo), calcium, chromium(Cr), cobalt(Co), molybdenum(Mo), nickel(Ni), silicon(Si), strontium(St), titanium(Ti), vanadium(Va), Selenium(Se) and zinc(Zn) (Ghedira *et al*., 2009).

4 Chemical diversity of propolis

The classification of propolis based on its plant sources is of major significance since it is associated with its biological activity and might serve as the basis for its standardization (Christov, 2004). In the following years, analysis of a large number of samples from different

geographic areas revealed that the chemical composition of propolis is highly diversified which makes it difficult to standardize. Furthermore, the association between propolis bioactive compound(s) and their respective therapeutic potential is of great interest.

To better understand the immense variability of the chemical composition of propolis, it is important to establish the plant origin of propolis. For the production of propolis, bees use substances secreted by plants or substances exuded from plant wounds: these are lipophilic materials found on leaves and leaf buds, gums, or resins. Thus, the composition of the plant source determines the chemical composition of propolis. Combined with the knowledge of active principles, it gives clues to standardization and quality control, allowing the specification of propolis types that have distinct chemical compositions (Bankova, 2005).

4.1 Variability and botanical origin of propolis in the world

Propolis standardization is crucial for ensuring chemical consistency and, as a result, efficacy (Nada *et al*., 2022). The first papers analyzing the chemical composition of propolis appeared in the 1970s when Russia analyzed the flavonoid composition of propolis and compared it to poplar. Recently, it has been well documented that in temperate zones worldwide, in Europe, North America, and non-tropical regions of Asia, the main source of propolis is the poplar (Populus. sp) (Bankova *et al*., 2000), whose major components are polyphenols: flavonoids, phenolic acids, and their esters (Seidel *et al*., 2008). Studies on propolis from Bulgaria, Italy, and Switzerland have made it possible to study the chemical composition of these samples and to identify more than 80 compounds, the main botanical source of which was the poplar: they contained pinocembrin, pinobanksine, chrysine, galangine, caffeic and ferulic acids, among others (Bankova *et al*., 2002).

Several studies on the chemical composition of propolis from Turkey, cited by Silici *et al*. (2007), confirmed that its botanical origin presents a typical pattern of "poplar" propolis, it contains a mixture of different components such as pinocembrin, pinobanksin, and its acetate, prenyl esters of caffeic acid, ferulic acids, and cinnamic acid. The propolis sample was found to contain mostly the same components present in poplar species exudates.

By its geographical position, Algeria presents a great diversity of biotopes occupied by an important floristic wealth. Its forest ecosystems are characterized by a remarkable floristic richness (Benabadji *et al*., 2007). According to the botanical flora available in Algeria, it can be deduced that the Algerian propolis is of pine (pinus sp), oak (Quercus suber and Quercus canariensis), chestnut, cypress, casuarina, and poplar origin (Debab *et al*., 2017).

According to a study made on samples of Algerian propolis, which focused on the chemical composition of the latter. The results showed the presence of many components,

including pinocembrin, pinobanesin, and its acetate, chrysin, apigenin, pectolinarigenin, pilosin, ladanein, galangin, naringenin, tectochrysin, methoxychrysin, prenyl esters of caffeic acids, ferulic acids, labdan and clerodan. In addition, the results of the chemical investigation carried out on the samples of propolis revealed the presence of two different types of propolis in the northern region of Algeria, one called Algerian polyphenol propolis (PAP) which displays as a trademark an array of polyphenolic compounds typical of propolis produced from Populus resins, suggesting that these plants could be one of the main sources of Algerian propolis (Piccinelli *et al*., 2013).

In tropical regions, there are no Populus species, so bees use other plant sources for propolis production. Thus, the chemical composition of propolis in tropical areas is very different from that in temperate areas due to the difference in vegetation. Several studies on the chemical composition of tropical propolis have been initiated recently and suggest great diversity and new compounds being reported every year: prenylated benzophenones, prenylated organic acids, and isoflavonoids are examples of secondary chemical compounds identified from tropical propolis (Cuesta-Rubio *et al*., 2012).

Brazilian propolis has been the subject of several chemical studies, the main botanical source is the alecrim tree *(Baccharis dracunculifolia)* (Moise & Bobiș, 2020). Other genera such as *Schinus, Vernonia, Diclenia, Hyptis, Myrcia, and Weinmanias* are also documented as the main botanical source of Brazilian propolis. According to its physico-chemical characteristics and botanical source, Brazilian propolis was classified into 12 groups: five in the southern Brazilian group, one in the south-eastern Brazilian group, and six in the north-eastern Brazilian group (Park *et al*., 2002).

5 Biological activities

Propolis has long been associated with various medicinal properties and has been utilized for its potential health benefits. Some of the attributed medicinal properties of propolis include antimicrobial, antifungal, antiviral, antioxidant, anticancer, anti-inflammatory, immunomodulatory, etc.

5.1 Antibacterial properties

The antibacterial properties of propolis are the best-known and one of the most widely studied characteristics (Wieczorek *et al*., 2022; Chavda *et al*., 2023). Several experiments have reported that propolis has a very broad antibacterial spectrum, with strong activity on both Gram-positive and Gram-negative, this activity is allotted to numerous phenolic compounds, mainly flavonoids, phenolic acids, and their esters (Sa-Eed *et al*., 2023). The germs whose growth is inhibited by propolis include many species such as *S.aureus,*

Streptococcus (S.pyogenes), S.mutans and S. sobrinus, Bacillus 'B. larva, B. subtilis, B. alvei, E. coli, P. mirabilis, P.vulgaris, P. galangin, Helicobacter pylori, Enterococcus faecalis, Pseudomonas aeruginosa, Listeria monocytogenes, Salmonella (S.gallinarum, S. pullorum and S. Dublin), Klebsiella sp … ect (Przybyłek & Karpiński, 2019).

Numerous studies demonstrate the antibacterial activity of propolis. For example, Mokrani *et al*. (2023) confirmed the inhibitory effect of Algerian propolis on oral pathogenic bacteria *Enterococcus faecalis*. A further study conducted in 2014, on propolis from Algeria showed strong inhibitory efficacy on *S. aureus* and non-hemolytic streptococcus, while the latter appeared less active on gram-negative strains such as *E. coli, P. aeruginosa, K. pneumonia,* and *P. mirabilis* (Segueni *et al*., 2014).

On the other hand, propolis from Tanzania has an antibacterial activity with a MIC equal to 15.62 µg/ml against several Gram-positive strains, including *S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Enterococcus faecalis, and Bacillus subtilis* (Runyoro *et al.*, 2017).

On the other hand, Brazilian green propolis, whose most important plant source is *B. dracunculifolia*, has been widely studied. Several studies have shown the activity of green propolis against several pathogenic bacteria, including Gram-positive bacteria (*S. aureus, S. epidermidis, S. pneumoniae*, and *Kocuria rhizophila)* and Gram-negative bacteria *(Haemophilus influenzae, Porphyromonas gingivalis, Porphyromonas endodontalis, and Prevotella denticola*) (Berretta *et al*., 2017).

5.1.1 Antimicrobial activity of propolis against pathogenic bacteria responsible of UTI

Thanks to its high content of antimicrobial substances, propolis helps in the treatment of several pathologies such as UTI (Vică *et al*., 2022). The positive response of propolis to urinary tract infections has been reported in many cases, and several studies confirm the contribution of propolis to the inhibition of certain bacteria responsible for UTI. In general, it contributes to the elimination of germs (in particular Streptococcus, Proteus, and *E. coli*) (Al-Zahrani *et al*., 2019).

The research conducted by Lavigne *et al*. (2011) shows that propolis acts effectively on UTIs especially if taken with type A proanthocyanidins (PAC), as this compound is believed to be an important inhibitor of *E. coli*. The combination of propolis and PAC reduces the adhesion activity of bacteria on uroepithelial cells and offers some protection against bacterial growth, bacterial adhesion, and virulence in the urinary tract, which represents a preventive strategy against UTIs.

5.2 Antioxidant properties

A variety of synthetic antioxidants are commonly used. However, the use of these components has been restricted by law due to their toxic and carcinogenic effects. Plant-based products are a potential source of natural antioxidants, such as vitamin C, tocopherol, carotenoids, flavonoids, and phenolic acids that prevent damage caused by free radicals. Therefore, a growing interest in natural antioxidant products, and a growing trend among consumers to favor natural antioxidants, has given impetus to attempts to explore natural sources of antioxidants (Gülçin *et al*., 2011).

Propolis has always been noted for its antioxidant actions. It contains mainly flavonoids and phenolic compounds, which have been described as the main group with antioxidant properties. Due to its antioxidant effect, propolis can protect humans against deleterious oxidation processes, thus, preventing many related disorders such as cardiovascular diseases, Alzheimer's disease, and cancer. Indeed, propolis contributes to the lipoperoxidation inhibition of linoleic acid which results in antioxidant action (Ghedira *et al*., 2009; Bonvehí & Gutiérrez, 2011; Chavda *et al*., 2023).

Flavonoids play a crucial role in preventing the former issues since they reduce the formation of free radicals and therefore, could have a protective effect against oxidation (Tao *et al*., 2023). The antioxidant activity of propolis from different geographical origins (Argentina, Australia, China, Hungary, and New Zealand) was linked to the high content of polyphenols and flavonoids such as kaempferol and phenethyl caffeate (Kumazawa *et al*., 2004). Similarly, the study of aqueous extracts of propolis from western Algeria gave the same results (Debab *et al*., 2017).

Propolis has a protective effect against CCl4 toxicity which causes kidney damage via oxidative stress. The studies of Bhadauria, (2012) were conducted to confirm the protective effect of propolis against oxidative stress, in which rats were exposed to CC14 for 12 weeks with a dose of 0.15ml/kg, and the symptoms that were observed were renal disorders (uremia) and an increase in lipo-peroxidation in the kidneys. Doses equal to 200mg/kg of propolis extract were given to the rats for 2 consecutive weeks. The results proved that propolis therapy prevented the above-mentioned alterations. Thus, propolis plays an important role as a kidney support agent, due to its strong antioxidant activity.

5.3 Antifungal properties

Propolis possesses potential antifungal activity and plays an important role as a potential inhibitor of the filamentous form that characterizes most fungal species (Tobaldini-Valerio *et al*., 2016). The spectrum of antifungal activity of propolis includes several species:

Candida albicans, Aspergillus parasiticu, Cryptococcus neoformans … etc. The antifungal activity of propolis is related to certain compounds such as flavonoids and phenol esters, a study conducted by Agüero *et al*. (2010) showed the inhibition of *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *Trichophyton rubrum* species by different propolis extracts, the effectiveness of the antifungal activity is due to the presence of dihydroxy-methoxy chalcone and dihydroxychalcone compounds. In addition, many isolated compounds such as galangin, pinocembrin, and hydroxy-methoxyflavanone presented moderate antifungal activity. Furthermore, the antifungal potential of various extracts of propolis has been examined against several yeasts, such as *C. albicans, C. glabrata, C. parapsisolis, Saccharomyces cerevisiae, Aspergillus niger, Botrytis cinerea, Cladosporium spp., Fusarium solani, Penicillium digitatum, Penicillium expansumn* (Ożarowski *et al*., 2022).

5.4 Antiviral properties

The pharmacological activity of propolis against several viral infections has been demonstrated in several reviews, e.g. influenza virus, HIV, adenovirus, and herpes simplex virus. In addition, therapeutic benefits have been reported from propolis against respiratory tract viral infections in children and genital herpes (Schnitzler *et al*., 2010). Canadian propolis has proven to be active against the herpes simplex virus. Many compounds isolated from propolis including, caffeic acid, galangin, and chrysin appear to be the constituents that exert this virucidal action (Bankova *et al*., 2014). Propolis has also secured a place among the substances that contribute to the elimination of the AIDS virus, the phenyl ethyl ester of caffeic acid (CAPE) is one of the most potent anti-integrating agents of HIV (Ghedira *et al*., 2009). On the other hand, several studies showed that propolis possesses great potential in inhibiting the recent pandemic virus (SARS-CoV-2 virus) (Ali & Kunugi, 2021; H Elwakil *et al*., 2021; Karaoğlu *et al*., 2023).

5.5 Antitumour properties

Recently, the anti-tumor properties of propolis have been widely documented in different works (Forma & Bryś, 2021), and the results have shown an antiproliferative effect on several cancerous diseases such as human leukemia, colon cancer, cervical cancer, lung cancer, pancreatic cancer, and breast cancer (Xuan *et al*., 2014). In the study by Motomura *et al*. (2008), propolis was found to inhibit the proliferation of human leukemic U937 cells in a dose-dependent manner by inducing apoptosis. On the other hand, Brazilian red propolis extract has a cytotoxic effect against colon cancer cell lines, bladder cancer cells (T24), and prostate cancer cells (PC-3). Furthermore, ethanolic extract of Turkish propolis has been shown to have cytotoxic activity towards hormone-resistant prostate cancer PC-3 cells.

Egyptian propolis ethanolic extract prompted cytotoxic effects in HCT-116 (colon cancer), MDA-MB-231, MCF-7 (breast cancer), and HeLa (cervical cancer) cancer cell lines (Forma & Bryś, 2021). The study of Boulechfar *et al*. (2023) demonstrated the potential apoptotic effect of Algerian propolis against cancer cells. Oršolić *et al*. (2005) reported that polyphenolic compounds exert direct cytotoxic effects on tumor cells. Caffeic acid (CA) and CAPE cause a significant delay in the formation of mammary carcinoma tumor cells transplanted into mice and an increase in life span from 29.30 to 51.73%.

5.6 Anti-inflammatory properties

Propolis is a potential low-cost anti-inflammatory agent for acute and chronic stages of inflammation. These properties are mainly used for muscle and joint inflammation, as well as other types of inflammation, infections, and rheumatism. Studies have shown that the ethanolic extract of propolis possesses anti-inflammatory activity after topical, injectable, or even oral administration (Ramos & Miranda, 2007).

The anti-inflammatory activity of propolis is apparently due to the presence of flavonoids. This latter has proven to inhibit certain enzymes involved in the metabolic pathway of inflammation: cyclooxygenase (COX) and lipooxygenase (LOX). In addition, propolis was shown to reduce the level of prostaglandin E2 (PGE2)- a potent inflammatory mediator in the peritoneal exudates and TNF-α in the liver of epirubicin-induced hepatotoxicity in rats (Zulhendri *et al*., 2022). Moreover, Xool-Tamayo *et al*. (2020) reported that Mayan propolis demonstrated anti-inflammatory effect through reducing the expression of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and increasing the anti-inflammatory cytokines (Boulechfar & Zellagui, 2023).

5.7 Antiparasitic properties

Propolis also acts against certain parasites, ethanolic extracts of propolis have demonstrated antiparasitic action against *Toxoplasma gondii, Trichomonas spp, Giardia lamblia, and Trypanosoma* (Dantas *et al*., 2006). The potency of propolis was evaluated against giardiasis disease caused by the flagellate protozoan *Giardia duodenalis* which causes severe abdominal symptomatology with chronic diarrhea syndrome. The level of suppression varied with the concentration of the extract. Propolis equally suppressed the adhesion of the parasites (Freitas *et al*., 2006).

5.8 Other activities

Besides the above-mentioned pharmaceutical proprieties, propolis exhibits a plethora of other benefits. Propolis was reported to possess immunomodalatrice activity, in a study using Brazilian propolis, it was demonstrated that propolis administration for 3 days to male

BALB/c mice modulated the activation of the initial steps of the immune response by upregulating toll-like receptor- (TLR-) 2 and toll-like receptor-4 expression and pro-inflammatory cytokines (IL-1 and IL-6) production by macrophages and spleen cells (Silva-Carvalho *et al*., 2015). Additionally, Several preclinical studies demonstrated the therapeutic effects of propolis extracts against allergic inflammation, asthma, allergic rhinitis, atopic dermatitis, and food allergy, which may be partly attributed to their inhibitory effects on the activation of mast cells and basophils (Liew *et al*., 2022).

6 Mechanisms of action of propolis and its polyphenolic compounds against bacteria

The science behind propolis biological mechanism is still a little unclear. Currently, very few studies documented the molecular mechanisms behind propolis's biological effects. Several underlying mechanisms for propolis' antimicrobial activity have been proposed by various research groups, including inhibition of cell division, nucleic acid synthesis, protein synthesis, impeding cytoplasmic membrane function, altering membrane permeability, reducing the ability to form biofilms, bacteriolysis, inhibiting the energy generation pathway, and decreasing bacterial resistance to certain conventional antibiotics (Bouchelaghem, 2022). The study of Torres *et al.* (2018) reported the mechanism of Brazilian propolis against *S. aureus* and *E.coli*, PEE exerted irreversible damage to the bacterial cell membrane, leading to cell death. Similarly, the activity of Russian propolis ethanol extract (RPEE) against *S. aureus* and *E. coli* causes cell lysis and bacterial cell membrane damage within mature biofilms at a concentration of 2–4 μg/mL (Ambi *et al*., 2017).

On the other hand, the effect of propolis on the physiology of *Bacillus subtilis*, *E. coli,* and *R.sphaeroidesa* has been demonstrated by the ethanolic extract of propolis which has an antibacterial effect due to the presence of very active components such as cinnamic acid and flavonoids which uncouple energy transduction from the cytoplasmic member and inhibit bacterial motility (Banskota *et al*., 2001). Some components present in propolis extracts, such as quercetin, galangin, pinocembrin, caffeic acid, benzoic acid, and cinnamic acid probably act on the microbial membrane or cell wall, causing functional and structural damage (Scazzocchio *et al*., 2006).

7 Synergy of propolis with natural or semi-synthetic products

7.1 Synergism with main components

The biological activity of propolis is certainly due to a synergistic effect between its main active components. Generally, the combination of different components of propolis proves to have effective results as a biologically active antimicrobial agent. Many studies reported that propolis exhibited significantly higher efficiency than a single isolated

constituent (Schnitzler *et al*., 2010). Furthermore, Kujumgiev *et al*. (1999) noted that the antibacterial action of individual substances isolated from propolis has lower activity and none of them had greater activity than that of the total extract.

7.2 Synergism with antibiotics

Propolis, in addition to possessing direct antibacterial properties, functions synergistically with conventional antibiotics, increasing their efficiency. In this regard, the combination of propolis with drugs for commercial use is of great interest for the development of new products by the pharmaceutical industry (Sforcin and Bankova, 2011). Several studies have confirmed that propolis exerts a stimulating effect on antibacterial activity when taken in combination with certain antibiotics. The study of Oksuz *et al*. (2005) approved the synergistic activity between ciprofloxacin and propolis against *S. aureus*. Similarly, Lavigne *et al*. (2020) proved that propolis significantly enhances the efficiency of antibiotics used in UTIs.

7.3 Synergism with natural product

Numerous studies have demonstrated the therapeutic properties of propolis combined with natural products. Considering that, the study of Takzaree *et al*. (2016) demonstrated that the application of combined honey and propolis accelerated the wound healing process, shortened the inflammatory phase, and increased tissue granulation and angiogenesis, and thus confirmed the synergetic effect between propolis and honey. Furthermore, The combination of cranberry and propolis supplements significantly reduces the frequency of urinary infections and delays the occurrence of an episode of cystitis (Bruyere *et al*., 2017).

8 Cytotoxicity

Unlike many "natural" remedies, allergic reactions to propolis are unusual; propolis is relatively non-toxic and has a very low level of side effects (Braakhuis, 2019). In a rat model, the median lethal dose of a concentrated propolis extract was estimated at 15 g/kg. Furthermore, the AFSSA (referral 2007-SA-0231) reported that the highest dose without adverse effects (NAOEL) is 1.4 g/kg in animals and that supplementation of 1.95 g/day for 30 days has no side effect (Boisard, 2014). In addition, oral administration of 5g/kg of ethanolic propolis extract to a rat model for 8 weeks didn’t exert any harmful effects and showed no mortality and no toxic effect (Ramadan *et al*., 2012).

Chapter III

Experimental

1 Propolis sampling, extraction process

The current study analyzes eight samples of propolis that were harvested from different northeastern Algerian areas between 2017-2021. The hallmarks of each sample including the collection period, site, and geographical origin are presented in figure 9. The hand-collected propolis samples were ground and stored in freezing conditions (−4 ◦C) until extraction processing.

A proportion of 20 g of each propolis sample was cut and extracted with 80% ethanol and shaken at room temperature for 24 hours. To extract the maximum amount of bioactive compounds, the extraction process was repeated four times. Following that, the generated extracts were filtered and evaporated using rotavapor (45 C°). The extracts were stored under dry conditions at 4 °C until analyzed.

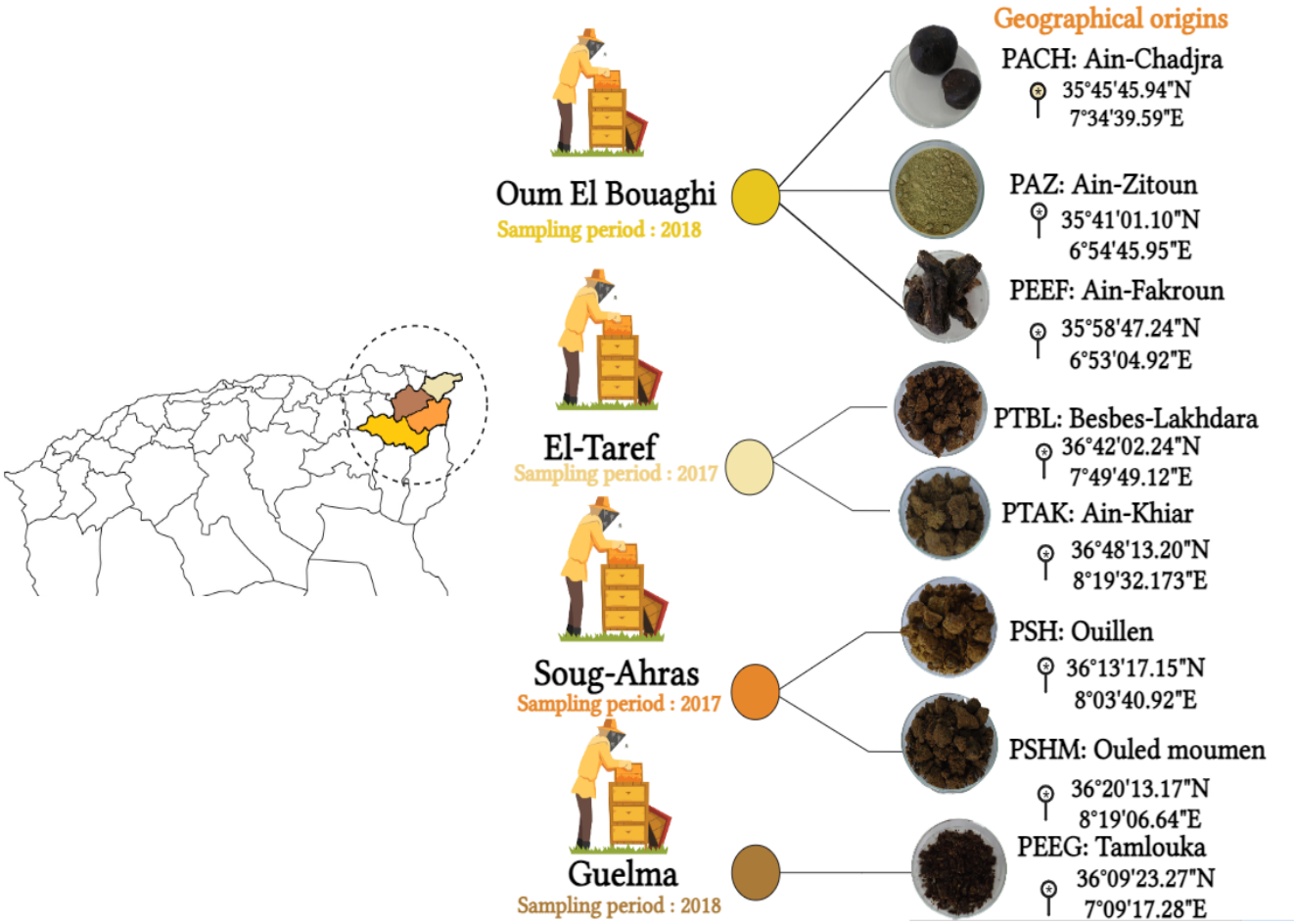


Figure 9. The hallmarks of propolis samples (Collection period, site, and geographical origins).

2 Chemical profile

2.1 Total phenolics content (TPC)

The quantification of TPC is based on the use of the Folin-Ciocalteu reagent which contains the complex phosphomolybdic acid ($H_3PMO_{12}O_{40}$)/ phosphotungstic acid ($H_3PW_{12}O_{40}$). In alkaline medium, this complex is reduced after the transfer of electrons from phenolic compounds to form a blue chromophore which is a mixture of blue oxides of tungsten (W_8O_{23}) and molybdenum (Mo_8O_{23}). This latter can be easily detected spectrophotometrically where the maximum absorption is proportional to the concentration of polyphenols (Lamuela-Raventós, 2018).

A volume of 200 µL of propolis ethanolic extract (PEE) (0.5 mg/mL) was mixed with 1 ml of FC reagent. After thorough shaking and 10 min of incubation in darkness, 800 µl of 7.5% sodium carbonate was added. Then, the mixture was subjected to a dark incubation for 90 min. The absorbance measurements were performed at a wavelength of 760 nm. The TPC of PEE was expressed as µg of gallic acid equivalents per milligram of extract (µg GAE/mg E) from a calibration curve with gallic acid (Singleton & Rossi, 1965)

2.2 Total flavoinds content (TFC)

TFC was quantified according to the described methodology of Topçu *et al*. (2007). The TFC coloring approach used the basic concept of the aluminum chloride colorimetric method. The complexation of flavonoids with aluminum chloride ($AlCl_3$) and the spectrophotometric measurement of the generated yellow complex is a frequently used technique for flavonoid quantification (Silva-Carvalho *et al*., 2015). A volume of 50 µl of the PEE at a final concentration of 0.5mg/ml was mixed with 130 µl of methanol,10µl of $AlCl_3$, and 10 µl of potassium acetate. The mixture was allowed to stand at room temperature for 40 min. The absorbance of the reaction mixture was measured at 415 nm. The results were expressed as µg of quercetin equivalent per milligram of extract (µg QE/mg E).

2.3 HPLC-DAD: High-performance liquid chromatography

The phenolic content was determined using HPLC-DAD analyses. The PEEs were separated using HPLC a Shimadzu high-performance liquid chromatography (Shimadzu Cooperation, Japan). The separation was carried out on an Inertsil ODS-3 (4 µm, 4.0 mm x 150 mm) analytical column and an Inertsil ODS-3 guard analytical column was used for content separation at 35°C. The solvent program was as follows: the mobile phases were aqueous acetic acid, 0.1% (A) and methanol (B). The flow rate was set at 0.6 mL/min, and 20µl of PEE was injected and filtered through a 0.45µm filter. The propolis extracts (8 mg)

were dissolved in 1 mL (8 $mg.ml^{-1}$). Detection was carried out using a diode array detector (DAD) at a 254 nm wavelength. The identification of phenolics was confirmed by comparing the retention times and UV–Vis spectral data to 42 known previously injected standards (Appendix 1). Results were expressed as milligrams per gram of dry weight (Tokul-Ölmez *et al*., 2020).

3 Determination of antioxidant activity

The antioxidant activity of propolis extracts was tested using six complementary tests; namely, DPPH radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC), ferric reducing power assay, ABTS cation radical decolorization assay, galvinoxyl radical (GOR) scavenging assay, and phenanthroline assay.

3.1 DPPH free radical scavenging assay

The DPPH assay is one of the most widely employed methods used to assess antioxidant activity since it is useful and requires mild experimental conditions. Generally, this method is based on the reduction of purple DPPH radical by an antioxidant via a hydrogen atom transfer mechanism to cause a change in the color to stable pale yellow DPPH molecules. To evaluate the antioxidant activity, a UV-Vis spectrophotometer is used to quantify the remaining purple DPPH radical at 515 - 520 nm (Sirivibulkovit *et al*., 2018).

DPPH radical scavenging activity was achieved using the method described by Blois, 1958. A volume of 40 µl of different dilutions of propolis extract solubilized in methanol was added to 160 µl of DPPH solution (60 µM) prepared in methanol. Then, the obtained mixtures were incubated for 30 min in obscurity. DPPH scavenging results were expressed as 50% inhibition concentration (IC_{50}), and compared with the antioxidant standards (BHA and BHT). The lower the IC_{50}, the higher the "antiradical efficiency". The DPPH radical scavenging was calculated using the equation given below:

DPPH free radical scavenging activity (%) = [(OD Control-OD Sample)/(OD Control)]x100

3.2 ABTS scavenging activity

The ABTS•+ is created by reacting a strong oxidizing agent such as potassium persulfate with the ABTS salt, resulting in a green–blue ABTS chromophore. The reduction of blue-green ABTS•+ by hydrogen-donating antioxidant is assessed by the suppression of its characteristic long-wave absorption spectra; during this reaction, the blue ABTS radical cation is transformed back to its colorless neutral state (Boligon *et al*., 2014).

The spectrophotometric analysis of ABTS scavenging activity was done following the method of Re *et al*. (1999). In short, the ABTS solution was prepared by reacting 7 mM

ABTS in water and 2.45 mM potassium persulfate, the mixture was then stocked for 16 hours in darkness at room temperature. Before use, the absorbance of ABTS solution was adjusted using H_2O to 0.700±0.020 at 734 nm. Then, 160 µl of the diluted ABTS•+ solution was mixed with 40 µl of each propolis extract at different concentrations prepared in methanol. After 10 min, the absorbance was measured at 734 nm. the results were established as a 50% inhibition concentration (IC_{50} = µg/ml).

3.3 Cupric ion reducing antioxidant capacity (CUPRAC)

The cupric-reducing antioxidant capacity is founded on the ability of the antioxidants to reduce the complex neocuproine-copper, therefore resulting in a chromogenic complex Cu(I)-Nc which absorbs at 450 nm (Saci *et al*., 2020). In this reducing assay, the reactive Ar-OH groups of polyphenols and other antioxidants are oxidized to the corresponding quinones and Cu^{2+}–neocuproine is reduced to the Cu^{+}–neocuproine complex, which is intensely colored in yellow–orange.

The antioxidant capacity using the CUPRAC assay was determined following the method described by Apak *et al*. (2004). In a 96-well plate, 50 µl of $CuCl_2$ (10 mM) solution was mixed with 50 µl of the neocuprine solution (7.5 mM) and 60 µl of ammonium acetate buffer solution (NH_4Ac, 1 M, pH = 7.0). Subsequently, a volume of 40 µl of the PEE prepared in methanol at different concentrations was added to the initial mixture. The microplate was then incubated in the darkness for 60min. The absorbances were measured at 450 nm and the results were expressed as $A_{0.5}$ (µg/ml). BHT and BHA were used as antioxidant standards for comparison of the activities.

3.4 Ferric reducing power assay

The reducing power of PEE was performed using Oyaizu, (1986) method. The principle behind the reducing power assay method is that compounds with reduction potential combine with potassium ferricyanide (Fe^{3+}) to generate potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric-ferrous complex with an absorbance maximum at 700 nm. Depending on the reducing power of each molecule, the yellow color of the test solution changes to various colors of green and blue in this assay.

An aliquot of 10 µl of each propolis extract solution at different concentrations was mixed with 40 µl of phosphate buffer (0.2 M, pH 6.6), and 50 µl of potassium ferricyanide (1%). After 20min of incubation at 50C°, 50 µl of trichloroacetic acid (10%), 40 µl of distilled water, and 10 µl of ferric chloride solution (0.1%) were added to the previous mixture. The absorbance was read at 700 nm, while BHA and BHT were used as a standard.

3.5 GOR scavenging assay

Galvinoxyl is a stable free radical with a dark yellow color and high absorption. Because of its interaction with antioxidant chemicals, which transfer electrons or protons to it, this radical loses its native color. The reduced form of galvinoxyl produces a bright yellow solution with low absorbance. 40µl of different concentrations of propolis sample was added to 160 µl of 0.1 mmol.L^{-1} methanol solution of Galvinoxyl. The obtained solutions were incubated for 2h at room temperature. The absorbance was read at 428 nm. BHA and BHT were used as antioxidant standards (Shi *et al*., 2001).

3.6 Phenanthroline assay

The phenanthroline method is based on the reduction of Fe^{3+} to Fe^{2+} ion by an antioxidant. The Formed Fe^{2+} ion then reacted with ortho-phenanthroline to a red-orange complex (YEFRIDA). The reduction activity of the extracts in terms of the production of the Fe+2-phenanthroline complex was determined using the method published by Szydłowska-Czerniak *et al*. (2008).

A volume of 10µl of PEE was added to 50 µl of FeCl (0.2%), and 30 µl of 1,10-phenanthroline solution (0.5%), the volume was made up to 200µl with methanol. Incubation was done in the dark for 20min. The mixture was incubated for 20 min at 30 ◦C. The absorbance was determined at 510 nm. The results were calculated as $A_{0.5}$ (µg/ml), corresponding to the concentration indicating 0.50 absorbance.

4 Microbiological investigations

4.1 Study population

Samples were collected from the community medical Hospital in the province of Oum El Bouaghi, Algeria, between September 2017 and January 2022. All patients were referred to the laboratories by urologists. Interviews of all the suspected cases of $rUTI_s$ were performed using a structured questionnaire based on the target population (Appendix 2). It demonstrates the identification number of the patient, age, and urinary signs and symptoms. The study was conducted on 104 female patients, ages 18–55 year, with symptoms of $rUTI_s$. The patients in the target population are characterized by ≥2 episodes of UTI within six months or 3 infections within the preceding 12 months with different symptoms; dysuria, pelvic pain, lower abdominal discomfort, and no concurrent major health concerns. However, certain criteria were excluded, including pregnancy and the history of antibiotic intake within 2 weeks before urine sampling.

4.2 Sample collection and bacterial identification

Urine samples were collected in sterile plastic bottles and transmitted to a laboratory within 2 hours. Only patients with positive urine cultures ($\geq 10^3$ CFU/ml) were studied. Identification was done based on culture characteristics, gram stain, and biochemical tests by the API kit (Biomerieux, France). API results were simuled using the UPBM online identifier (https://lab.upbm.org/) (Appendix 3).

4.3 Susceptibility to antibiotics and selection of resistant strains

This part of the study aims to select MDR uropathogens. Therefore, the antimicrobial susceptibility test was performed by the disc diffusion method using Muller-Hinton agar according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST, 2020). Twenty-seven antimicrobial agents were tested according to the type of isolated bacteria.

Amikacine(AK:30µg),Amoxicillin(AML;30µg),Ampicillin(AMP;10µg),Aztreonam(AT;30µg),Cefotaxim(CTX;30µg),Cefoxitin(FOX;30µg),Ceftazidime(CAZ;30µg),Chloramphenicol(C;30µ),Ciprofloxacin(CIP;5µg),Colistin(CL;10µg),Cotrimoxaole(COT;25µg),Erythromycin(E;15µg),Fosfomycin(FOS;50µg),Gentamicin(GEN;10µg),Kanamycin(K;30µg),Nitrofurantoine(FTN;300µg),Ofloxacin(OF;5µg),Oxacillin(OX;1µg),PenecilliG(P;10µg),Rifampicin(RIF;5µg),Streptomycin(S;10µg),Tetracyclin(TE;30µg),Ticarcilinclavulanicacid((TCC;75/10);,Ticarcillin(TI;75µg),Tobramycin(TOB;10µg),Trimethoprim.sulfamethoxazole(SXT;25µg),Vancomycin VA;30µg).

Using agar disc diffusion method, against the isolated uropathogens. Overnight cultures were used and the optical density was adjusted to 10^8 UFC/ml (0.5 McFarland). Petri plates containing sterile Mueller Hinton (MH) agar were inoculated with the isolated uropathogens using a sterile swab. Then, sterile antibiotic discs were deposited on the inoculated Mueller Hinton agar plates. Finally, the plates were incubated at 37C° for 24h. The diameters of the inhibition zone were measured. The interpretation of the results was performed according to EUCAST, and the bacterial strains were categorized as (R) resistance or susceptible (S).

To select the multi-drug resistant uropathogens isolates resistant to more than three different groups of antibiotics were considered as MDR (Magiorakos *et al*., 2012). Moreover, the MAR index (MARI) was calculated. The MARI was defined as a/b, where "a" is the number of antibiotics to which a particular isolate was resistant and "b" is the total number of antibiotics tested (Krumperman, 1983).

4.4 Antimicrobial activity of propolis ethanolic extract

4.4.1 Disc diffusion method

The antibacterial assay was assessed following the disc diffusion method of the National Committee of Clinical Laboratory Standards (NCLLS) against 25 MDR uropathogens. Overnight bacterial cultures (0.5 McFarland) were spread evenly on Mueller-Hinton (MH) agar. Then, sterile filter discs (diameter 6 mm) were impregnated with 20 µl of propolis extract (20mg/ml) prepared in dimethylsulfoxide (DMSO). All the experiments were conducted in triplicate. Finally, the plates were incubated at 37C° for 24h. The diameters of the inhibition zone were measured. DMSO was used as a negative control. Ampicillin (Amp, 10µg/disc) and Amikacin (AK, 30µg/disc) were used as a positive control (Murray, 1995).

4.5 Determination of MIC and MBC

A total of 25 resistant uropathogens were used to evaluate the minimal inhibition concentration (MIC) and the minimal bactericidal concentration (MBC) using the microdilution assay. Bacterial suspensions were prepared according to 0.5 McFarland's standard (10^8 UFC/ml). PEE was dissolved in dimethylsulfoxide (DMSO) and serial dilutions were made (20 to 0.625 mg/ml). 10µl of bacterial suspensions were inoculated into the wells of 96-well microtitre plates containing Muller-Hinton broth (170µl), then 20µl from the serial dilutions of PEE were transferred to each well. The lowest concentration of extract showing no visible bacterial growth is called MIC.

MBC was determined by overlying 10µl of samples from wells with no growth on nutrient agar. After incubation, the lowest concentration with no bacterial growth was defined as MBC. Several wells were reserved in each plate for negative control (inocula + medium), sterility (no inocula added), and the DMSO inhibitory effect (Simionatto *et al*., 2009)

4.6 Assay of biofilm formation

4.6.1 Classification of adherence

A semi-quantitative adherence assay was conducted on a 96-well plate to determine each MDR strain's potential to create a biofilm. Accordingly, each isolate was grown in trypticase-soy broth (TSB) supplemented with 0.5% glucose. After 24h of incubation at 37 °C, 200 µl of the diluted cell suspension (1:100) was dispensed into each well and incubated for 48h without shaking at 37 °C. After incubation, the wells were gently rinsed three times with distilled water to remove the planktonic bacteria and dried. Then, the adherent biofilm was stained with 200 µl of 0.1% crystal violet solution for 15 min. Once again, the wells were rinsed three times. Finally, the plates were air-dried, and 200 µl of 95% ethanol and 33%

glacial acetic acid were added to each Gram-negative and Gram-positive bacteria wells, respectively. The optical density (OD) was measured at 570 nm (Mack *et al*., 2001).

Biofilm formation was classified into four categories: non-adherent (OD < ODc); weakly-adherent (ODc < OD < 2xODc); moderately-adherent (2xODc < OD < 4xODc); strongly-adherent (4xODc < OD); with ODc: the cut-off OD (three standard deviations above the mean OD of the blank test) (Stepanović *et al*., 2000).

4.6.2 Influence of propolis extract on biofilm formation

A microplate anti-biofilm assay was used to examine the anti-adhesion activity of PEE. Only high biofilm producers were selected for this test. An aliquot of 10 µl of overnight isolate cultures was dispensed into each well of 96-well plates in the presence of 170 µl of MHB supplemented with 2% glucose (w/v) and containing 20 µl of PEE at concentrations ranging from 20 to 0.625 mg/mL. The test was carried out using the crystal violet assay (Merritt *et al*., 2005). The OD was measured at 550 nm, and the percentage of biofilm inhibition was calculated using the Eq. (1):

$$\text{Biofilm inhibition (\%)} = [(\text{OD Control-OD Sample})/(\text{OD Control})]\text{x}100$$

4.7 Anti-urease activity

Urease inhibitory activity was conducted following the method described by Taha *et al*. (2018). In a 96-well microplate, 10 µl of PEE, 25 µl of urease enzyme, and 50 µl of urea substrate were mixed. After 15 min of incubation at 30 °C, 45 µl of phenol reagent and 70µl of alkaline reagent (2.85% NaOH and 4.7% NaOCl) were added. The thiourea was used as a standard. After 50 min, the absorbance of the solution was measured at 630 nm by the use of 96- well microplate reader. The urease inhibitory activity was calculated using the following equation:

$$\% \text{ Inhibition} = [(\text{AbsControl} - \text{AbsSample})/\text{AbsControl}] \times 100$$

4.8 Quorum-sensing

4.8.1 Violacein inhibition assay

Chromobacterium violaceum 12475 (CV12475) was used to test the effect of PEE on violacein production. 10 µl of an overnight culture of CV12472 (adjusted to 0.4 OD at 600 nm) was added into wells containing 170 µl of luria bertani broth (LBB) and incubated in the presence of 20 µl of PEE. Plates were incubated at 30 °C for 24h (Choo *et al*., 2006). LB broth containing CV12472 was used as a positive control. Plates were incubated at 30°C for 24 h. The inhibition of violacein production was observed and measured using a microplate

reader (OD=585nm) and the percentage of violacein repression was calculated by following the formula below:

$$\textit{Violacein inhiition (\%)} = \frac{OD\ Control - OD\ Sample}{OD\ Control} x100$$

4.8.2 Bioassay for QSI activity using CV026

Chromobacterium violaceum 026 (CV026), this mutant strain is enable to produce violacein in response to the addition of exogenous N-hexanoyl-L-homoserine lactone (C6-HSL). To achieve this test, PEE was employed to disrupt bacterial communication mechanisms following the method specified by (Koh & Tham, 2011). Briefly, 5 ml of warm molten soft agar was mixed with 100 µl of an overnight culture of CV026, 20 µl of C_6HSL, and 10 µl of kanamycin. After thoroughly mixing, CV026 was poured over the surface of a solidified LBA plate. Six-millimeter wells were made through the LBA and filled with 50 µl of sub-MIC concentrations of PEE. The plates were incubated at 30 °C for 3 days. DMSO was used as a negative control. The QSI is detected by the appearance of a white or cream-colored halo around the wells, surrounded by a purple lawn.

5 Anti-inflammatory activity

The anti-inflammatory activity of PEE was assessed using the bovine serum albumin (BSA) denaturation approach described by (Kandikattu *et al*., 2013). Diclofenac sodium was incorporated as an anti-inflammatory standard. A reaction mixture composed of 0.5 ml of BSA solution (0.2%) prepared in Tris-HCl Buffer Saline (pH 6.6) and 0.5 ml of different PEE concentrations (250-2000µg/ml) was prepared and incubated at 37°C for 15 min and then heated in a water bath at 70 °C for 5 min. Each experiment was released in triplicate and the absorbances were measured at 660 nm. The test samples were replaced with double distilled water for the preparation of the control solution, and the percentage inhibition of protein denaturation was determined using the following equation:

% I= [[Ac-(As-Aw)]/Ac] x 100.

As: Absorbance of samples: 0.5 ml extract + 0.5 ml BSA

Aw: Absorbance of white 0.5 ml extract + 0.5 ml Tris-HCL (pH: 6.8)

Ac: absorbance of control: 0.5 H2O + 0.5 ml BSA (The control represents 100% of proteins denaturation).

6 Cytotoxicity

Brine Shrimp Lethality assay was used to evaluate the toxicity of PEE. The cytotoxicity effect of four selected extracts was carried out against *Artemia salina* eggs. This latter was revived in a lighted incubator at 30°C in a becher glass containing one liter of

lukewarm seawater. The fraction in various concentrations (0.5, 1, 2, 4 mg/ml) was dissolved in DMSO-seawater (4%). Afterward, 100 µl of each extract dilution was transferred into a glass tube containing 4.9 ml of seawater and 10 vials larvae of brine shrimps. After 24 hours of incubation under illumination, the survived larvae were counted and the percentage of mortality (death) at each dose and also the control (blank) were determined (Meyer *et al*., 1982).

Chapter IV
Results and discussion

1 Phytochemical screening

1.1 Extraction yield

The extraction process and the used solvent play critical roles in increasing extract yield and bioactivity. This procedure allows the isolation of specific components from crude natural products and provides an indication of the products extractability under various conditions (Adam *et al*., 2019). The extraction yields of the studied propolis extracts are presented in table 2.

Table 2. Extraction yield of different propolis extracts.

Samples	Extraction yield (%)
PTBL	79.50%
PTAK	46.80%
PSH	38.25%
PSHM	25.35%
PEEG	33.30%
PEEF	16.35%
PACH	56.25%
PAZ	17.55%

Abbreviations: **PTBL:** Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

As can be noted, propolis extraction showed variable yields, a dense brown extract was obtained within the extracts PTBL, PTAK, PSH, PSHM, PEEG, and PACH, while a yellowish extract was obtained within PAZ and PEEF extracts. The yield of propolis ethanolic extracts ranged from 16.35 to 79.50%. The optimal extraction yield was obtained with PTBL extract with a value of 79.50%, while the lowest yield was recorded within PEEF extract (16.35%).

Propolis extracts showed variable yields, this results were different from those reported in different local regions. (Boulechfar & Zellagui, 2023) reported a lower yield of PEE ranging from 9.50 to 39.00%. In addition, a yield ranging from 14% to 37% was reported with propolis from Constantine, Tebessa, Tipaza, and El-Oued regions. Moreover, a different yield was recorded within propolis harvested from different Algerian local regions such as Boumerdes, Mostaganem, Bejaia, and Ghardaia, with a yield ranging from 15.57 % to 41.10%.

The extraction yield of the active components from propolis materials is well-known to be affected by several conditions. The fact that the studied propolis extracts were harvested from different geographical regions suggests that the characteristics of these propolis samples were diverse thus underlying different yields. Also as Abduh *et al*., (2023) indicated that

extraction yield is strongly affected by the extraction method, the concentration of ethanol affects the type of compounds extracted. Furthermore, the properties of the solvent, the temperature and duration of the extraction process, the composition and physical characteristics of propolis samples can all greatly affect the yield and quality of the final extracts. Additionally, these variations could be attributed mostly to environmental factors such as botanical origins, and bee species (Boulechfar & Zellagui, 2023).

1.2 Quantification of total phenolic and flavonoid contents

The total phenolic contents of PEE were performed in order to reveal the amounts of propolis bioactive components. In this study, the presence and the amounts of phytochemicals were assessed depending on colorimetric assays which were based on the visual observation of color change. The folin-ciocalteu procedure was performed to determine the amount of TPC, whereas TFC was quantified using the aluminum chloride method.

The quantification of TPC is based on the use of the FC reagent which contains the complex phosphomolybdic/ phosphotungstic acid. In alkaline medium, this complex is reduced after the transfer of electrons from phenolic compounds to form a blue chromophore. This latter can be easily detected spectrophotometrically where the maximum absorption is proportional to the concentration of polyphenols. Gallic acid is commonly used as the reference standard substance (Lamuela-Raventós, 2018).

A calibration curve of gallic acid showing a linearity of the absorbance according to the concentrations was employed to determine the TPC of propolis extracts (Figure 10). Each sample was analyzed in triplicates and the results were expressed as µg gallic acid equivalent per milligram of propolis extract (µg GAE/mg extract). The concentrations of the total polyphenols are calculated from the regression equation of the calibration range established with gallic acid of type: $y = 0.0034 x + 0.1044$ knowing that $R^2 = 0.9972$.

According to the obtained results shown in table 3, all propolis samples represented high amounts of phenolic content except for PAZ sample. Interestingly, The PTBL extract showed the highest phenolic content with an amount of 1117.23 ± 2.03 µg GAE/mg E compared to the remaining extracts.

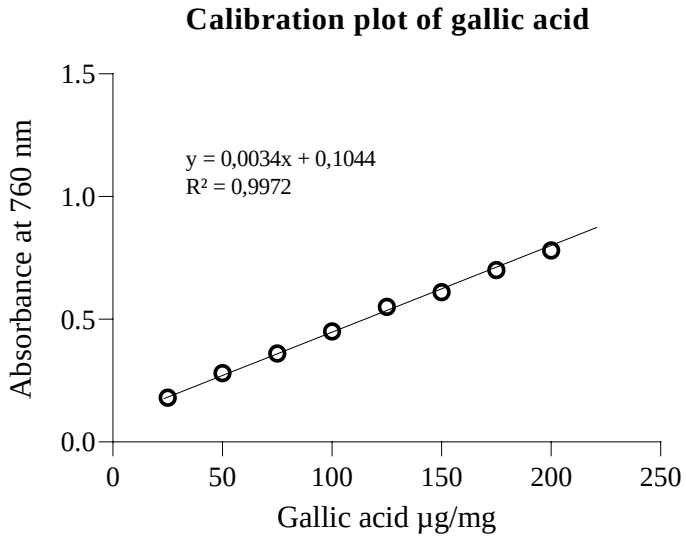


Figure 10. Calibration curve of gallic acid for total polyphenol content.

Following that, propolis from the same region (PTAK) recorded the second-highest amount with a value of 791.64±6.11 µg GAE/mg E. Furthermore, PSH, PSHM, and PACH extracts showed a significant varying level of polyphenolic content, and the amounts of TPC in decreasing order were: 698.80±2.88 µgGAE/mg E, 477.33±2.71 µgGAE/mg E, 415.67± 2.80 µgGAE/mg E. The PEEF and PEEG extracts gave low content compared to the former extracts. Whereas, PAZ extract gave the lowest content (111.54±4.49 µgGAE/mg E).

Table 3. TPC and TFC of propolis ethanolic extracts.

Propolis extracts	TPC (µg GAE/mg E)	TFC (µg QE/mg E)
PTBL	1117.23 ± 2.03	321.31±4.86
PTAK	791.64±6.11	260.83± 3.97
PSH	698.80±2.88	211.04±2.06
PSHM	477.33±2.71	160.55±2.94
PACH	415.67± 2.80	169.37±5.89
PAZ	111.54±4.49	96.45±1.76
PEEF	136.35±3.56	126.38± 1.62
PEEG	188.50±0.33	144.23± 1.03

Data are expressed as Mean±SD of three parallel measurements ($p < 0.05$). Abbreviations: TPC: Total phenolic content is expressed as µg Gallic acid equivalent/mg of extract. TFC: Total flavonoid content is expressed as µg Quercetin equivalent/ mg of extract. Abbreviations: **PTBL:** Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

For total flavonoid content (TFC), the TFC coloring approach used the basic concept of the aluminum chloride colorimetric method. The complexation of flavonoids with aluminum chloride ($AlCl_3$) and the spectrophotometric measurement of the generated yellow complex is a frequently used technique for flavonoid quantification (Silva *et al*., 2015).

The employed standard used for the establishment of the calibration curve for TFC quantification was quercetin. The obtained results were expressed as µg of quercetin equivalent per milligram of propolis extract (µg QE/mg extract). The calibration curve was established with a linear regression coefficient. The flavonoid levels of the aqueous and ethanolic extracts of propolis were obtained from the calibration curve (Figure 11) which follows an equation of type: y = 0.0048x knowing that R^2 = 0.997.

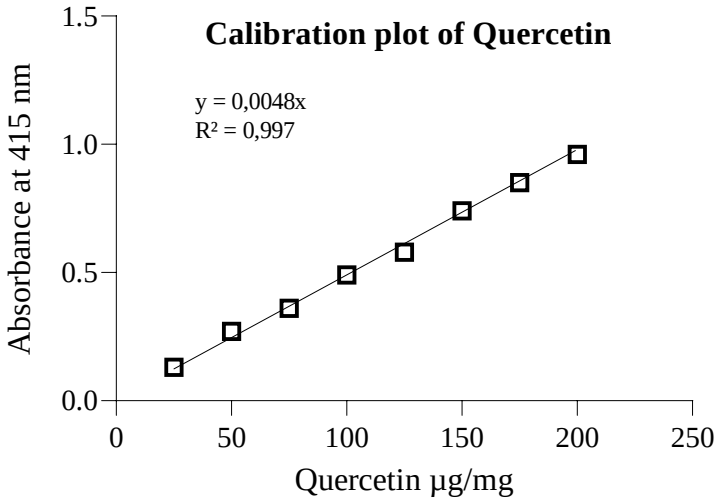


Figure 11. Calibration curve of quercetin for TFC quantification.

Regarding the results of TFC, PTBL extract dominated showing a significant amount of flavonoids (321.31±4.86 µg QE/mg E) followed by PTAK extract (260.83± 3.97 µg QE/mg E). The remaining extracts gave less content showing variable amounts with values ranging from 96.45±1.76 µgQE/mg E to 211.04±2.06 µgQE/mg E.

The examination of the essential active components in natural products is an important step in determining the quality of raw materials and validating the efficacy and safety of their pharmaceutical benefits.

As can be noted from the obtained results these samples exhibited high TPC and TFC concentrations thus underlying a high-quality category. Indeed, studies dealing with Algerian propolis demonstrated variable polyphenolic content showing significantly higher and less

concentrations compared to the studied extracts. For instance, PTBL and PTAK extracts from EL-Taref region recorded the best TPC, this extract shows the highest content compared to propolis from different locations in Algeria, e.g., Annaba (Nedji & Loucif-Ayad, 2014), El-Menia and Skikda (Boulechfar *et al*., 2022), Tlemcen and Mascara (Benhanifia *et al*., 2013). Moreover, the content of TPC of PACH extract collected from the Oum El Bouaghi region showed a double content compared to the study of Boulechfar and coworkers. (2022) who studied propolis from the same region. Conversely, the content recorded within PAZ (111.54±4.49 µg GAE/mg E) contradicts the results of Boulechfar *et al*. (2022) who reported high TPC (270.62±1.91 µg GAE/mg E) which show the intense variability in propolis active contents. Furthermore, these findings demonstrate the high TPC of Algerian propolis when compared to propolis from other parts of the world, such as Brazil, Bulgaria, and China (Shehata *et al*., 2020).

Regarding TFC, the results also showed that all extracts exhibited high content, greater than those reported by Boulechfar *et al*. (2022) ranging from 46.66±0.98 to 76.98±0.26 µg QE/mg E. Similarly, these extracts showed greater TFC compared to propolis from different regions of Annaba ranging from 58.99±2.49 to 91.44±4. µg QE/mgE (Nedji & Loucif-Ayad, 2014). On the other hand, a study carried out by Bouaroura *et al*. (2021) on methanolic extracts of propolis from different local regions of Algeria: Constantine, Boumardess, Mila, and Tebessa where the total flavonoid content ranged between 6.22±0.20 to 73.26±2.98 µg QE/mg E. Overall, the results show a high flavonoid concentration, indicating that these extracts might be considered a valuable reservoir of bioactive chemicals.

Despite the fact that the propolis samples were extracted using the same procedure and were collected from separate areas of the same region; PTBL and PTAK (El-Taref), PSH and PSHM (Souk-Ahras), PEEF, PAZ and PACH (Oum Bl Boughi), a remarkable variability in polyphenolics contents was recorded. Indeed, these differences also included all samples when compared to each other's.

Referring to previous research dealing with the possible explanation of these differences, many studies stated that this variation is mainly attributed to several factors such as the type of botanical flora on which the bees forage (Bayram *et al*., 2020); this predominant factor is most likely responsible for the considerable disparity in polyphenolic contents. Indeed, this factor has a direct impact on propolis proprieties. For instance, based on physical characteristics several research has shown that the intensity of propolis extracts color changes according to its polyphenolic content; specifically, dark propolis color reveals a high

concentration of polyphenolics and vice versa (Revilla *et al*., 2017; Boke Sarikahya *et al*., 2022). Considering that, the studied extracts are characterized by a dark brown color (Figure 9) which proves their exhibition of high polyphenolic content. Furthermore, this significant variance reflects the very extensive spectrum of plant resins foraged by honey bees. In addition, honey bee genetics, food availability, hive structure, and environmental factors, are among the contributing factors (Auley *et al*., 2021).

1.3 Identification and quantification of phenolic content using HPLC-DAD analyses

The identification of the chemical content of propolis extracts is of paramount importance for determining their beneficial properties. In this regard, the most commonly employed method for the determination of polyphenolic content is the performance of High-Performance Liquid Chromatography (HPLC) coupled with Diode Array Detection (DAD). This reliable technique allows the separation, identification and quantification of natural complex products (Dimcheva *et al*., 2019). Furthermore, this approach facilitates revealing chemical similarities and differences among samples. The identification of phenolic was confirmed by comparing the retention times and UV–Vis spectral data to 42 known previously injected standards. Results were expressed as milligrams per gram of dry weight of propolis.

The HPLC–DAD chromatograms are presented in figure 12, whereas the identified compounds are listed in table 4. By comparing the different peaks of different propolis extracts in terms of the retention time of different known compounds, we can remarkably note that the studied extracts have distinctive profiles showing a qualitative-quantitative phytochemical differences. Overall, 19 out of the 42 reference standards employed were detected among the studied extracts. Regarding the content of PSH extract, 14 chromatographic peaks were identified with an abundance of cynarin (12.68 mg/g) and caffeic acid (11.36 mg/ml). On the other hand, 11 compounds were detected in PTAK, PSHM, and PACH extracts, with an abundance of cynarin (5.99 mg/g) and ellagic acid (5.30 mg/g) for PTAK, chrysin (10.50 mg/g) and cynarin (6.23 mg/g) for PSHM, cynarin (6.05 mg/g) and chrysin (4.32 mg/g) for PACH. While in PTBL extract 10 compounds were detected and the highest amount referred to caffeic acid (20.36 mg/ml) and cynarin (7.33 mg/g). Whereas only 6 chemicals were identified in PAZ extract with an abundance of cynarin (6.02 mg/g). It is worth noting that in terms of qualitative contents, almost all the studied extracts shared similar contents such as protcatechic acid, cynarin, quercetin, apigenin, naringenin, hesperitin, and kampferol. However, many identified components were exclusively detected only in specific

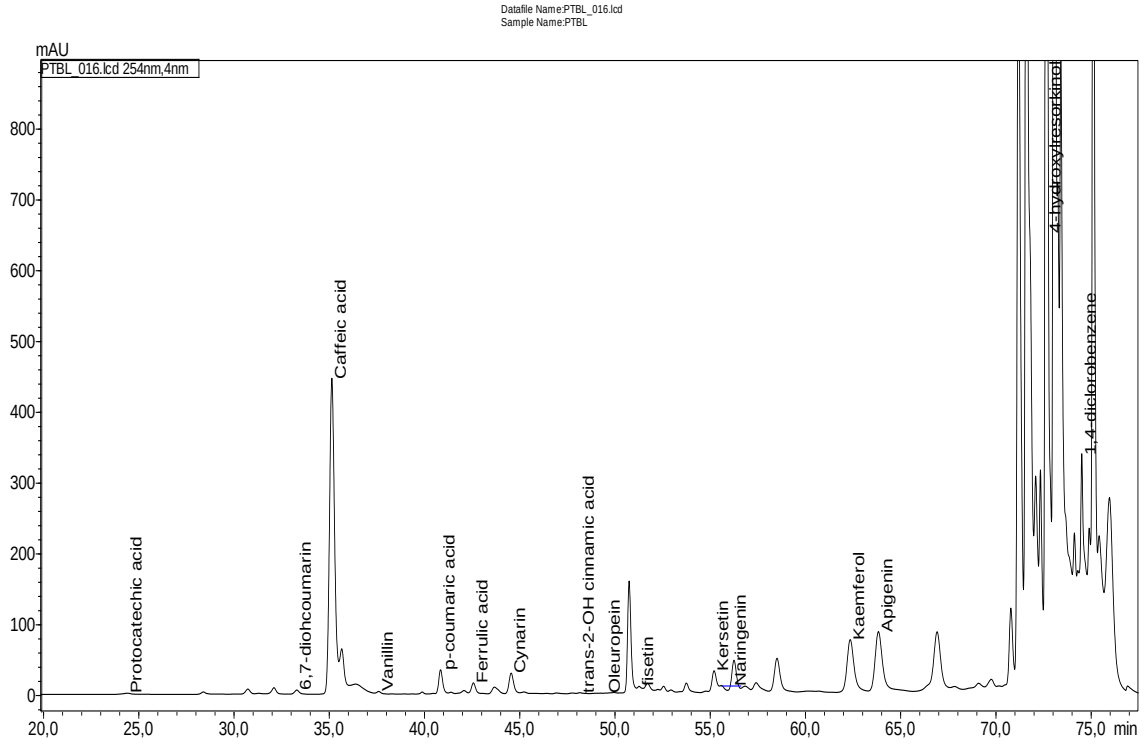
samples, notably chlorogenic acid and oleuropein were only detected in PSH extract. While vanillic acid and luteolin were only detected among PSHM extract content. Otherwise, p-coumaric acid and fisetin were found only in PTBL and PSH extracts. Interestingly, cynarin was among the most abundant components in all studies extracts.

Table 4. Quali-quantitative analysis of polyphenols of PEE using HPLC-DAD analyses.

	Compound	RT	PTBL	PTAK	PSH	PSHM	PACH	PAZ	PEEF	PEEG
1	Protocatechic acid	22.39	0.10	0.29	0.07	0.24	0.07	TR	0.04	0.03
2	Theophlline	29.44	-	TR	TR	-	-	-	-	TR
3	4-hydroxy-benzoic acid	31.69	-	-	0.00	0.01	TR	TR	-	TR
4	6,7-dihydroxycoumarin	33.43	TR	-	TR	Tr	-	-	-	-
5	Vanilic acid	34.68	-	TR	TR	0.08	TR	TR	TR	TR
6	Caffeic acid	35.19	20.36	0.92	11.36	TR	TR	TR	TR	1.14
7	Vanillin	37.12	TR	TR	TR	TR	TR	TR	TR	-
8	Chlorogenic acid	38.88	-	-	0.18	-	-	-	-	-
9	P-coumaric acid	40.81	1.14	-	2.60	TR	TR	-	-	TR
10	Ferrulic acid	42.92	0.42	0.11	1.33	TR	0.26	TR	-	-
11	Cynarin	43.85	7.33	5.99	12.68	6.23	6.05	6.02	5.96	6.12
12	Coumarin	44.91	-	-	-	TR	TR	-	-	-
13	Prophylgallate	46.98	-	-	-	-	-	-	-	-
14	Rutin	47.52	-	0.16	-	TR	0.07	-	0.74	-
15	Trans-2-cinnamic acid	48.07	TR	TR	-	TR	TR	-	-	TR
16	Ellagic acid	50.00	-	5.30	-	3.78	3.78	-	-	-
17	Fisetin	51.24	0.18	-	0.41	-	-	-	-	-
18	Quercetin	55.42	0.90	0.66	0.90	1.35	0.52	0.52	0.34	0.38
19	Trans cinnamic acid	55.92	-	-	-	TR	-	-	-	-
20	Luteolin	57.87	-	-	-	0.32	-	-	-	-
21	Kaempferol	62.48	2.70	0.24	0.90	0.07	0.11	0.05	0.03	0.93
22	Apigenin	64.07	4.54	-	1.57	0.27	0.19	0.17	TR	0.04
23	Chrysin	72.77	-	4.18	9.07	10.50	4.32	-	0.59	-
24	Hesperedin	47.38	-	-	-	-	-	-	-	0.58
25	Oleuropein	49.54	TR	-	0.26	-	-	-	-	-
26	Naringenin	55.51	3.92	1.99	7.30	-	1.42	2.87	-	1.04
27	Hesperetin	57.47	-	2.04	7.30	3.62	2.34	0.96	3.70	0.68

RT: Retention Time. - : Not Detected. TR: < 0.01 mg/g. **PTBL:** Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

(A)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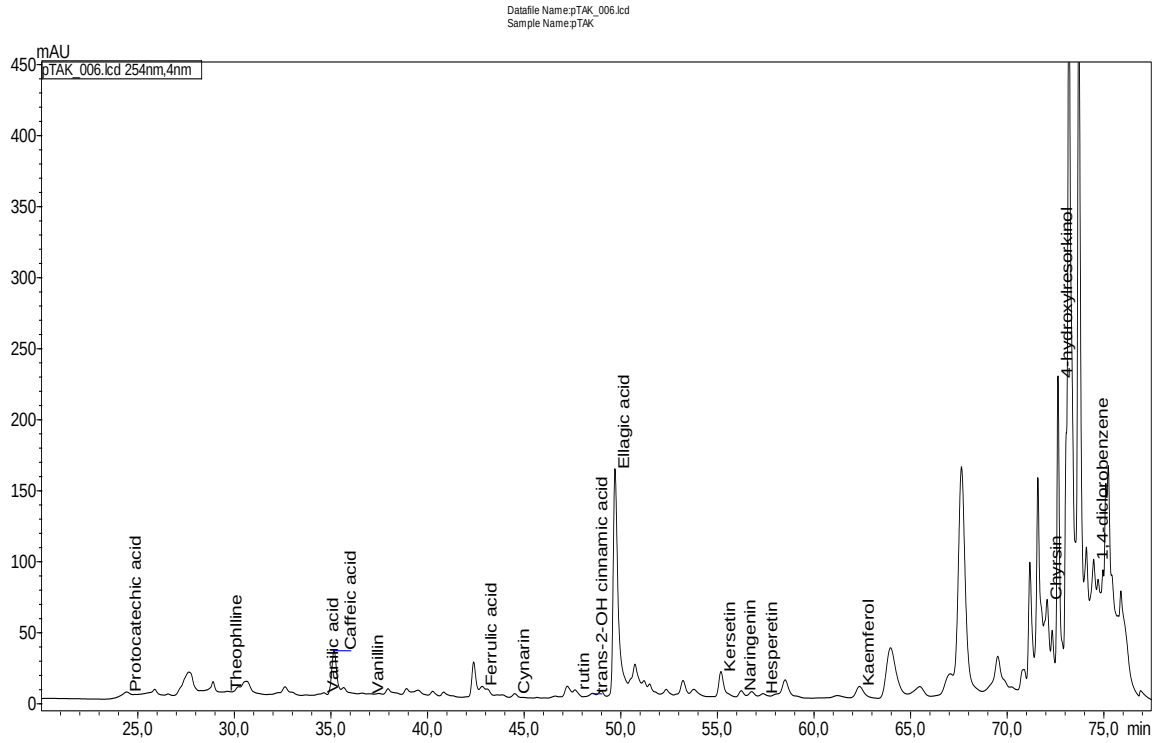
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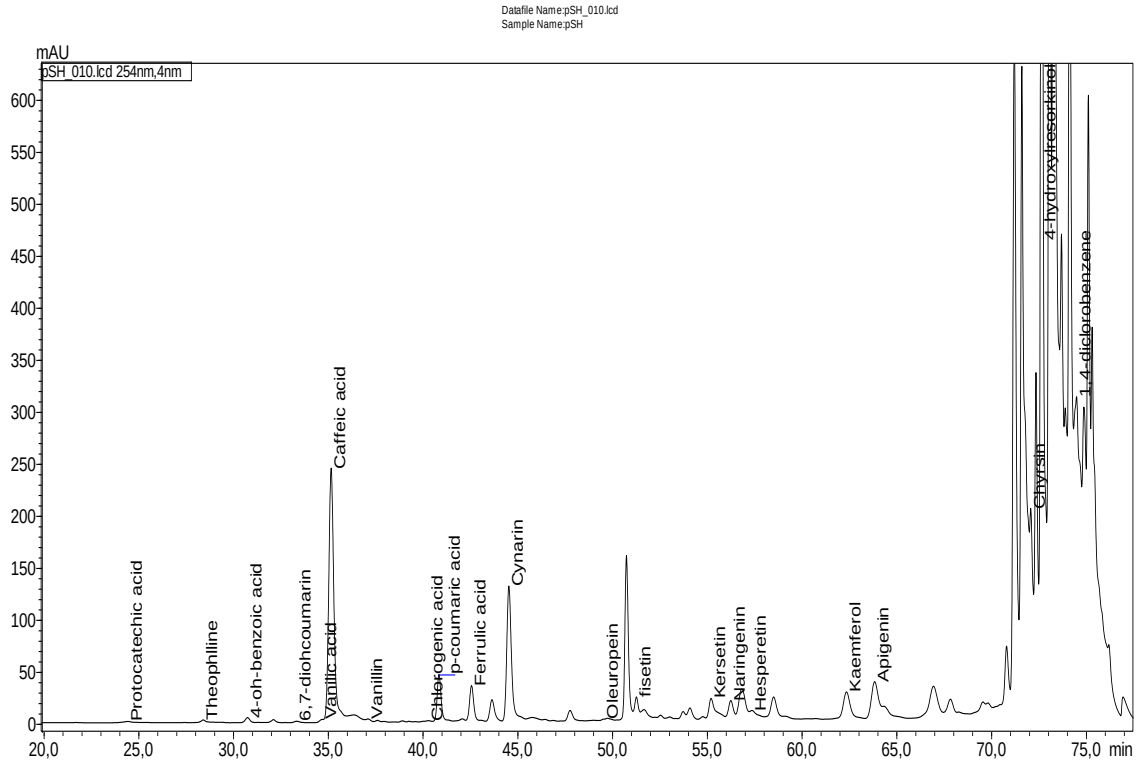
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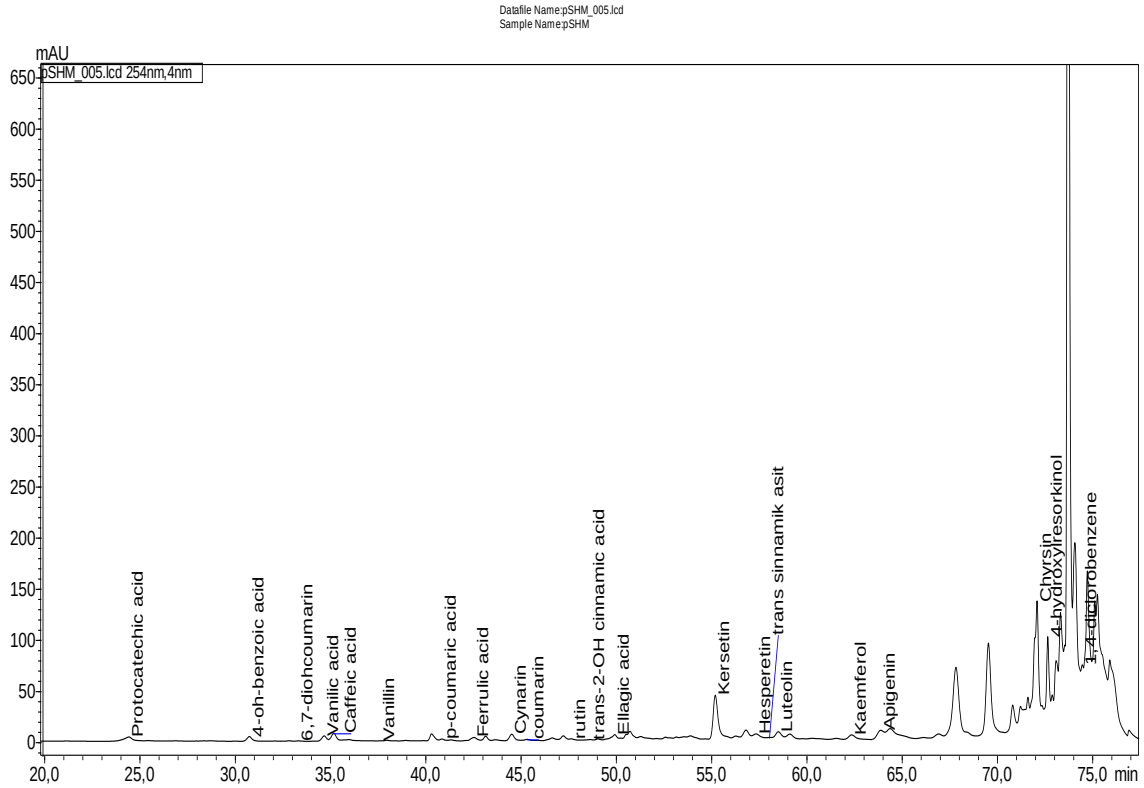
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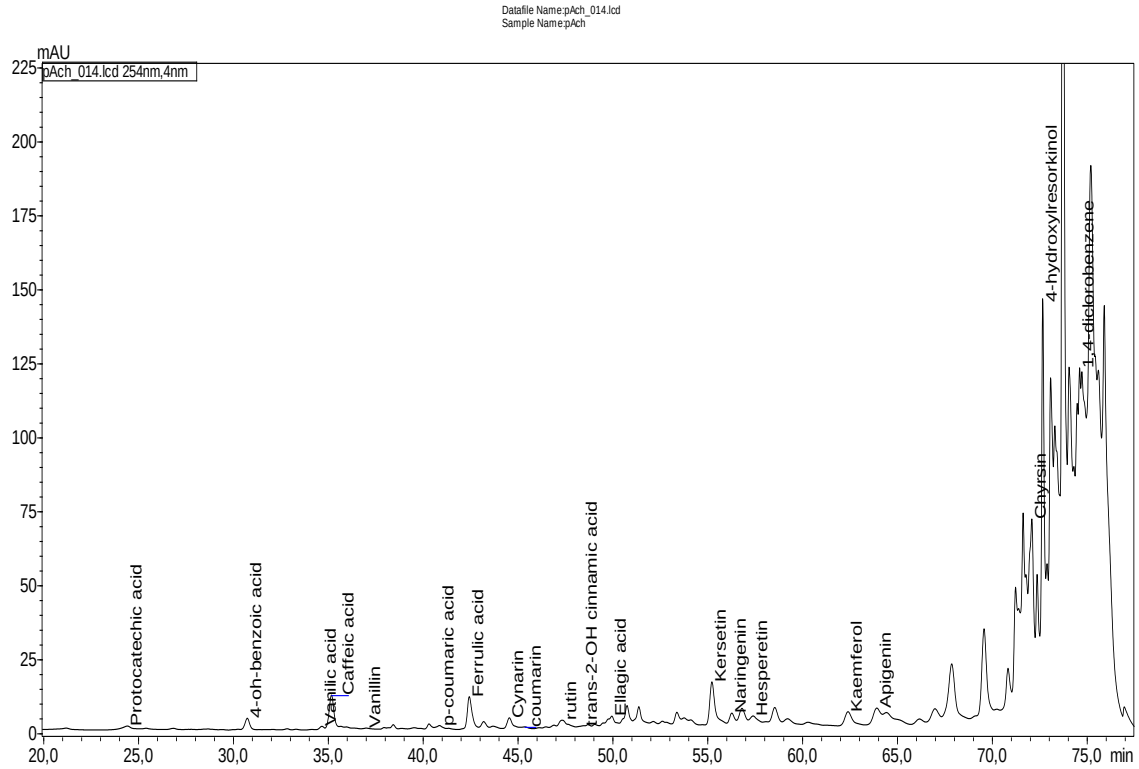
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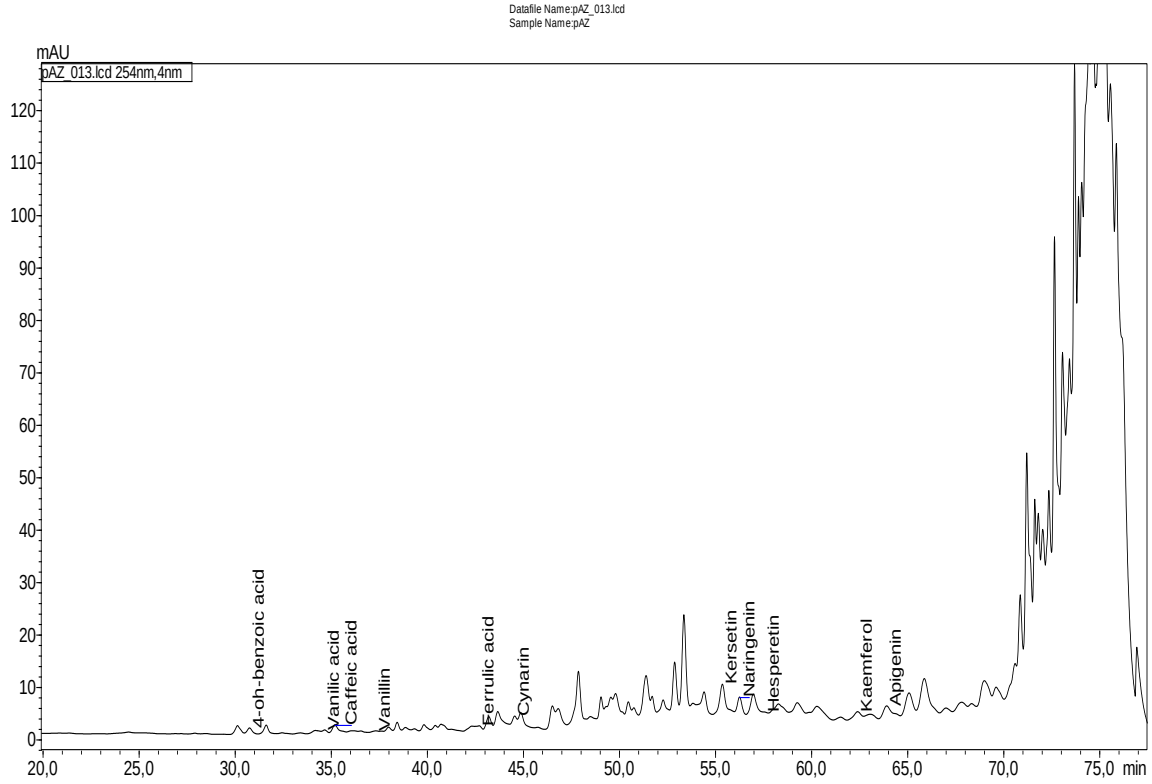
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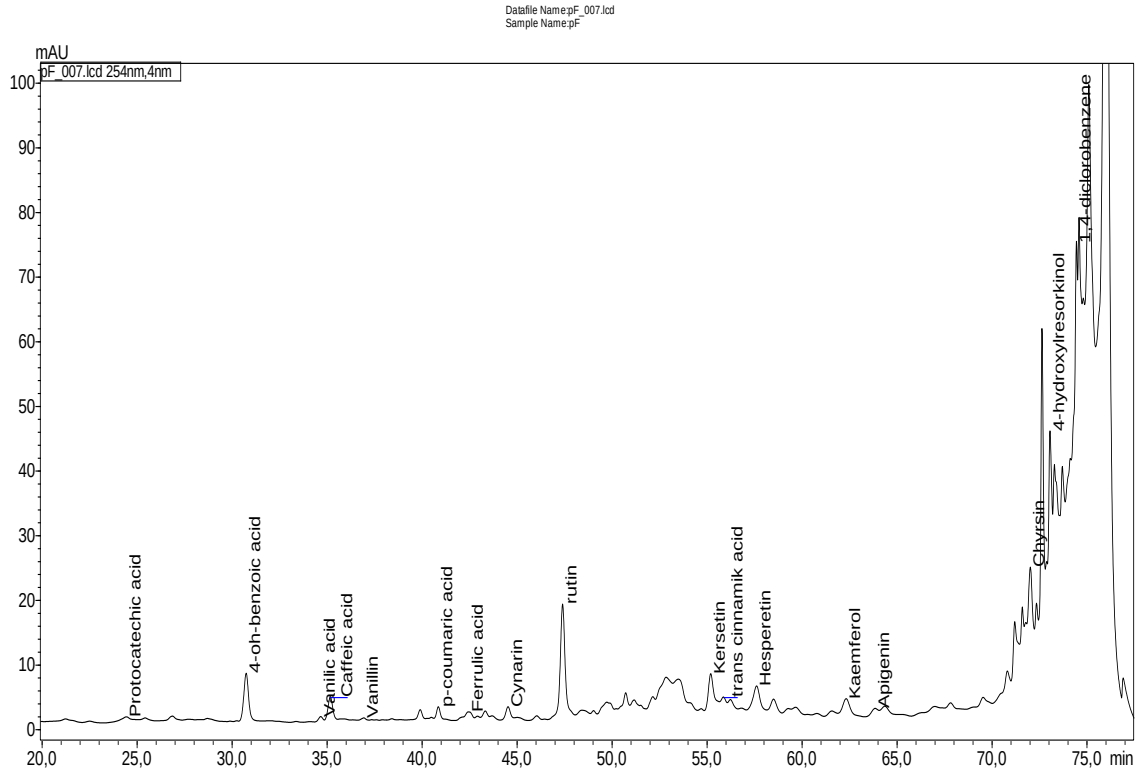
(G)



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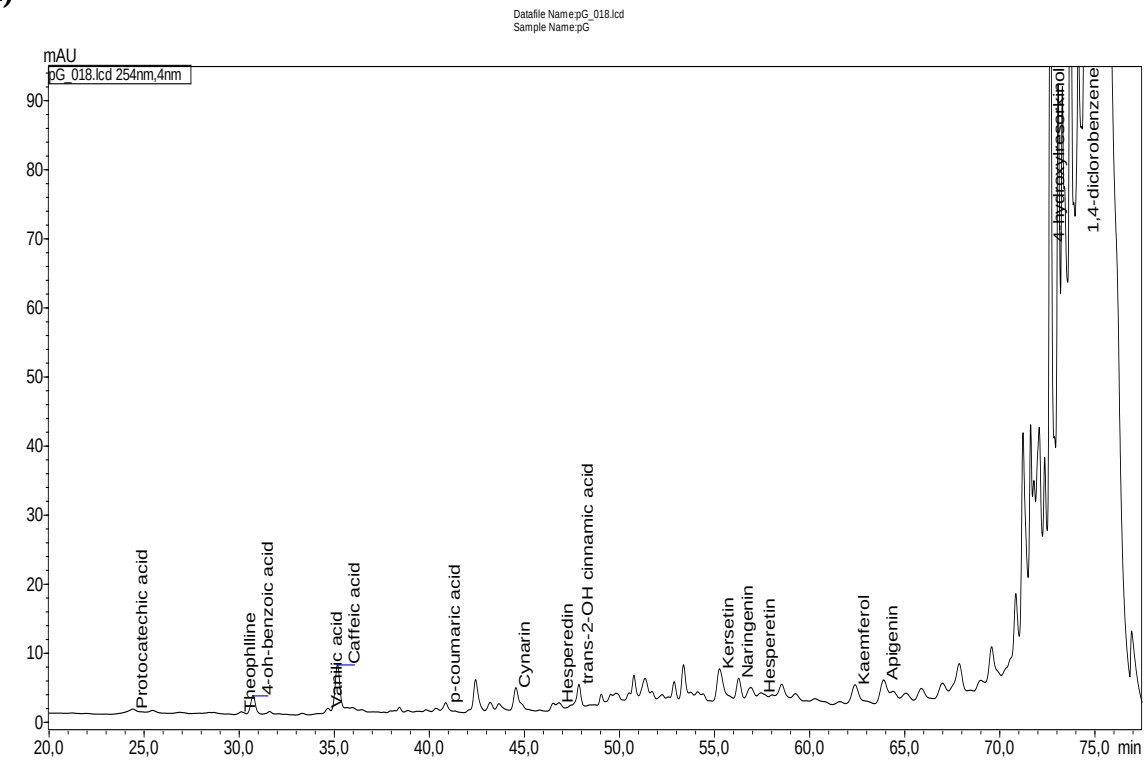

Figure 12. HPLC-DAD chromatogram of different propolis extracts. (A): PTBL, (B): PTAK, (C): PSH, (D): PSHM, (E): PACH, (F): PAZ, (G): PEEF, (H): PEEG.

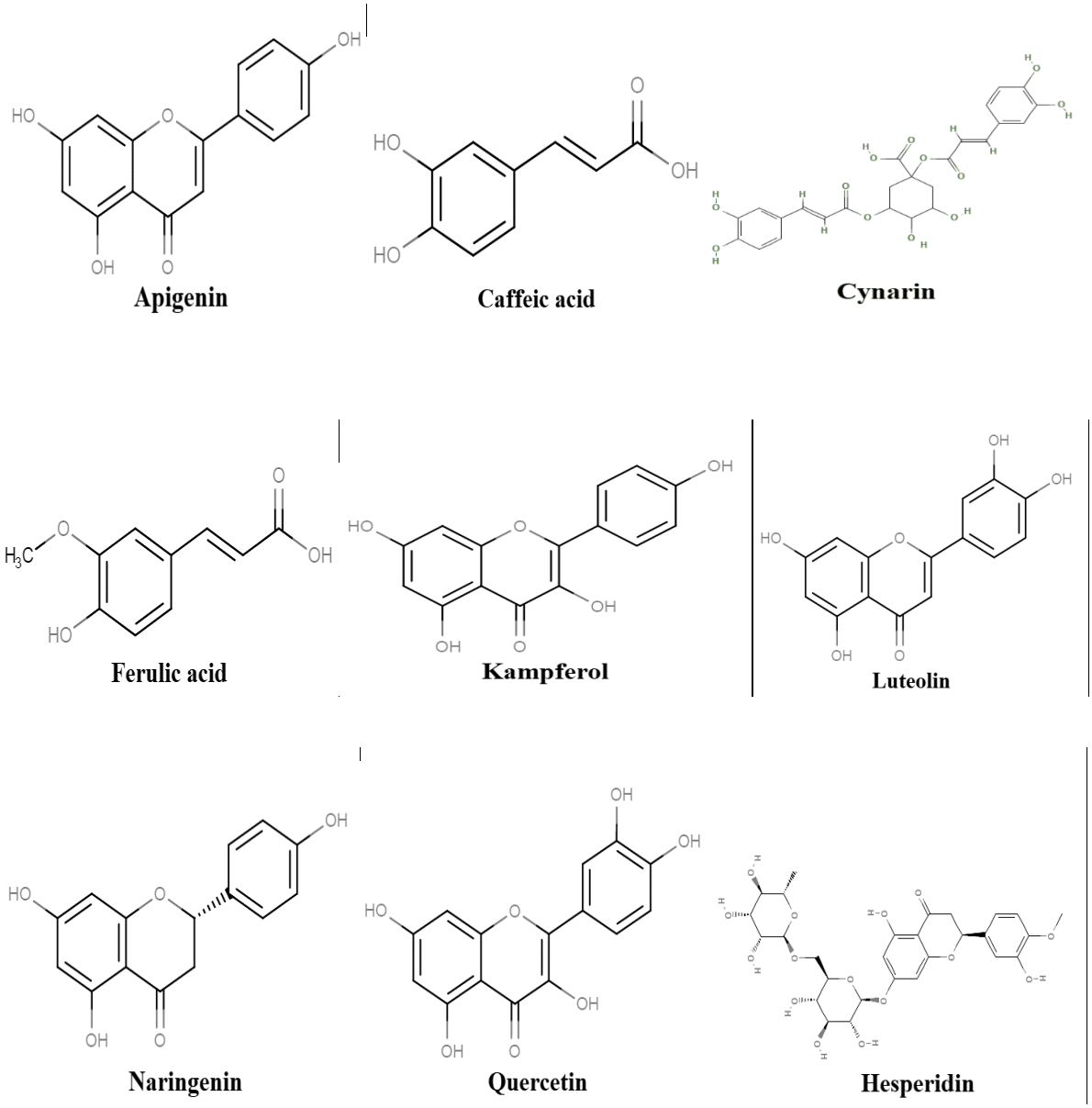

Figure 13. Chemical structures of some abundant compounds detected in the different studied propolis extracts.

According to our literature survey, a very limited number of studies have been encountered so far on the chemical composition of Algerian propolis. It's clearly shown that our propolis samples are characterized by the abundance of flavonoids, approximately similar content such as chrysin, apigenin, luteolin, kampferol, quercetin, fisetin, hesperedin, naringenin, has been observed in samples from different location of Algeria (Velikova et al., 2000; Piccinelli et al., 2013; Segueni et al., 2017; Daikh et al., 2019; El-Guendouz *et al*., 2019; Bouaroura *et al*., 2021; El Menyiy *et al*., 2021; Rebiai *et al*., 2021; Wieczorek *et al*., 2022). Likewise**,** the phenolic compounds were relatively identical to those identified in propolis from the west side of Romania (Coneac *et al*., 2008). Similar content has been also

observed as the main chemical content in samples from Greece (Kasiotis *et al*., 2017) and Tunisia (Gargouri *et al*., 2019).

It is important to emphasize that the identified chemical compounds are distinct components of temperate zone propolis, mainly derived from poplar and characterized by a high flavonoid concentration (Bankova *et al*., 1998; Piccinelli *et al*., 2013; Bouaroura *et al*., 2020) which demonstrates the plant's widespread distribution at the collection site. Moreover, chrysin was identified in most studied extracts, this compound is the reference flavonoid in poplar propolis (Mărghitaş *et al*., 2013). Several investigations have been conducted to demonstrate the relationship between the chemical composition of poplar propolis and bud exudates of trees in the genus *Populus*; all authors have agreed that the flavonoids in the studied samples were the same as in the well-known poplar type (Debbache *et al*., 2014; Dezmirean *et al*., 2020). On the other hand, cynarin was detected in all studied extracts, the emergence of this compound among the chemical content of Algerian propolis is unusual since this isolated compound was not previously identified in Algerian propolis. The botanical source of this compound was unknown, but it was hypothesized from a chemotaxonomic standpoint that cynarin would be collected by bees from exudates of plants belonging to the *Asteraceae* family, specifically *Cynara cardunculus*. This species is found not just in the apiaries' surrounding areas, but also in numerous northeast Algerian localities (Issasfa *et al*., 2015).

Caffeic acid was identified in high concentration in PTBL and PSH extracts, this compound although is officially a chemotaxonomic marker of propolis, its presence in relatively high quantities among propolis chemical content has been widely described in the literature (Tolba *et al*., 2013). On the other hand, it can be noted that the difference in chemical content included even the samples harvested from the same regions which indicates that propolis chemical content is complex and varies according to phytogeographical conditions.

The considerable amount of phenolic compounds in propolis has been extensively studied, revealing a wide variation of compositions worldwide. Considering that, the diversity of the flora foraged by the bees is considered the main reason behind these variations. It is important to note that this diversity has both beneficial and negative consequences. Taking into account the positive side, the variation in the chemical contents of propolis may considerably enhance the discovery of multiple compounds which increases its value. When it comes to the negative side; This diversity is the most impediment to propolis standardization

since it significantly impacts the possibility of fingerprinting propolis according to its original location. For instance, Algerian propolis has a multivariate chemical profile, and hence, maintaining its contents on a single regional platform remains challenging. As mentioned above an abundance of flavonoids was detected within all the tested extracts. It is worth noting that some identified components such as caffeic acid, rutin, quercetin, naringenin, apigenin, and kampferol, were detected in several northeastern Algerian regions (Daikh *et al*., 2019; Bouzahouane *et al*., 2021; Boulechfar *et al*., 2022;). Thereby, it can be primarily concluded that these components are considered one of the qualitative hallmarks of propolis from the northeastern regions of Algeria.

Considering this, we hypothesis that each newly analyzed extract should be categorized based on its bioactive profile. The application of this approach as a preliminary quality control measure may facilitate the standardization of propolis at least at the regional level.

2 Antioxidant activities

The most common sources of free radicals in the organism are reactive forms of oxygen (ROS) that are continuously produced as a result of many biological processes including protein phosphorylation, activation of several transcriptional factors, apoptosis, immunity, and differentiation. A large body of evidence demonstrates that the excess production of free radicals leads to many harmful effects on important cellular structures like proteins, lipids, and nucleic acids. However, this disorder found to be involved in the onset and the progression of several diseases (Russo *et al*., 2002; Rzepecka-stojko *et al*., 2014).

Table 5. Antioxidant activities of propolis ethanolic extracts.

Extracts	Antioxidant activity					
	DPPH assay IC50 (µg/mL)	ABTS assay IC50 (µg/mL)	Reducing power assay A0,5 (µg/mL)	CUPRAC assay A0,5 (µg/mL)	GOR IC50 (µg/mL)	Phen (A0,5 µg/mL)
PTBL	4.67±0.56	3.11±0.13	10.61±0.39	7.94±0.36	3.81±0.04	3.03±0.10
PTAK	7.91±1.54	4.04±0.75	25.63±2.83	13.30±0.18	4.17±0.92	5.63±2.19
PSH	17.99±2.03	4.51±0.31	12.39±0.59	11.19±0.01	4.97±0.16	4.80±0.68
PSHM	52.59±3.37	21.94±0.19	NA	58.28±0.42	35.55±3.92	21.44±0.19
PACH	23.15±3.08	19.56±0.97	NA	35.96±0.38	29.79±1.06	NA
PAZ	32.31±7.65	67.93±3.32	NA	89.92±4.25	80.43±7.32	NA
PEEG	73.55±6.35	10.46±1.40	NA	20.61±2.93	41.68±5.61	22.26±0.13
PEEF	NA	24.29±2.05	NA	68.87±1.10	46.30±2.79	20.91±1.39
BHT	22.32±1.19	1.59±0.03	8.41±0.67	9.62±0.87	3.32±0.18	2.24±0.17
BHA	5.73±0.41	1.03±0.00	9.01±1.46	3.64±0.19	5.38 ±0.06	0.93±0.07

IC_{50} and $A_{0.50}$ values are defined as the concentration of 50% inhibition percentages and the concentration at 0.50 absorbance, respectively. IC_{50} and $A_{0.50}$ were calculated by linear regression analysis and expressed as Mean±SD (n=3). Abbreviations: BHA: butylatedhydroxyanisole, BHT: butylatedhydroxytoluene, NA: no absorbance. **PTBL:** Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis

from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

There are multi-assay approaches to evaluate the antioxidant capacities of natural products. In this regard, six different complementary assays namely; DPPH radical scavenging activity, cupric ion reducing antioxidant capacity (CUPRAC), Ferric reducing power assay, ABTS cation radical decolorization assay, galvinoxyl radical (GOR) scavenging assay, phenanthroline assay were performed to evaluate the antioxidant activities of PEE. The results are summarized in table 5 and the inhibition percentage for each assay is presented in figure 14. The results were given as 50% inhibition concentration (IC_{50}) for DPPH, ABTS, and GOR assays, while the results were given as absorbance at 0.5 for reducing power, phenanthroline, and CUPPRAK assays. The extract concentrations providing 50% antioxidant activity (IC_{50}) and ($A_{0.5}$) were calculated using the graph of antioxidant activities percentage against extract concentrations. BHT and BHA were used as powerful antioxidant standards for comparison of the activities.

The anti-radical activities of propolis extracts were evaluated for their ability to quench DPPH radicals. The synthetic free radical's DPPH can be converted into a more stable and unreactive species molecule by accepting an electron or a hydrogen atom from a donating antioxidant compound (Laskar *et al*., 2010; Asem *et al*., 2020). The results of the DPPH assay showed variable scavenging capacities among propolis extracts ranging from 4.67±0.56 µg/mL to 73.55±6.35 µg/ml. The strongest antioxidant activity was recorded for PTBL extract showing a more potent scavenging capacity than the employed standards (BHA and BHT). Following that, PTAK and PSH extracts also revealed a high capacity to scavenge the radical DPPH better than the standard BHT. While lower activities were recorded within the remaining extracts compared to the antioxidant standards. In contrast, the PEEF extract was the only extract that failed in trapping DPPH radicals. According to figure 14, we can see that the best inhibition was recorded within the highest concentrations, which indicates that the inhibition of free radicals is dose-dependent. the highest inhibition was recorded for the PTBL sample with an inhibition of 89.95±7.20 at the highest concentration of 100µg/ml. Interestingly, PTAK, PSH, PSHM, and PACH recorded a potent inhibition higher than 80% at the same concentration.

Another *in vitro* approach that allows us to estimate the antioxidant activity of propolis is a method using ABTS radical cation to measure the relative ability of propolis to scavenge the ABTS radicals. This method is a spectrophotometric method that uses the oxidized ABTS

radical cation (*ABTS*•+) to react with antioxidants to reduce the ABTS radical and lose its bluish-green color (Cano *et al*., 2023). Similar to the obtained results in the DPPH assay, PTBL extract showed a more potent scavenging capacity using ABTS assay in comparison with the other extracts, with IC_{50} value of 3.11±0.13µg/mL, very close to BHT and BHA standards (IC_{50}= 1.59±0.03 and 1.03±0.00µg/ml), respectively. Furthermore, PTAK and PSH extracts followed the same trend as PTBL extract showing strong and approximately closer activities with an IC_{50} value of 4.04±0.75 µg/mL, 4.51±0.31 µg/ml, respectively. Whereas, PAZ extract had proved to be the weakest in neutralizing ABTS radicals. On the other hand, the remaining extracts showed varying antioxidant capacities but none of them exhibited stronger activity than the employed standards. As can be noted from figure 14, the higher the percentage of inhibition, the greater the antioxidant propolis properties. Moreover, figure 14 shows that ABTS•+ cation radical was significantly inhibited at all the tested concentrations. However, at the highest concentration (100 µg/mL), the percentage inhibition by the PTAK, PTBL, PSH, and PACH extracts reached an inhibition greater than 90%. This outcome is close to that of BHT and BHA (94.87±0.87%, and 95.39±2.62%, respectively).

Reducing power activity is employed to evaluate the contribution of natural antioxidants to donate electrons (Aissous *et al*., 2021). This property is assessed when the antiradical compound reacts with potassium ferricyanide (Fe^{3+}) to generate potassium ferrocyanide (Fe^{2+}), which then react with ferric chloride to form ferric–ferrous complex that has an absorption maximum at 700nm (Bhadauria, 2012). In this study, only PTBL, PTAK, and PSH had the potency to reduce ferric ferricianide through increasing the absorbance via concentration. PTBL extract showed a strong activity (10.61±0.39µg/ml) compared to the well-known standards BHA (9.01±1.46µg/ml) and BHT (8.41±0.67µg/ml). On the other hand, PSH extract also showed good antioxidant protection against reactive radicals in reducing power assay. The $A_{0.5}$ of PSH extract (12.39±0.59 µg/ml) was very close to the standards BHA (9.01±1.46 µg/ml), and the differences were considered non-significant. Furthermore, the absorbance increased with an increasing amount of the propolis extracts, it exhibited an absorbance of 0.78±0.01, 0.50±0.02, and 0.81±0.07 at the highest concentration (25 µg/ml), while the positive standards BHT and BHA exhibited an absorbance of 0.28±0.05, 1.74±0.07, respectively, at the same concentration.

The cupric-reducing antioxidant capacity is founded on the ability of the antioxidants to reduce the complex neocuproine-copper, therefore resulting in a chromogenic complex of Cu(II)-Nc which absorbs at 450 nm (Saci *et al*., 2020). As illustrated in table 5, the results

were compared with those of BHT and BHA, PTBL exhibited higher activity than BHT but lower activity than BHA. Also, PSH and PTAK extracts indicated high ability to reduce copper with a value of 11.19±0.01 and 13.30±0.18 µg/ml, respectively, very close activity to that of BHT (9.62±0.87 µg/ml). In contrast, PSHM, PAZ, and PEEF revealed a weak CUPRAC-reducing antioxidant capacity. The comparison of PEEG and PACH to the reference molecules revealed moderate activity with an $A_{0.5}$ of 20.61±2.93 and 35.96±0.38 µg/ml, respectively. Hence, none of these extracts exhibited higher activity than those of antioxidant standards (BHT and BHA). As can be seen in figure 14, the maximum absorbances were recorded within the highest concentration. In this regard, at a concentration of 100µg/ml, almost similar absorbance's at 450 nm were observed via PTBL extract and BHA (3.76±0.03), followed by PSH (2.58± 0.15). However, less absorbance's was obtained with the other extracts at the different tested concentrations of propolis.

Galvinoxyl is an oxygen-centered radical rather stable, in the presence of an antioxidant agent galvinoxyl accepts an electron or hydrogen to become a stable and diamagnetic molecule, it can be reduced irreversibly (Niki, 2011). The analysis data of the GOR assay showed that all the extracts have the ability to sequester the galvinoxyl radical in the following order: PTBL, PTAK, PSH, PACH, PSHM, PEEG, PEEF, and PAZ, with IC_{50} values ranging from 3.81±0.04 to 80.43±7.32 µg/ml). Compared to standards, PTBL extract exhibited higher trapping power than BHA standard (5.38 ±0.06 µg/ml), and closer activity to that of BHT (3.32±0,18 µg/ml). Similarly, the results demonstrate that PTAK and PSH have a strong redox property towards the GOR test showing close activity to standards. Based on the results of figure 14, it is worth noting that all extracts exhibited a high percentage of inhibition better than the standards (BHA and BHT) at the concentration of 100µg/ml and the inhibitions were up to 70% except for PAZ extract.

The phenanthroline method is based on the reduction of Fe^{3+} to Fe^{2+}ion by an antioxidant. The Formed Fe^{2+}ion then reacted with ortho-phenanthroline to form a red-orange complex (YEFRIDA) (Szydłowska-Czerniak *et al*., 2008). The antioxidant activity using phenanthroline assay shows that almost all extracts showed positive results as antioxidant agents. The $A_{0.5}$ of PTBL extract (3.03±0.10 µg/ml) was the highest among extracts and very close to the standard BHT (2.24 ± 0.17 µg/ml), followed by PSH extract (4.80±0.68 µg/ml), and PTAK (5.63±2.19 µg/ml). While PEEF, PSHM, and PEEG showed lower activity. Conversely, PACH and PAZ did not exhibit any activity using the phenanthroline method. The results shown in figure 14 demonstrate that the potential to reduce phenanthroline

increases with the rise of propolis concentrations. At a concentration of 25 µg/ml, PTBL extract had a significant phenanthroline activity, the absorbance reached a value of 1.85± 0.18. which was lower compared to BHA and BHT (OD: 0.93±0.07 and 2.24±0.17, respectively).

The novel tendency to prevent the early onset of oxidative stress-related disorders is the consumption of natural compounds. In this regard, Propolis' longevity-promoting characteristics have been consistently highlighted by scientists since it possesses a great capacity to diminish the harmful effects of free radicals. Considering the highest antioxidant activity recorded within all tested propolis extracts it can be positively reported that propolis is a potent agent capable of countering free radicals. According to Jun *et al.* (2003), the antioxidant activity is classified into 5 groups: highly active (IC_{50}<50 ppm), active (IC_{50}: 50-100 ppm), moderate (IC_{50}:101-250 ppm), weak (IC_{50}: 250-500 ppm) and inactive (IC_{50}> 500 ppm). In accordance with the data obtained in most approaches, almost all tested extracts fall into the category of strong antioxidants (IC_{50}<50 ppm).

The powerful antioxidant properties of propolis are continuously documented. In this study, the IC_{50} DPPH radical scavenging activity of different propolis extracts ranged from 4.67±0.56 to 73.55±6.35µg/ml. Boulechfar & Zellagui, (2023) have also studied the antioxidant activity of Algerian propolis and found IC_{50} values within the range 22.24 to 72.08±0.43 µg/ml. Similarly, the study conducted by Bouaroura *et al*. (2021) on Algerian propolis revealed good activity with an IC_{50} ranging from 10.03±0.26 to 17.00±0.24 µg/ml. Furthermore, these results are in line with those reported by Piccinelli *et al*. (2013) who studied the antioxidant activity of propolis from North Algeria. Compared to propolis worldwide, this outcome seems superior to the Brazilian Propolis those reported by Reis *et al*. (2019) ranged from 47.42 to 103.85 µg/ml, and Indonesia propolis found those reported by Hasan *et al*. (2014) ranged from 68.94 to 4162.61 µg/ml. Moreover, these results are significantly inferior to many studied propolis from various regions of the world (Wang *et al*., 2014; Araújo *et al*., 2016; Nina *et al*., 2016). Compared the results of the remaining assays with many research variables antioxidant activities were noticed (Béji-Srairi *et al*., 2020; Bouaroura *et al*., 2021).

This anti-radical efficiency most of the time is related to phenolic compounds which are considered one of the major constituents of propolis acting as primary antioxidants of free radicals by providing a hydrogen atom. It is worthy noting, that propolis revealed variable trapping activity compared to propolis samples from different local regions in Algeria,

proving that this activity is region-dependent. Of equal importance, it is vital to establish the reasons behind the differences in antioxidant activities among the tested samples, which are probably caused by the distinct quenching mechanisms of free radicals and the differences in the phenolic profile.

As previously mentioned, the results showed that PTBL extract had the highest polyphenol content, showing high antiradical activity. In contrast, PAZ extract showed the lowest antioxidant activity and low levels of polyphenol content. These findings highlight the strong and significant positive correlation between antioxidant activity and total phenolic content. A large body of research is in agreement with these findings, Asem *et al*. (2020) reported a positive and strong correlation between the phenolic and flavonoid content of propolis and its antioxidant properties. Kothai and Jayanthi. (2014), found the same behavior in an Indian stingless bee propolis sample. Furthermore, a positive influence of the polyphenols and flavonoid contents on antioxidant activities in Brazilian green propolis has been reported by Mello and Hubinger. (2012). Additionally, Kumazawa *et al*. (2004) reported that flavonoids are the most abundant and most effective antioxidant in propolis, and demonstrated the positive correlation between antioxidant activity and total flavonoid contents of PEE.

Referring to the bioactive content detected in the studied extracts the potent antioxidant activity recorded within all propolis extracts may be ascribed to the abundance of bioactive compounds such as caffeic acid (Zheng *et al*., 2020), ellagic acid, Ferrulic acid (Zduńska *et al*., 2018), and cynarin (Topal *et al*., 2016) which has been identified in several reports as a potent phenolics capable of decreasing and eliminating the damaging effects of free radicals. Interestingly, PTBL extract was the most active within all antioxidant approaches, this extract was characterized by a high level of caffeic acid with an amount of 20.36 mg/g. This constituent could be responsible for the highest antiradical activity of this sample. Furthermore, several studies have been carried out to correlate the impact of caffeic acid with antioxidant activity (Russo *et al*., 2002; Gülçin, 2006; Gregoris & Stevanato, 2010; Tajner-Czopek *et al*., 2020). Additionally, many other detected bioactive compounds such as quercetin and luteolin have proven to possess great antioxidant capacities (De Martino *et al*., 2012) which suggests that propolis can be considered as an alternative to avoid the side effects of oxidative stress and thus limiting the onset of related diseases.

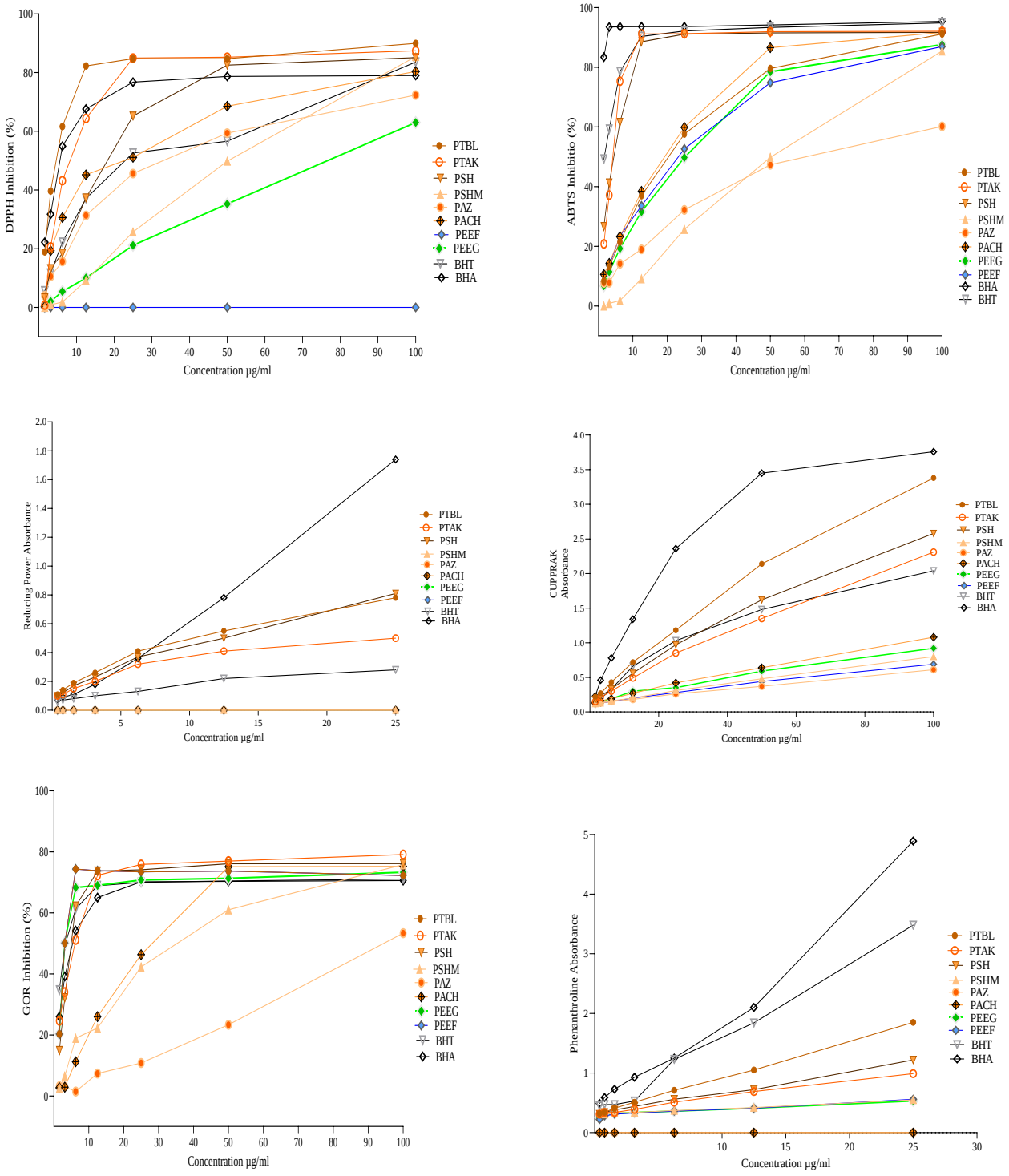

Figure 14. Concentration-dependent antioxidant activities of propolis ethanolic extracts and standards (BHA and BHT).

3 Cohort description and isolated uropathogens

Laboratories and clinicians mainly rely on the generation of accurate results from the laboratory to make therapeutic recommendations for the management of patients with UTI

(Burd & Kehl, 2011). During the collecting period, 82 bacterial isolates (78.8%) were collected from 104 women classified as having rUTI. Female patients included in this study were aged between 18-55 years, presenting multiple symptoms of acute cystitis such as dysuria, urgency, frequency, hematuria, or suprapubic pain and a history of three or more UTI episodes in the past year or two or more episodes in the past 6 months.

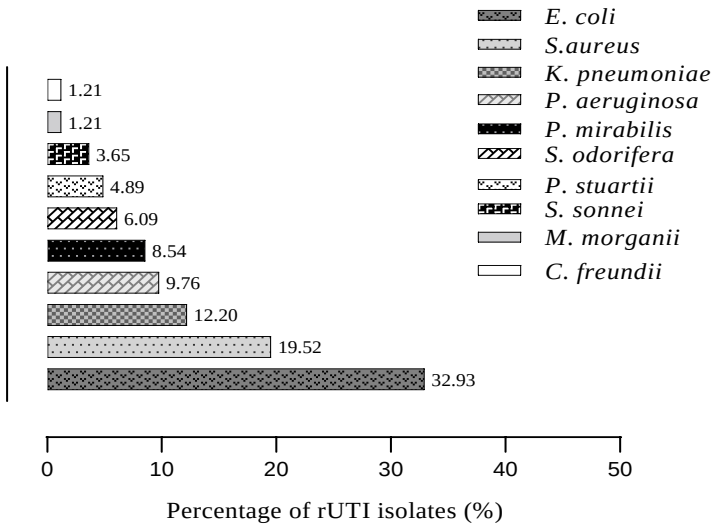

Figure 15. Distribution of uropathogens isolated from 82 urine samples of women with recurrent UTI.

Out of 104 women examined for rUTI, only 82 (78.8%) were found positive for significant bacteriuria. The microscopic examinations of 82 mid-stream urine samples of women with rUTI revealed the presence of white cells blood, epithelial cells, phosphate crystals, bacteria, yeast cells, and red blood cells thereby indicating the presence of bacterial infection.

On the other hand, the results of the morphological and biochemical identification of bacterial strains (Appendix 4) revealed notable prevalent of gram-negative bacteria which accounts for 80.48% of the total positive isolates, nine Gram-negative species were isolated, while Gram-positive bacteria accounted for only one species, the frequency of the different isolated uropathogens were illustrated in figure 15. Relevantly, *E. coli* was the most predominant isolate (n=27, 32.9%), followed by *Staphylococcus aureus* (n=16, 19.5%), *Klebsiella pneumonia* (n=10, 12.2%,), *Pseudomonas aeruginosa* (n= 8, 9.7%), *Proteus mirabilis* (n=7, 8.5%), *Serratia odorifera* (n=5, 6.09%). In contrast, other isolates were found in small numbers like *Shigella sonnei*, *Morganella morganii*, and *Citrobacter freundii.*

Despite having no anatomical abnormalities in the lower and upper urinary tracts, and being otherwise healthy individuals, the current study revealed high rates of infection. Almost all tested cultures were positive which is a high proportion indicating the overwhelming diffusion of rUTI. The high prevalence rate of UTI obtained in this study was not surprising; since many studies documented that women are extremely exposed to urinary tract infections because of their genital anatomy allowing easier access of the bacteria to the urethra (Minardi *et al*., 2011). Furthermore, current evidence indicates that the rate of recurrence following an initial UTI is high. A study from Denmark revealed that for women aged between 16 to 65 years, about 25 to 35 % of women diagnosed with a primary UTI will suffer a recurrence within 3–6 months. Another study on recurrence on Finnish women showed that about 44% of women will experience recurrence within 12 months (Glover *et al*., 2014).

Enterobacteriaceae are considered the major cause of UTI, accordingly, this study shows that *E.coli* was the most common uropathogens isolated from patients and was responsible for 32.9% of rUTI cases, these findings correlate with many studies that showed *E. coli* dominance in UI within female (Odoki *et al*., 2019; Al-Zahrani *et al*., 2019; Kot *et al*., 2021). *E. coli* is not only the number one cause of UTIs in women, but it also increases the likelihood of rUTI. A study of women ages 17–82 years suggested that recurrent *E. coli* cystitis occurs in 53% of women over the age of 55 years and 36% of younger women. This feature is due to many virulence factors that increase the colonization and invasion of the urinary epithelium (Ejerssa *et al*., 2021). On the other hand, Nosseir *et al*. (2012) stated that *E.coli* is the leading uropathogen isolated (80%) in rUTIs in women followed by *Staphylococcus saprophyticus* (10%–15%). Other potential but less common uropathogens include Klebsiella, Enterobacter, Serratia, Proteus, and Pseudomonas.

In most cases, the development of rUTI starts with bladder colonization with enteric flora, specifically uropathogens such as *E. coli*. This etiologic agent enters an interim phase of peri-urethral and distal urethral colonization. This peri-urethral colonization along with the presence of a shortened urethra subsequently encourages bladder colonization and infection (Franco, 2005).

S. aureus was the second most common isolated uropathogens (19.50%), Therefore, these findings prove that *S. aureus* is an important etiologic agent in rUTI. However, other isolated uropathogens in this study were *P. aeruginosa*, *P. mirabilis*, *S. odorifera*, *P. stuartii.*

4 Susceptibility to antibiotics and selection of resistant strains

Among the 82 urine isolates, 25 (30.4%) uropathogens were found to exhibit high rates and different patterns of resistance against the tested antibiotics. The antimicrobial resistance of MDR urine isolates has been depicted in table 6 and 7. As shown all *S. aureus* MDR isolates were 100% resistant to 8 antibiotics namely ampicillin, cefoxitin, ticarcillin, colistin, kanamycin, penicillin, ticarcilin-clavulanic acid, and streptomycin.

Likewise, a high percentage of resistance (83.3%) was remarkable towards amikacine, amoxicillin, erythromycin, and ofloxacin. Furthermore, a moderate resistance (66.6%) was observed for the tested antibiotics oxacillin, cefotaxim, chloramphenicol, and tetracyclin, followed by ciprofloxacin (50%). While, a low rate of resistance (16.7%) were recorded for gentamicin, tobramycin, and nitrofurantoin. However, all *S. aureus* MDR isolates show great sensitivity (100%) to rifampicin, vancomycin, fosfomycin, trimethoprim-sulfamethoxazole, and cotrimoxaole.

Maximum resistance of *E.coli MDR* strains 100% was seen toward ampicillin, amoxicillin ticarcylin, ticarcilin-clavulanic acid, vancomycin, streptomycin, penecillin, followed by cefotaxim (90%), trimethoprim.sulfamethoxazole and erythromycin (80%). Also, an important resistance (60%) was detected against ceftazidime, cotrimoxazole, and kanamycin, tetracyclin, and colistin (50%). Conversely, most *E. coli* MDR isolates show significantly high sensitivity to amikacin (90%), and chloramphenicol, fosfomycin, cefoxitin, aztreonam, tobramycin, ciprofloxacin **(**80%). Most of the isolates were sensitive to gentamicin (70%), nitrofurantoin**,** and ofloxacin (60%).

As expected, *P. aeruginosa* MDR isolates were approximately fully resistant to the different tested antibiotics. Multidrug resistance for about 14 antibiotics was observed among 100% of the isolates, in addition, high rates of resistance were also recorded for cotrimoxazole, fosfomycin, cefoxitin, and ciprofloxacin (66.7%). Interestingly, only amikacin and erythromycin exhibited high levels of anti-pseudomonal activity (100%). In the case of *K. pneumonia* MDR strain, the results show high incidence of resistance to 16 antibiotics namely, ampicillin tetracyclin, cotrimoxaole, amoxicillin, penicillin, cefotaxim, trimethoprim-sulfamethoxazole**,** ticarcylin, streptomycin, aztreonam, tobramycin, erythromycin, gentamycin, ceftazidime, ciprofloxacin, ticarcillin-clavulanic acid, while sensivity was recorded toward the remaining antibiotics.

On the other hand, *S. odorefera* MDR was resistant to different drugs such as rifompycin, penicillin, streptomycin, erythromycin, ticarcilin-clavulanic acid. The frequencies of resistance in *M. morganii* MDR were similar to those of *P. stuartii* MDR regarding

ampicillin, chloramphenicol, tetracyclin, cefoxitin, colistin, amoxicillin, penicillin, and ticarcilin-clavulanic acid, also *M. morganii* MDR was not susceptible to cotrimoxaole, fosfomycin, trimethoprim.sulfamethoxazole, streptomycin, erythromycin. *P. stuartii* was also found resistant to both cefotaxim and tobramycin. While *S. sonnei* isolate was resistant toward ampicillin, kanamycin, fosfomycin, penicillin, nitrofurantoine, trimethoprim. sulfamethoxazole, streptomycin, and ticarcilin-clavulanic acid.

Table 6. Detailed antimicrobial susceptibility results for the 25 MDR isolated uropathogens.

Strains	RIF	AMP	VA	K	C	TE	COT	FOS	AK	AML	OX	P	FTN	CTX	FOX	SXT	TI	S	CL	AT	TOB	E	GEN	CAZ	OF	CIP	TCC
***S.aureus** (MDR1)*	S	R	S	R	R*	R	S	S	R	R	R	R	S	R	R	S	R	R	R	-	R	R*	R	-	R*	R*	R
***S.aureus** (MDR2)*	S	S	S	R*	S	S	S	S	S	S	R	R	S	S	R	S	R	R	R	-	S	S	S	-	S	S	R
***S.aureus** (MDR3)*	S	R	S	R*	R*	R*	S	S	R	R	R	R	R	S	R	S	R	R	R	-	S	R*	S	-	R*	S	R
***S.aureus** (MDR4)*	S	R	S	R	R	S	S	S	R	R	R	R	S	R	R	S	R	R	R	-	S	R	S	-	R	R	R
***S.aureus** (MDR5)*	S	R	S	R	S	R	S	S	R	R	S	R	S	R	R	S	R	R	R	-	S	R*	S	-	R	R	R
***S.aureus** (MDR6)*	S	R	S	R	R*	R	S	S	R	R	S	R	S	R	R	S	R	R	R	-	S	R	S	-	R*	S	R
***P.aeruginosa** (MDR1)*	-	R	-	R*	R	R	S	R	S	R	R	-	R	R*	S	R	R	R*	R	S	S	-	R	-	R	R	S
***P.aeruginosa** (MDR2)*	-	R	-	R*	R	R	R	R	S	R	R	-	R	R*	R	R	R	R*	R	S	R	-	S	-	R	R	S
***P.aeruginosa** (MDR3)*	-	R	-	R*	R	R	R	S	S	R	R	-	R	R*	R	R	S	R*	R	S	S	-	S	R*	R	S	S
***E.coli** (MDR1)*	-	R	R	S	S	R	S	S	S	R	-	R	S	R	S	R	R	R	S	R	S	R	S	R	S	S	R
***E.coli** (MDR2)*	-	R	R*	S	R	R	R	S	S	R	-	R	R	R	S	S	R	R	S	S	S	R	S	S	R	S	R
***E.coli** (MDR3)*	-	R	R	R*	S	S	S	S	S	R	-	R	S	R*	R	S	R	R	R	S	S	R	R	R	R	S	R
***E.coli** (MDR4)*	-	R	R*	S	R	S	R	S	S	R	-	R	S	R	S	R	R	R*	R	S	S	S	S	R*	S	S	R
***E.coli** (MDR5)*	-	R	R*	S	S	R	R	S	S	R	-	R	R	S	S	R	R	R	R	S	S	S	R	S	S	S	R
***E.coli** (MDR6)*	-	R	R	R*	S	R	R	S	S	R	-	R	S	R	S	R	R	R	S	S	S	R	S	R*	R	R	R
***E.coli** (MDR7)*	-	R	R*	R	S	R	S	S	S	R	-	R	S	S	S	R*	R	R	S	S	S	R	S	S	S	S	R
***E.coli** (MDR8)*	-	R	R	R	S	S	R	R	S	R	-	R	R	R	S	R	R	R*	S	R	R	R	R	R	R	R*	R
***E.coli** (MDR9)*	-	R	R	R	S	S	S	R	R	R	-	R	R	R	R*	R*	R	R	R	S	R	R	S	R*	S	S	R
***E.coli** (MDR10)*	-	R	S	R*	S	S	R	S	S	R	-	R	S	R*	S	R	R	R	R	S	S	R	S	S	S	S	R
***K. pneumonia** (MDR)*	-	R	R	S	S	R	R	S	S	R	-	R	S	R	S	R	R	R	S	R*	R	R	R	R	S	R	R
***S. odorefera** (MDR)*	R	S	-	S	S	S	S	S	S	S	-	R	S	S	S	S	R	R*	S	S	S	R*	S	S	S	S	S
***M.morganni** (MDR)*	-	R	-	S	R	R	R	R	S	R	-	R	S	S	R	R	R	R	R	S	S	R	S	S	S	S	S
***P. stuartii** (MDR1)*	-	R	-	S	R	R	S	R	S	R	-	R	R	R*	R	S	R	R	R	S	R	R	R	R*	R*	S	S
***P. stuartii** (MDR2)*	-	R	-	S	R*	R	S	S	S	R	-	R	R	R	R	R	R	S	R	S	S	-	S	S	S	S	S
S. sonnei *(MDR)*	-	R	-	R*	S	S	S	R	S	S	-	R	R	S	S	R	R	R	S	S	S	R	S	S	S	S	S

Note: R*: Isolates with Intermediate category "I" were all interpreted as resistant. The value of "I "was closer to the breakpoint of "resistant". The organism is inhibited *in vitro*, but the therapeutic effect is uncertain. The therapeutic success should be enhanced by adjusting the dosing regimen.

Amikacine(AK:30µg),Amoxicillin(AML;30µg),Ampicillin(AMP;10µg),Aztreonam(AT;30µg),Cefotaxim(CTX;30µg),Cefoxitin(FOX;30µg),Ceftazidime(CAZ;30µg),Chloramphenicol(C;30µ),Ciprofloxacin(CIP;5µg), Colistin(CL;10µg),Cotrimoxaole(COT;25µg),Erythromycin(E;15µg),Fosfomycin(FOS;50µg),Gentamicin(GEN;10µg),Kanamycin(K;30µg),Nitrofurantoine(FTN;300µg),Ofloxacin(OF;5µg),Oxacillin(OX;1µg),Penecill iG(P;10µg),Rifampicin(RIF;5µg),Streptomycin(S;10µg),Tetracyclin(TE;30µg),Ticarcilinclavulanicacid((TCC;75/10);,Ticarcillin(TI;75µg),Tobramycin(TOB;10µg),Trimethoprim.sulfamethoxazole(SXT;25µg),Vanco mycin VA;30µg). -: Not tested. R: Resistant. S: Susceptible. MDR: Multi-drug resistant.

Table 7. Percentages of antimicrobial resistance of the 25 MDR isolated uropathogens.

	***S. aureus* MDR**		***P. auruginosa* MDR**		***E. coli* MDR**		***K. pneumonia* MDR**		***S. odorefera* MDR**		***M. morganii* MDR**		***P. stuartii* MDR**		***S. sonnei* MDR**	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**	**R**	**S**
RIF	0(0)	6(100%)	-	-	-	-	-	-	1(100%)	0(0)	-	-	-	-	-	-
AMP	6(100%)	0(0)	3(100%)	0(0)	10(100%)	0(0)	1(100%)	0(0)	0(0)	1(100%)	1(100%)	0(0)	2(100%)	0(0)	1(100%)	0(0)
VA	0(0)	6(100%)	-	-	10(100%)	(0)	0(0)	1(100%)	-	-	-	-	-	-	-	-
K	6(100%)	0(0)	3(100%)	0(0)	6(60%)	4(40%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	1(100%)	0(0)
C	4(66.6%)	2(33.3%)	3(100%)	0(0)	2(20%)	8(80%)	0(0)	1(100%)	0(0)	1(100%)	1(100%)	0(0)	2(100%)	0(0)	0(0)	1(100%)
TE	4(66.6%)	2(33.3%)	3(100%)	0(0)	5(50%)	5(50%)	1(100%)	0(0)	0(0)	1(100%)	1(100%)	0(0)	2(100%)	0(0)	0(0)	1(100%)
COT	0(0)	6(100%)	2(66.7%)	1(33.3%)	6(60%)	4(40%)	1(100%)	0(0)	0(0)	1(100%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)
FOS	0(0)	6(100%)	2(66.7%)	1(33.3%)	2(20%)	8(80%)	0(0)	1(100%)	0(0)	1(100%)	1(100%)	0(0)	1(50%)	1(50%)	1(100%)	0(0)
AK	5(83.3%)	1(16.7%)	0(0)	3(100%)	1(10%)	9(90%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	1(100%)
AML	5(83.3%)	1(16.7%)	3(100%)	0(0)	10(100%)	0(0)	1(100%)	0(0)	0(0)	1(100%)	1(100%	0(0)	2(100%)	0(0)	0(0)	1(100%)
OX	4(66.6%)	2(33.3%)	3(100%)	0(0)	-	-	-	-	-	-	-	-	-	-	-	-
P	6(100%)	0(0)	-	-	10(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	1(100%)	0(0)
FTN	1(16.7)	5(83.3%)	3(100%)	0(0)	4(40%)	6(60%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	2(100%)	0(0)	1(100%)	0(0)
CTX	4(66.6%)	2(33.3%)	3(100%)	0(0)	9(90%)	1(10%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	2(100%)	0(0)	0(0)	1(100%)
FOX	6(100%)	0(0)	2(66.7%)	1(33.3%)	2(20%)	8(80%)	0(0)	1(100%)	0(0)	1(100%)	1(100%)	0(0)	2(100%)	0(0)	0(0)	1(100%)
SXT	0(0)	6(100%)	3(100%)	0(0)	8(80%)	2(20%)	1(100%)	0(0)	0(0)	1(100%)	1(100%)	0(0)	1(50%)	1(50%)	1(100%)	0(0)
TI	6(100%)	0(0)	1(33.3)	2(66.7%)	10(100%)	0(0)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	1(100%)
S	6(100%)	0(0)	3(100%)	0(0)	10(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(50%)	1(50%)	1(100%)	0(0)
CL	6(100%)	0(0)	3(100%)	0(0)	5(50%)	5(50%)	0(0)	1(100%)	0(0)	1(100%)	1(100%)	0(0)	2(100%)	0(0)	0(0)	1(100%)
AT	6(100%)	0(0)	3(100%)	0(0)	2(20%)	8(80%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	1(100%)
TOB	1(16.7)	5(83.3%)	1(33.3)	2(66.7%)	2(20%)	8(80%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	1(50%)	1(50%)	0(0)	1(100%)
E	5(83.3%)	1(16.7%)	0(0)	3(100%)	8(80%)	2(20%)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	0(0)	1(100%)
GEN	1(16.7)	5(83.3%)	1(33.3)	2(66.7%)	3(30%	7(70%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	1(50%)	1(50%)	0(0)	1(100%)
CAZ	-	-	3(100%)	0(0)	6(60%)	4(40%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	1(50%)	1(50%)	0(0)	1(100%)
OF	5(83.3%)	1(16.7%)	3(100%)	0(0)	4(40%)	6(60%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	1(50%)	1(50%)	0(0)	1(100%)
CIP	3(50%)	3(50%)	2(66.7%)	1(33.3%)	2(20%)	8(80%)	1(100%)	0(0)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	1(100%)
TCC	6(100%)	0(0)	1(33.3)	2(66.7%)	10(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	1(100%)	0(0)	2(100%)	0(0)	1(100%)	0(0)

Amikacine(AK:30µg),Amoxicillin(AML;30µg),Ampicillin(AMP;10µg),Aztreonam(AT;30µg),Cefotaxim(CTX;30µg),Cefoxitin(FOX;30µg),Ceftazidime(CAZ;30µg),Chloramphenicol(C;30µ),Ciprofloxacin(CIP;5µg), Colistin(CL;10µg),Cotrimoxaole(COT;25µg),Erythromycin(E;15µg),Fosfomycin(FOS;50µg),Gentamicin(GEN;10µg),Kanamycin(K;30µg),Nitrofurantoine(FTN;300µg),Ofloxacin(OF;5µg),Oxacillin(OX;1µg),Penecill iG(P;10µg),Rifampicin(RIF;5µg),Streptomycin(S;10µg),Tetracyclin(TE;30µg),Ticarcilinclavulanicacid((TCC;75/10);,Ticarcillin(TI;75µg),Tobramycin(TOB;10µg),Trimethoprim.sulfamethoxazole(SXT;25µg),Vanco mycin VA;30µg). -: Not tested. R: Resistant. S: Susceptible. MDR: Multi-drug resistant.

The wide spread of MDR bacteria is increasing continuously, endangering the potency of antibiotics. In the present study, 25 MDR uropathogens exhibited a high degree of resistance. For instance, *E. coli* MDR isolates were resistant to β-lactam antibiotics (cephalosporins of 3^{ed} generation and penicillins) this feature is usually due to Extended-Spectrum Beta-Lactamases (ESBLs) encoded by ESBL genes; bla_{TEM} and bla_{CTX}, responsible for penicillins and cephalosporins (C3G)-resistance, respectively. Moreover, all the strains showed resistance to the penicillin group, especially ampicillin, amoxicillin, and penicillin G. Similar results have been reported in many parts of the world (Jafri *et al*., 2014; Kot *et al*., 2021; Mouanga Ndzime *et al*., 2021). This can be attributed to the indiscriminate use of these antibiotics usually used for the treatment of UTIs (Gajdács & Albericio, 2019).

As mentioned above, *E.coli* MDR strains demonstrated high resistance to vancomycin. Considering that, *E.coli* possesses intact outer membrane, which serves as a boundary that limits access of antibiotics rendering this strain more resistant (Walsh *et al*., 1996). The resistance to vancomycin reported in this study is consistent with the data reported by Iseppi *et al*. (2020). In our case, 50% of *E. coli* MDR strains were resistant to colistin, which proves the overuse of this antibiotic. The fast dissemination of polymyxin resistance is an emerging public-health concern since it is considered the last-resort antimicrobial agent for MDR gram-negative bacteria (Johura *et al*., 2020; Nation & Li, 2009). Similar findings were previously reported in Bangladesh and Lebanon (Mikhayel *et al*., 2021). According to Stoesser *et al.* (2016), this resistance is attributed to *mcr-1* gene isolates from humans, environment, and animals.

Besides the high rate of resistance to penicillin groups, *K. pneumonia* MDR isolate showed significant resistance to different antimicrobial agents, such as C2G (FOX) and C3G (CTX/CAZ). Accordingly, these results highlight the increasing dissemination of Extended-Spectrum Beta-Lactamases (ESBLs), in particular, cephalosporin-resistant in *K. pneumonia.* Furthermore, the resistance to aminosids (GEN/TOB, and S), quinolone (CIP), macrolid (E), and sulfonamides were also detected. Undoubtedly, this isolate seems to be an MDR-phenotype which can be considered as a serious problem for public human health (Effah *et al*., 2020). Our findings are completely similar to surveys conducted in different parts of the world like Morocco (El Bouamri *et al*., 2015), and Portuguese (Carvalho *et al*., 2021).

Alarmingly, Pseudomonas strains show the highest intrinsic resistance to almost all the varieties of antibiotics tested. Therefore, antibiotics are losing efficacy. This remarkable feature of Pseudomonas strains refers to their ability to develop resistance through chromosomal gene mutation selection and the increasing prevalence of transferable resistance

determinants, particularly those encoding extended-spectrum -lactamases (ESBLs) (del Barrio-Tofiño *et al*.,

2020). On the other hand, Pseudomonas isolates show high resistance to tobramycin and streptomycin. Unambiguously, aminoglycoside resistance is achieved by inactivation of the antibiotic involving different mechanisms such as phosphorylation, acetylation, or adenylation (Poole, 2011). The results are consistent with a similar study carried out in India (Javiya *et al*., 2008). In contrast, the results of this study are contrary to a similar study conducted in a hospital in South China where Pseudomonas showed high susceptibility to beta-lactam, aminoglycosides, and polymyxin (Yang *et al*., 2015).

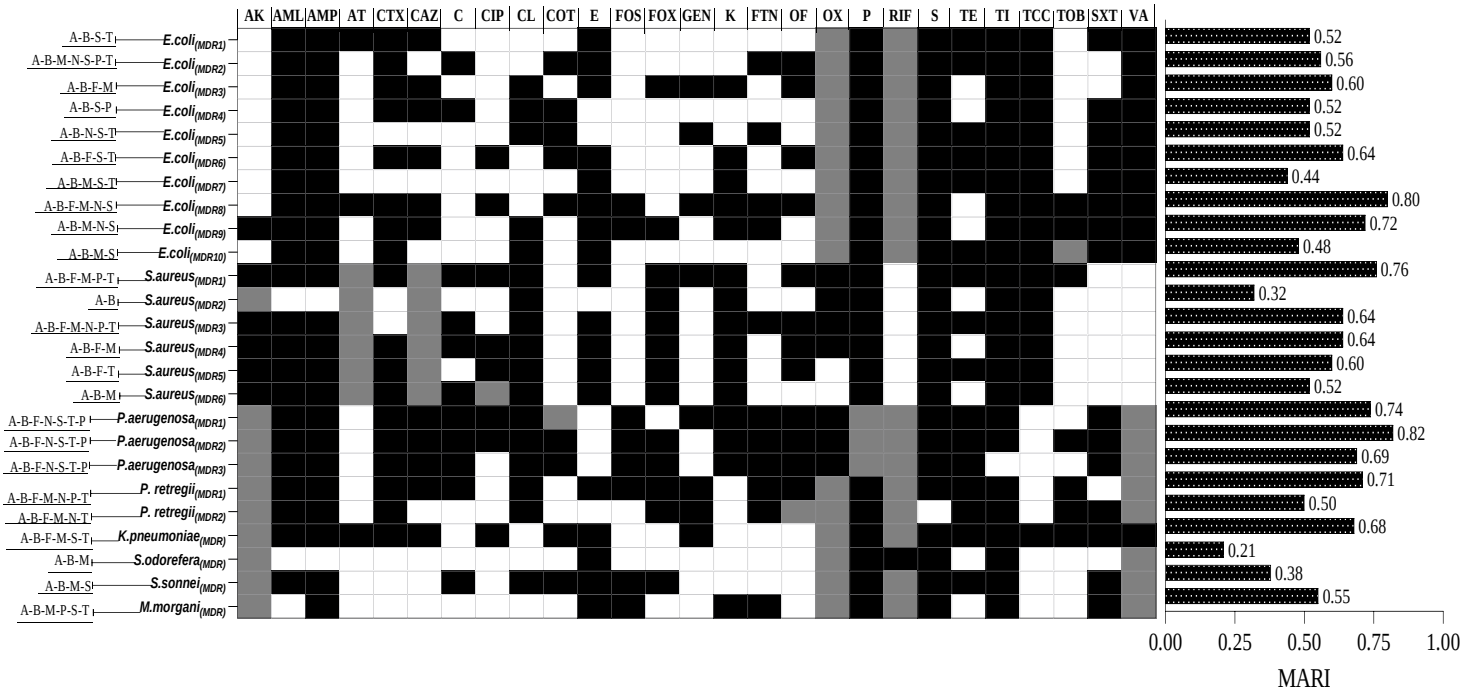

Figure 16. Antibiotic susceptibility profiles of the 25 MDR isolated uropathogens. (Black; Resistant), (White; Susceptible); (Grey = Data not available, antibiotics not tested). Resistance to different antibiotic groups are indicated by successive codes in alphabetical order; Key: A = Aminoglycoside (Amikacin; AK, Gentamycin; GEN, Tobramycin; TOB, Streptomycin; S, Kanamycin; K), B = Beta-lactam: (Amoxicillin; AML), (Ampicillin; AMP), (Aztreonam; AT), (Ceftazidime; CAZ), (Colistin; CL), (Cefotaxim; CTX), (Fosfomycin; FOS), (Cefoxitin; FOX), (Oxacillin; OX), (Penecillin-G; P), **(**Ticarcilin-clavulanic acid; TCC), (Ticarcilin; TI), (Vancomycin; VA). M = Macrolides (Erythromycin; E). F = Fluoroquinolone (Ciprofloxacin; CIP), (Ofloxacin; OF). S= Sulfamid (Cotrimoxaole; COT), (Trimethoprim.sulfamethoxazole; SXT). N = Nitrofurantoin (Nitrofurantoin ; FTN), T= Tetracyclines (Tetracycline; TE). P = Phenicol (Chloramphenicol; C). MARI: Multiple Antibiotic Resistance Index.

Among the 82 isolated uropathogens, 25 (30.4%) were identified by antimicrobial susceptibility testing as MDR (Figure 16). Out of 10 genera, only 8 genera were detected as

MDR uropathogens organisms, namely; *E. coli* (n=10), *S. aureus* (n=6), *P. aeruginosa* (n=3), *P. stuartii* (n=2), *K. pneumonia* (n=1), *S. sonnei* (n=1), *S. odorifera* (n=1), *M. morganii* (n=1).

The calculated MAR index values ranged from 0.65 to 0.82 for *P. aeruginosa* strains, for *E. coli* strains the MAR index ranged from 0.44 to 0.80, whereas for *S. aureus* it ranged from 0.32 to 0.76. Interestingly, the highest MARI values were found for the *P. aeruginosa* strain (*P. aeruginosa* $_{(MDR2)}$/MARI = 0.82), followed by the *E. coli* strain (*E. coli* $_{(MDR8)}$/MARI = 0.80) which indicates that these isolates are highly resistant.

The MAR index values of the 82 isolates tested in this study ranged between 0.00 and 0.82 with 30.4% (25/82) of isolates having MAR indices >0.20. The high MARI value was obtained for *P. aeruginosa* $_{(MDR2)}$ with a value of 0.82, and was resistant to 19/24 antibiotics, which is higher than the study documented by Ehinmidu, (2003) were the highest MARI for Pseudomonas isolates were 0.70. While for *E. coli* almost similar values were obtained by Akinjogunla *et al*. (2010). Regarding the MARI calculated for *S. aureus* $_{(MDR1)}$ isolate, this value is alarmingly high compared to other previous studies (Subramani & Vignesh, 2012; Kasote *et al*., 2015; Deyno *et al*., 2017; Noumi *et al*., 2017;). Overall, the results indicate that these isolates originate from high-risk sources, which may be attributed to the inappropriate or overuse of antibiotics among the target population (Adzitey *et al*., 2021; Hosu *et al*., 2021) and thus require urgent action to stop its spread.

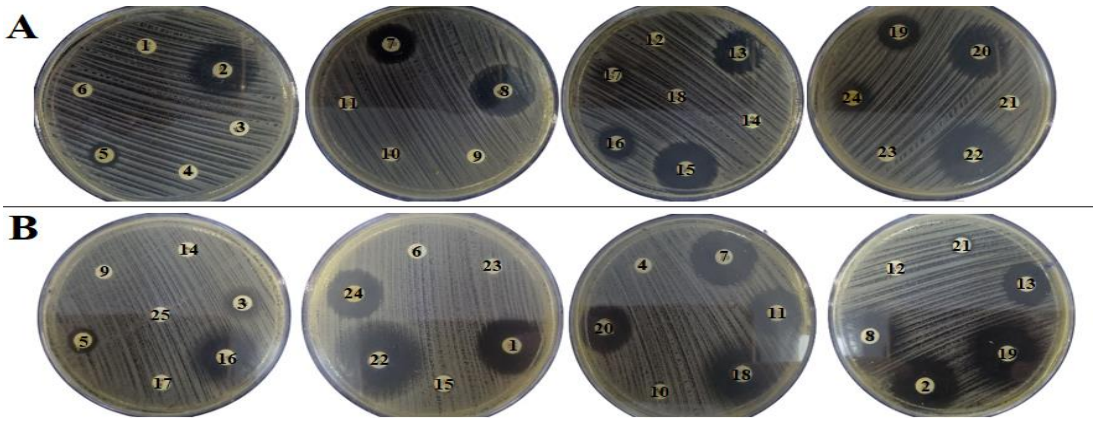

Figure 17. Detection of MDR uropathogens using antibiotic susceptibility test. Antibiotics are listed from 1 to 25; (1) :(CTX), (2): (AT),(3):(VA) , (4): (P), (5): (TE), (6): (COT), (7): (FOS),(8):(AK),(9): (S), (10): (AML),(11): (CAZ),(12): (SXT),(13): (CL),(14): (AMP),(15): (OF),(16):(K),(17): (E),(18): (GEN),(19): (TOB),(20): (FOX),(21): (CIP),(22): (C),(23): (TI),(24):(FTN),(25):(TCC).

5 Antibacterial activity

The susceptibility of the 25 MDR uropathogens responsible for rUTI in women was tested against propolis extract. The obtained disc diffusion data are depicted in table 8. As can be noted, all tested extracts showed variable antibacterial activity against different strains. Interestingly, the majority of all MDR uropathogens were sensitive to PTBL extract. Furthermore, this extract presented the highest antibacterial efficiency against *S. aureus (MDR3)* with an inhibition zone of 28.50±0.87 mm. Furthermore, this extract showed a wide spectrum against a panel of 10 MDR *E. coli* strains with the highest activity recorded against *E.coli* (MDR6).

Coming in the second range of potency, PSHM extract also showed strong inhibition, and the highest antibacterial effect was recorded against *S. aureus* (MDR1), with a zone of inhibition of 25.33±2.08 mm. Moreover, this extract appears to be the only extract that exhibited antibacterial activity against the most resistant strain *P. aeruginosa* (MDR2). According to the results, PSHM showed less activity against *E. coli* strains compared to PTBL, only two MDR *E. coli* strains were susceptible, namely *E. coli* (MDR4) and *E.coli* (MDR5), whereas variable activity was recorded against the remaining strains. As significant, PSH extract also showed a wide antibacterial spectrum against all MDR *S. aureus* strains, with the inhibition zone diameter ranging from 12.33±0.58 to 23.00±1.00mm. Similarly, PTAK and PEEG extracts exhibited potent activity in inhibiting *S. aureus* MDR strains with the greatest inhibition zone of 24.23±1.96 and 18.67± 1.53 mm, respectively.

On the other hand, PACH, PAZ, and PEEF showed variable activity against different *S. aureus* MDR strains, while no activity was recorded against gram-negative bacteria. The PAZ and PEEF extracts demonstrated the weakest activity, in which 5 out of 6 and 4 out of 6 *S. aureus* strains present no zone of growth inhibition, respectively.

According to the micro-dilution results, the sensitivity of bacteria to propolis varied among the tested strains and the propolis extracts used (Table 9). Overall, the MIC values ranged between 0.625 to 20 mg/ml. The lowest MIC value was recorded within the PTBL extract against *S. aureus* (MDR1). Regarding gram-negative strains, all propolis samples showed some effect, but MIC values were significantly higher in comparison to gram-positive bacteria. Whereas the MBC values ranged from 2.5 to 20 mg/ml. The lowest MBC was found in PTBL and PTAK with a value of 2.5 against *S. aureus* (MDR1) and *S. aureus* (MDR3), respectively.

Table 8. Antibacterial activity of different propolis extracts against 25 MDR uropathogens.

	PSH	PSHM	PACH	PAZ	PEEF	PTBL	PTAK	PEEG	Ampicillin	Amikacin
	Mean ± SD* (mm)	Mean ±SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)	Mean ± SD (mm)
S.aureus *(MDR1)*	12.33±0.58	25.33±2.08	16.00±1.73	-	10.34±2.52	22.83±0.35	24.23±1.96	18.67± 1.53	13	14
S.aureus *(MDR2)*	13.67±0.56	18.00±1.00	-	-	-	24.85±0.40	15.07±0.21	13.33± 0.58	31	19
S.aureus *(MDR3)*	23.00±1.00	16.00±0.00	20.67±1.15	14.33±2.08	-	28.50±0.87	20.27±2.01	11.00 ±2.00	11	11
S.aureus *(MDR4)*	17.00±0.00	-	14.67±0.58	-	13.00±1.00	15.00±0.00	-	15.00 ±0.00	10	18
S.aureus *(MDR5)*	22.00±1.00	20.67±1.15	13.33±1.53	-	-	21.07±1.10	20.40±0.36	-	17	17
S.aureus *(MDR6)*	13.33±1.15	-	-	-	-	15.47±1.21	13.00±0.00	-	9	12
P. aeruginosa *(MDR1)*	11.00±0.00	12.00±0.00	-	-	-	12.27±0.13	19.57±1.60	9.33± 0.58	R	20
P. aeruginosa *(MDR2)*	-	13.33±0.58	-	-	-	-	-	-	R	18
P. aeruginosa *(MDR3)*	-	-	-	-	-	14.23±0.39	12.0±00.00	10.00± 0.00	R	22
E.coli *(MDR1)*	13.33±1.53	-	-	-	-	14.00±0.00	-	9.67± 2.08	R	20
E.coli *(MDR2)*	-	-	-	-	-	8.50±0.35	-	-	R	20
E.coli *(MDR3)*	-	-	-	-	-	10.33±0.71	-	-	R	17
E.coli *(MDR4)*	13.67±1.15	10.33±1.15	-	-	-	9.00±0.00	-	-	R	21
E.coli *(MDR5)*	-	12.00±0.00	-	-	-	11.00±0.00	-	-	R	17
E.coli *(MDR6)*	-	-	-	-	-	17.00±0.80	-	-	R	18
E.coli *(MDR7)*	13.00±1.73	-	-	-	-	13.77±1.17	-	-	R	17
E.coli *(MDR8)*	12.00 ±0.00	-	-	-	-	9.00±0.00	-	-	R	20
E.coli *(MDR9)*	-	-	-	-	-	13.03±2.05	-	-	R	R
E.coli *(MDR10)*	-	-	-	-	-	13.00±0.00	-	-	R	20
K. ***pneumonia*** **(MDR)**	-	12.33±1.53	-	-	-	-	15.23±1.17	14.00± 1.73	R	21
S.*odorefera* **(MDR)**	-	11.00±0.20	-	-	-	23.67±1.02	-	-	R	23
M.morganii **(MDR)**	10.67±0.58	13.00±00.00	-	-	-	14.00±0.00	14.00±0.00	-	R	22
P. stuartii *(MDR1)*	-	-	-	-	-	-	-	-	R	18
P. stuartii *(MDR2)*	-	-	-	-	-	-	-	10.00±0.92	R	20
S.sonnei *(MDR)*	-	11.00±2.00	-	-	-	20.10±1.35	-	-	R	26

Note: Data are presented as mean±SD (n=3). *: Inhibition zone around the disc with the PEE (20 µL/disk; 200µg/disc) expressed as mean of three replicates (mm ± SD), -: No inhibition, NT: NOT tested. R: resistant. AMP: Ampicilline, AK: Amikacine. R: resistant.

Table 9. MIC and MBC of propolis extracts against the 25 MDR uropathogens.

Strains	PSH		PSHM		PACH		PAZ		PEEF		PTBL		PTAK		PEEG	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S.aureus *(MDR1)*	20	+20	2.5	+20	5	+20	+20	NT	20	+20	0.625	2.5	10	20	2.5	5
S.aureus *(MDR2)*	5	10	10	+20	10	20	20	+20	+20	NT	5	+20	10	+20	5	10
S.aureus *(MDR3)*	1.25	10	10	20	20	+20	10	+20	20	+20	1.25	10	1.25	2.5	20	+20
S.aureus *(MDR4)*	10	+20	20	+20	2.5	20	+20	NT	10	+20	5	10	10	20	5	20
S.aureus *(MDR5)*	5	+20	10	20	10	+20	20	+20	20	+20	1.25	+20	10	+20	10	20
S.aureus *(MDR6)*	2.5	5	+20	NT	10	20	+20	NT	+20	NT	10	20	20	+20	+20	NT
P. aeruginosa *(MDR1)*	20	+20	20	NT	20	+20	+20	NT	+20	NT	20	+20	20	+20	10	20
P. aeruginosa *(MDR2)*	20	NT	20	NT	20	NT	+20	NT	+20	NT	20	+20	20	NT	+20	NT
P. aeruginosa *(MDR3)*	20	NT	20	+20	20	NT	+20	NT	+20	NT	10	+20	10	+20	20	+20
E.coli *(MDR1)*	5	+20	5	+20	+20	NT	+20	NT	+20	NT	10	20	20	+20	20	+20
E.coli *(MDR2)*	20	+20	10	+20	10	+20	20	+20	+20	NT	20	+20	10	+20	+20	NT
E.coli *(MDR3)*	+20	NT	+20	NT	+20	NT	+20	NT	+20	NT	10	20	20	+20	+20	NT
E.coli *(MDR4)*	20	+20	10	+20	20	NT	20	+20	+20	NT	5	10	20	NT	20	NT
E.coli *(MDR5)*	10	+20	20	+20	+20	NT	+20	NT	+20	NT	20	+20	+20	NT	+20	NT
E.coli *(MDR6)*	10	20	+20	NT	20	+20	+20	NT	+20	NT	20	+20	10	+20	+20	NT
E.coli *(MDR7)*	10	+20	20	+20	+20	NT	+20	NT	+20	NT	10	+20	20	+20	+20	NT
E.coli *(MDR8)*	+20	NT	20	+20	+20	NT	+20	NT	+20	NT	20	+20	+20	NT	+20	NT
E.coli *(MDR9)*	+20	NT	+20	NT	+20	NT	+20	NT	+20	NT	20	+20	20	+20	+20	NT
E.coli *(MDR10)*	+20	NT	+20	NT	+20	NT	+20	NT	+20	NT	+20	NT	20	+20	+20	NT
K. pneumonia **(MDR)**	5	20	20	NT	20	NT	20	+20	+20	NT	5	+20	20	+20	20	NT
S.odorefera **(MDR)**	2.5	10	10	+20	10	20	+20	NT	+20	NT	10	+20	+20	NT	10	20
M.morganii **(MDR)**	20	+20	5	10	10	20	20	+20	+20	NT	10	20	10	20	+20	NT
P. stuartii *(MDR1)*	10	20	10	20	20	+20	20	+20	+20	NT	20	+20	20	+20	+20	NT
P. stuartii *(MDR2)*	10	+20	+20	+20	+20	NT	+20	NT	+20	NT	5	10	10	+20	+20	NT
S.sonnei *(MDR)*	5	+20	+20	NT	20	+20	20	+20	+20	NT	10	20	+20	NT	20	+20

Abbreviations: **MIC**: minimum inhibitory concentration, **MBC**: minimum bactericidal concentration, **NT**: not tested. **PTBL:** Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

The antibacterial features of propolis are documented from centuries showing a broad inhibition spectrum on bacterial growth. Considering that, our findings are consistent with those found in the literature. The current study's findings are quite encouraging because, despite the uropathogens' high antibiotic resistance profile, the propolis extracts exhibited valuable antibacterial activity, particularly against gram-positive bacteria. Moreover, the results of the disc agar diffusion method, MIC, and MBC indicate that the propolis extracts are more potent toward gram-positive than gram-negative bacteria, which agrees with several previous reports (Al-Ani *et al*., 2018; Przybyłek & Karpiński, 2019; Daraghmeh & Imtara, 2020). The studied propolis extracts showed high activity on MDR *S. aureus* strains, for instance, PTBL extract showed the highest bacteriostatic efficiency against *S. aureus* (MDR1). In this regard, the comparison of the efficiency of this extract to the commonly employed antibiotics including oxacillin, ampicillin, and cefoxitin, showed more potency than the tested antibiotics, thus proving its efficacy in inhibiting MDR bacteria. Similarly, Czech propolis from Moravia exerted a marked inhibition against MDR *S.aureus* (Astani *et al*., 2013). Additionally, Palestinian propolis exhibited good antibacterial activity against MDR *S.aureus* with MIC values ranging from 0.01 to 2.5 mg/ml (Daraghmeh & Imtara, 2020). In contrast, limited action was recorded against MDR *E. coli* strains. The lowest MIC value for MDR *E. coli* strains was 5 mg/ml for PTBL, PSH, and PSHM extracts, which is higher than the MIC obtained by Sforcin *et al*. (2000) of 8 mg/ml against the same strain. However, other authors have reported no effect on *E. coli* MDR strains (Ding *et al*., 2021; Mohdaly *et al*., 2015).

All the propolis extracts have shown slight or no antibacterial activity on Pseudomonas MDR strains. Such sensitivity is limited specifically to the most resistant strain *P. aeruginosa* (MDR2) against PSHM extract, which emphasizes the high effect of this extract. These results are in line with other findings stating that *P. aeruginosa* displays low sensitivity toward propolis (Al-Ani *et al*., 2018; Silici *et al*., 2007; Touzani *et al*., 2019). On the other hand, PSHM, PTAK, and PEEG extracts showed moderate activity against MDR *K. pneumonie*. These results correlate with the study of Pobiega *et al*. (2019) demonstrating the activity of polish propolis against *K. pneumonia*. Among the extracts, PSH and PSHM showed activity against *S. odorefera* MDR strain with a MIC value of 2.5 mg/mL and 10 mg/mL, respectively. These overall activities appeared to be superior to those reported by Sanpa *et al*. (2015) for the antibacterial activity of eastern Thai propolis on *Serratia marcescens* (MIC = 16 mg/ml).

Generally, this confliction in the antibacterial activity of propolis could be due to several factors; one of them is the difference in the outer membrane between gram-positive

and gram-negative bacteria since the structure of the cell wall of gram-positive bacteria is composed of a thick peptidoglycan layer, which makes it more permeable to any antibacterial compound such as propolis (Chen *et al*., 2018). Contrary to gram-positive cells, the gram-negative cell wall is more complex and contains a thin peptidoglycan layer that is surrounded by a thick plasma membrane composed of a double layer of phospholipids linked with the inner membrane by lipopolysaccharides, which grant impermeable to the bacteria (Nazzaro *et al*., 2013). Another main reason that can grant resistance to propolis is the possession of MDR pumps, which serve as a boundary that limits access of external poisons (Tegos *et al*., 2002; Ioannidis *et al*., 2015; Ding *et al*., 2021). The broad knowledge in this respect includes also the richness of propolis chemical profile with antibacterial substances such as phenolics and flavonoids (Almuhayawi, 2020; Dezmirean *et al*., 2020; Maroof & Gan, 2020; Nichitoi *et al*., 2021).

The results indicate that PTBL extract has the highest antibacterial activity. This feature may be explained by the high amounts of phenolic acids and flavonoids in PTBL extract, which correlate positively with the antibacterial activity, whereas PAZ has the weakest TFC and TPC content which explains the weak activity. Furthermore, several studies demonstrated that propolis antibacterial properties were attributable to its constituents such as caffeic acid. It is worth mentioning that among the constituents of the PTBL and PSH extract, caffeic acid was the major identified compound. Many previous studies have been carried out on the numerous antibacterial activities of caffeic acid against multiple pathogens (Magnani *et al*., 2014; Pinho *et al*., 2015). Similarly, research carried out by Velazquez *et al*. (2007) showed that Mexican propolis has antibacterial activity, and this activity was correlated to caffeic acid. Furthermore, quercetin was detected in all the tested extracts, this compound was shown to possess great bacterial inhibitory effect in multiple studies (Yang *et al*., 2020; Nguyen & Bhattacharya, 2022). In addition, the study carried out by Wang *et al*. (2018) showed that quercetin significantly inhibited *P.aeruginosa*, *S.aureus*, and *E.coli* by damaging the cell walls and membranes.

Moreover, the studied extracts are characterized by the abundance of flavonoids, in particular, chrysin, apigenin, luteolin, kaempferol, quercetin, fisetin, hesperedin, and naringenin, which have been proven in multiple studies to exhibit antibacterial activity (Nayaka *et al*., 2014; Iranshahi *et al*., 2015; Ming *et al*., 2017; Adamczak *et al*., 2019; Kharsany *et al*., 2019; Almuhayawi, 2020). The antibacterial activity of propolis may be due to the synergistic effects of these compounds.

6 Biofilm forming capacity of MDR isolated uropathogens

Bacterial biofilms are a serious global health concern since these unique niches protect bacteria not only from harsh conditions but also from toxic components such as antibiotics entering the bacterial biofilm community which increases bacteria's resistance, resulting in the establishment of MDR bacteria (Sharma *et al*., 2019).

The biofilm-forming potential of the 25 MDR uropathogens is investigated using table 10 and the results are shown in figure 18. In the current investigation, the OD_{avg} of negative control was calculated and found to be 0.12 ±0.03 whereas the OD_{cut} was found to be 0.21. According to the OD_{560} measurements of adherent cells, the 25 MDR tested strains were classified into four different categories (Figure 18); 9(36.0%) were high biofilm producers, 6 (24.0%) were moderate, while 10 (40.0%) isolates were classified as none or weak biofilm producers. The highest capacity of forming biofilm was detected within *P. aeruginosa* *(MDR2)* strain (2.81), followed by *K. pneumoniae* (MDR) (OD = 2.43) and *S. aureus* *(MDR3)* (OD=1.99).

Table 10. Classification criteria for biofilm formation with optical densities obtained in this study.

Cut-Off Value Calculation	Optical density values at 570nm	Criteria for classification
OD ≤ ODcut	**OD is** < 0.21	Non-biofilm producer (NBP)
ODcut < OD ≤ 2 × ODcut	0.21< **OD** ≤ 0.42	Weak biofilm- Producer (WBP)
2 ODcut < OD ≤ 4 × adequate	0.42< **OD** ≤ 0.84	Moderate biofilm-Producer (MBP)
OD >4 × ODcut	**OD** >0.84	High biofilm-Producer (HBP)

OD: optical density. The cut-off OD: Three standard deviations above the mean OD of the blank test.

The biofilm-forming capacity among uropathogens has been widely investigated. Bacterial strains employ this mechanism as a strategy of persistence to antibiotics (Holá *et al*., 2021). It is important to highlight that all these species have already been linked to biofilm formation (Flores-Mireles *et al*., 2015). In this study, almost all uropathogens tested strains produced biofilm 20/25 (80%), nevertheless, there is a significant variation in the biofilm-forming ability among the clinical isolates, which is distributed into different categories as mentioned before. For instance, all Pseudomonas strains demonstrated high rates of biofilm formation. These results corroborate with many recent findings that elucidate the great ability of Pseudomonas to form biofilm (Ciofu & Tolker-Nielsen, 2019). Moreover, these strains were MDR, suggesting that maybe there is a correlation between strong biofilm production and multidrug resistance. Like the results found by Tellis *et al*. (2017), MDR was found to be significantly higher among the strong biofilm producers than the non-producers. Likewise, *K.*

pneumoniae showed a high ability to form a biofilm with a greater average OD value than that reported by Leoney *et al*. (2020).

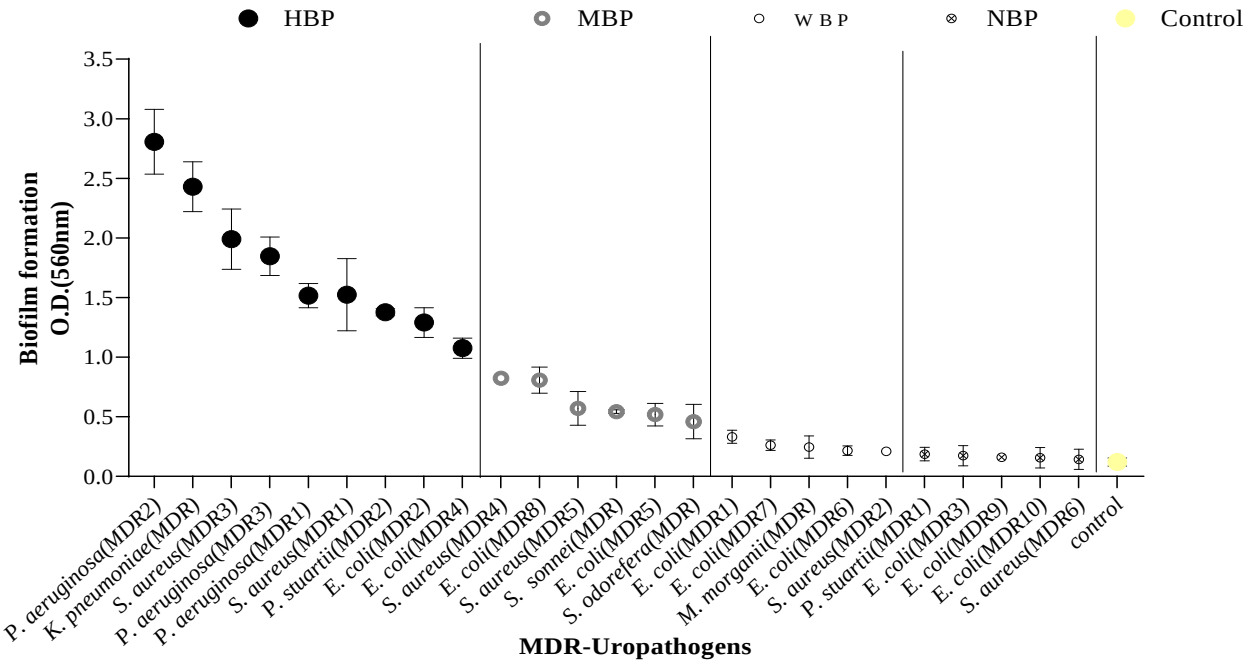

Figure 18. Biofilm formation of MDR uropathogens expressed by the mean values of absorbance at 560 nm. The results are expressed as the mean of three replicates (mm ± SD). OD: Optical Density. HBP: High Biofilm Producer, MBP: Moderate Biofilm Producer, WBP: Week Biofilm Producer, NBP: Non Biofilm Producer.

Only two strains of *S.aureus* namely *S. aureus* (MDR1) and *S. aureus* (MDR3) were high biofilm producers. The ability of *S. aureus* to form biofilm is a long-known fact. These data correlate well with those reported by Kwon *et al*. (2008), suggesting that the MDR clinical isolates of *S. aureus* have a greater likelihood of developing biofilms. When it comes to *E. coli* MDR strains, OD values ranged between 0.16-1.35, which seems almost similar to the results obtained by Leoney *et al*. (2020), in which OD values ranged from 0.4 to 1.5.

Although the general stages leading to biofilm formation are similar across pathogens, all the isolates in this study are highly variable in their ability to form biofilm. This may be associated with several factors such as adhesive fibers, proteins, nucleic acid, and exopolysaccharide material associated with a biofilm which can be distinct in a species or even strain-specific manner. Likewise, the architecture and regulatory components controlling biofilm formation vary from pathogen to pathogen (Floyd *et al*., 2017).

7 Antibiofilm activity of propolis extracts

In response to a range of stressful conditions, bacteria develop biofilms as a survival strategy. This form involves irreversible adhered bacterial clusters which are usually embedded in a protective extracellular polymeric matrix. Most chronic and recurring urinary tract infections have recently been linked to biofilm formation within the urinary system. This global health concern increases bacteria's resistance to antibiotics (Sharma *et al*., 2019), resulting in increased mortality and morbidity. Traditional antimicrobials confront considerable challenges due to the multifactorial nature of biofilm development and drug resistance. In this regard, the screening of natural resources as prospective anti-biofilm mitigation candidates has grown.

In this study, the antibiofilm activity of PEE was evaluated at MIC and sub-MIC concentrations and the results were reported in table 11. The examined strains were selected for their high biofilm formation potential. The antibiofilm activity results showed that the highest biofilm inhibitions were observed at MIC while lower biofilm inhibitions were detected in minimum concentrations, thus, this activity decreased in a concentration-dependent manner right down to the low-test concentration.

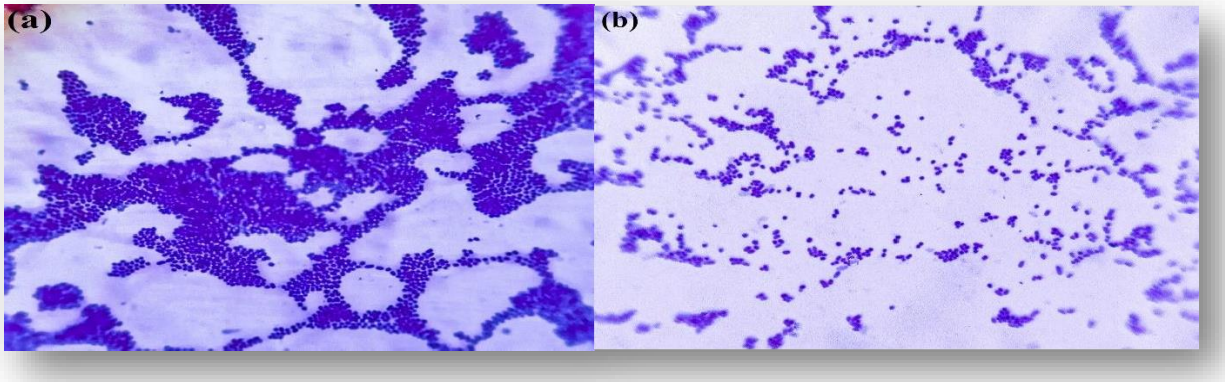

Figure 19. A representative image revealing the significant inhibition in biofilm formation of *S. aureus* (MDR1) using light Microscopic observation (magnification X40): (a) before treatment with PEEG and (b) after treatment with PEEG at MIC concentration by crystal violet staining assay.

As can be seen, PEE exhibited varying degrees of biofilm inhibition against the selected strains, PSH and PTBL extracts were highly active and inhibited the biofilm of all tested pathogens. The highest antibiofilm activity was recorded against *S. aureus*(MDR3) with inhibitions ranging from 16.03±3.38 to 74.56±0.69% under the activity of PSH extract. Similarly, the high ability to eliminate *S. aureus*(MDR3) biofilm by PACH extract was also recorded and the biofilm eradication varied from 72.43±2.88 at MIC to 8.89±0.62 % at MIC/8

while less activity was recorded within the remaining strains. Interestingly, good antibiofilm activity was recorded within the PSH extract against *P. aeruginosa*$_{(MDR3)}$ compared to the remaining extracts. Concerning PAZ extract no activity was recorded against all the tested strains. Overall, none of the extracts showed biofilm inhibition at MIC/16 and MIC/32.

Table 11. Antibiofilm activity of different propolis extracts against high biofilm producer.

Extracts	Concentration	*E. coli (MDR4)*	*E. coli (MDR2)*	*P.aeruginosa (MDR2)*	*P.aeruginosa (MDR3)*	*P.aeruginosa (MDR1)*	*S.aureus (MDR3)*	*S.aureus (MDR1)*	*K.pneumonia (MDR)*	*P. stuartii (MDR1)*
PSH	**MIC**	54.76±5.60	50.41±2.66	29.35±5.60	61.75±1.67	39.83±4.59	74.56±0.69	45.93±1.39	43.36±0.61	18.67±2.09
	MIC/2	-	42.24±2.68	21.14±1.20	48.43±0.71	22.94±3.45	60.86±1.83	31.18±3.64	29.20±4.65	-
	MIC/4	-	19.82±4.17	-	18.89±4.53	12.32±1.94	39.57±2.49	12.70±2.29	10.72±0.90	-
	MIC/8	-	-	-	9.53± 1.86	-	16.03±3.38	-	-	-
	MIC/16	-	-	-	-	-	-	-	-	-
	MIC/32	-	-	-	-	-	-	-	-	-
PSHM	**MIC**	58.46±1.02	21.72±6.98	-	26.40±1.77	-	43.54±1.70	21.62±4.59	35.85±1.01	42.57±3.60
	MIC/2	45.41±4.67	-	-	5.59±0.62	-	25.66±0.94	8.79±1.44	32.20±1.96	25.76±3.09
	MIC/4	13.62±2.09	-	-	-	-	5.96±1.02	-	-	18.97±2.03
	MIC/8	-	-	-	-	-	-	-	-	10.32±1.69
	MIC/16	-	-	-	-	-	-	-	-	-
	MIC/32	-	-	-	-	-	-	-	-	-
PAZ	**MIC**	-	-	-	-	-	-	-	-	-
	MIC/2	-	-	-	-	-	-	-	-	-
	MIC/4	-	-	-	-	-	-	-	-	-
	MIC/8	-	-	-	-	-	-	-	-	-
	MIC/16	-	-	-	-	-	-	-	-	-
	MIC/32	-	-	-	-	-	-	-	-	-
PACH	**MIC**	-	33.94±0.77	-	-	-	72.43±2.88	-	-	-
	MIC/2	-	7.43±3.72	-	-	-	46.68±1.64	-	-	-
	MIC/4	-	-	-	-	-	15.95±2.59	-	-	-
	MIC/8	-	-	-	-	-	8.89±0.62	-	-	-
	MIC/16	-	-	-	-	-	-	-	-	-
	MIC/32	-	-	-	-	-	-	-	-	-
PEEF	**MIC**	-	-	-	-	-		29.54±2.36	-	-
	MIC/2	-	-	-	-	-		17.20±1.83	-	-
	MIC/4	-	-	-	-	-		-	-	-
	MIC/8	-	-	-	-	-		-	-	-
	MIC/16	-	-	-	-	-		-	-	-
	MIC/32	-	-	-	-	-		-	-	-
PTBL	**MIC**	24.59±3.31	43.50±1.78	49.60±3.03	23.00±0.07	51.55±1.65	63.70±3.58	52.92±7.79	46.57±0.82	66.81±1.24
	MIC/2	13.17±1.28	14.16±1.21	20.34±2.64	10.06±2.77	37.97±0.08	44.48±1.47	38.26±1.44	13.64±1.02	51.90±2.85
	MIC/4	-	-	6.27±1.49	-	26.62±0.20	31.72±4.49	24.22±3.01	-	45.68±0.71
	MIC/8	-	-	-	-	10.91±2.38	21.49±1.21	11.90±1.09	-	29.64±1.33
	MIC/16	-	-	-	-	-	-	-	-	-
	MIC/32	-	-	-	-	-	-	-	-	-
PTAK	**MIC**	-	50.95±4.36	-	31.26±0.40	16.30±1.46	13.91±0.95	54.79±2.13	30.38±3.24	36.43±0.82
	MIC/2	-	29.42±0.43	-	24.59±0.00	12.19±1.72	-	42.78±5.83	22.86±1.44	15.90±1.12
	MIC/4	-	12.60±1.57	-	9.21±1.76	-	-	25.00±0.00		
	MIC/8	-		-		-	-	-		
	MIC/16	-		-		-	-	-		
	MIC/32	-		-		-	-	-		
PEEG	**MIC**	-	28.00±2.20	-	34..56±2.93	-	62.18±1.07	-	39.56±1.45	-
	MIC/2	-	-	-	14.75±1.74	-	48.41±2.58	-	15.76±1.31	-
	MIC/4	-	-	-	-	-	27.39±2.29	-	9.70±0.35	-
	MIC/8	-	-	-	-	-	-	-	-	-
	MIC/16	-	-	-	-	-	-	-	-	-
	MIC/32	-	-	-	-	-	-	-	-	-

MIC: Minimal inhibitory concentration**. PTBL:** Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

Uropathogenic biofilms provide numerous issues, as bacterial cells in biofilms are innately resistant to antimicrobial treatments, making infections more difficult to treat.

Furthermore, biofilm in the bladder's uroepithelium has been hypothesized as the mechanism causing recurrent cystitis.

The results of the antibiofilm activity indicate that the ethanolic extracts of propolis exhibited a variable capacity in biofilm inhibition against the tested strains. Although *S. aureus* *(MDR3)* reveals high levels of resistance to overall 16 antibiotics, PSH and PACH extracts showed high inhibition greater than 70% on biofilm formation which indicates that these extracts are of great interest. Similarly, the study of Daikh *et al*. (2019) demonstrated that the ethanolic extract of Algerian propolis possessed excellent antibiofilm activity against *S. aureus* strains. In another study, Bouchelaghem, (2021) evaluated the effect of Hungarian propolis samples on *S. aureus*, the PEE effectively inhibited the biofilm formation. Analysis of *P. aeruginosa* biofilms also revealed to be sensitive to different extracts. All MDR *P. aeruginosa* strains were affected by PSH and PTBL extracts, the highest biofilm reduction was observed on *P. aeruginosa*$_{(MDR3)}$ with a reduction of 61.75±1.67 %. Likewise, the results from Brazilian green propolis also showed high antibiofilm effect against eight MDR clinical strains of *P. aeruginosa* (Santos *et al*., 2020). Furthermore, Albanian propolis showed considerable anti-biofilm activity against *P. aeruginosa* strain (P1242) (Meto *et al*., 2020).

On the other hand, *E. coli* MDR strains showed week sensitivity. A previous study conducted by Ranfaing *et al*. (2018) showed that propolis had a positive activity on full biofilm development versus a panel of 12 MDR Uropathogenic *E.coli*. In addition, a study of Russian propolis showed complete inhibition of *E.coli* biofilm at a high concentration (20% w/v) (Bryan *et al*., 2015). The analysis of MDR *K. pneumoniae* biofilm demonstrated that it was susceptible to the various examined extracts. The investigation conducted by Santos *et al*. (2020) discovered that propolis extract significantly reduces biofilm viability of eight MDR *K. pneumonia*.

It is worthy to note that PTBL extract showed good biofilm inhibition against MDR *P. stuartii*. According to the literature, no published studies documented the impact of propolis extract on MDR Providencia biofilm, thus more investigations are needed.

Overall, these data reveal the efficacy of propolis extract in controlling biofilms formed by MDR bacteria. In fact, it is evident from the literature that chemical compounds from propolis such as polyphenols and flavonoids are responsible for its multiple pharmaceutical activities (Sforcin *et al*., 2000). As mentioned previously, propolis extracts exhibited a significant variance in biofilm inhibition among the different strains. The most likely explanation is that antibiofilm inhibition exerted by propolis extracts is the outcome of the activity of different components or a synergetic effect; thereby, the type of chemicals and

differences in concentration between bioactive contents of propolis may cause variation in anti-biofilm activities.

Apparently, the tested extracts share similarities in their flavonoid contents such as quercetin, cynarin, apigenin, hesperdine, and kampferol, conversely, distinct content was shown regarding their content in phenolic acids, for instance, PSH extract exhibited four phenol acids (caffeic acid, chlorogenic acid, P-coumaric acid, and ferulic acid), while, PSHM show only two (vanillic acid and ellagic acid). Whereas for PACH extract; mainly one phenolic compound was detected (ellagic acid), while PAZ were phenol acids free showing non-biofilm inhibition. As a result, it is believed that the antibiofilm activity is largely attributed to phenolic acids rather than flavonoids, or related to a synergic effect between the different detected phenolic acids. Furthermore, many detected compounds like apigenin, kaempferol, quercetin, rutin, ellagic acid, chrysin, fisetin, naringenin, and hesperidin, have been shown to exhibit a destructive capacity against biofilm formation (Slobodníková *et al*., 2016)

Actually, there are limited studies interested in the mechanism of action of propolis against bacterial biofilms. It has long been recognized that bacteria in mature biofilm are protected and attached to one another by an extracellular polymeric substance which limits the access of the antibacterial compounds through the biofilm community. The mechanism of anti-biofilm activity of Russsin PEE has been reported by Bryan *et al*. (2015), the results indicate that at high concentrations of RPEE, the diffusion of active compounds through the biofilm was achieved, the mechanism starts when anti-biofilm compounds in the RPEE damage the structural integrity of the extracellular polymeric matrix, which eliminates the outer layer from the surface of the biofilm. As a result, the biofilm loses its ability to protect the bacteria, allowing antibiofilm components access to the bacteria, thus leading to cell lysis and, finally, cell death.

8 Quorum-sensing inhibition

The anti-QS activity of PEE was performed using two assays; violacein inhibition on the strain *C. violaceum 12472* and Quorum-sensing inhibition on *C. violaceum 026*. The MIC values of all propolis extracts were evaluated on both strains and reported in table 12. As can be noted, all propolis extracts inhibited violacein production in CV12472 at MIC, MIC/2, MIC/4, and MIC/8. The PSH, PTBL, PTAK, and PACH extracts showed the highest inhibition with a percentage of 100 ± 0.00% at MIC and this inhibition decreased with the decrease in concentration. Interestingly, PSH extract was the only extract showing complete

violacein inhibition at MIC/2 (100%). No inhibition activity was observed under MIC/8 within PSHM, PACH, and PEEF.

Compared to all extracts, PAZ extract exhibited the lowest activity with an inhibition of 29.64±1.09% at MIC, and showed no activity beyond MIC/2.

Regarding the results of the anti-QS assay against CV026, only three extracts showed activities namely PTBL, PSH, and PTAK. The highest anti-QS inhibition diameter zone on CV026 was 14.00mm at MIC and 11.00mm at MIC/2 for PTBL extract (Appendix 5). PATK and PSH extracts exhibited similar activity at MIC with a diameter zone of 13mm. whereas the remaining extracts showed no inhibition of QS against CV026.

QS is a microbial cell-to-cell communication system that regulates the pathogenicity of several bacterial species. Generally, this bacterial communication depends on QS signaling molecules such as N-acylhomoserine- lactones (AHLs). Since this phenomenon plays an important role in microbial virulence, many scientists are trying to develop natural products that can disturb QS. The limitation of this virulence process leads to the attenuation of microbial virulence, as well as, the treatment of several infections (Asfour, 2018).

In this study, all propolis extracts showed a significant drop in violacein production. PSH extract was a significantly more powerful violacein inhibitor even at lower concentrations. These results are consistent with the findings of Sorucu & Ceylan, (2021) who showed the potent capacity of propolis in suppressing the production of violocein of *C. violaceum 12472* at MIC (12.5 mg/ml). Moreover, Ceylan & Halime. (2020) investigated the anti-QS of southwest Anatolian PEE against *C. violaceum 12472* and all tested extracts were potent in violacein inhibition. Similarly, Gemiarto *et al*. (2015) confirmed the strong capacity of manuka propolis to inhibit violacein production of *C. violaceum*. The study conducted by Bulman *et al*. (2011), documented that propolis contains many bioactive contents. These constituents disrupt QS autoinducers such as acyl homoserine lactone (HSL) production, which is the key mediator of the QS process and one of the major triggers of violacein production. Therefore, the deficiency of purple pigment in *C. violaceum* suggests that propolis could disrupt acyl-homoserine lactone (AHL) formation and, as a result, hinder violacein production.

On the other hand, the suppression of CV026 QS was demonstrated at MIC for PSH, PTBL, and PTAK extracts, with the development of a white or cream-colored halo surrounding the wells against a purple lawn of active CV026 bacteria. Although AHL were externally supplied in the assay, CV026 could not produce violacein which indicates that

propolis prevented the reception or the absorbance of AHL by CV026. Consequently, makes this strain unable to trigger violacein production.

Table 12. Violecein pigment inhibition on CV12472 and anti-quorum sensing on CV026 of propolis ethanolic extracts.

	PSH		**PSHM**		**PTBL**		**PTAK**		**PACH**		**PEEF**		**PAZ**		**PEEG**	
	VI	QSI	VI	QSI	VI	QSI	VI	QSI	VI	QSI	VI	QSI	VI	QSI	VI	QSI
MIC*	100±0.00	13	65.23±2.65	-	100±0.00	14	100±0.00	13	100±0.00	-	44.86±2.49	-	29.64±1.09	-	62.39±1.19	-
MIC/2	100±0.00	9	53.79 ±1.97	-	96.02±1.93	11	75.76±0.24	-	83.50±1.18	-	41.15±0.77	-	-	-	38.36±0.00	-
MIC/4	83.36±0.46	-	44.13±2.01	-	79.98±2.12	9	54.67±0.33	-	54.97±1.72	-	34.87±1.46	-	-	-	36.45±0.00	-
MIC/8	55.75±3.19	-	40.75±2.89	-	56.02±0.53	-	34.43±0.77	-	38.55±2.81	-	18.45±1.60	-	-	-	31.22±0.42	-
MIC/16	49.30±4.69	-	-	-	46.02±0.53	-	20.77±0.93	-	-	-	-	-	-	-	26.66±0.98	-
MIC/32	44.55±3.77	-	-	-	20.98±0.19	-	9.37±2.34	-	-	-	-	-	-	-	24.20±1.20	-

MIC: Minimal inhibitory concentration.**VI**: Violacein inhibition.**QSI**: Quorum-sensing inhibition.

*****: MIC values for CV12472: MIC(PSH)=10 mg/ml, MIC(PSHM)=20mg/ml, MIC(PTBL)=0.312mg/ml, MIC(PTAK)=1.25mg/ml, MIC(PACH)=20 mg/ml, MIC(PEEF)=20 mg/ml, MIC(PAZ)=20 mg/ml, MIC(PEEG)=20 mg/ml. MIC values for CV06 were 20mg/ml for all extracts except for PTBL (MIC=1.25mg/ml).

PTBL: Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

According to a literature investigation, only a few reports demonstrated the efficacy of propolis components as QS inhibitors. Phenolic acids and flavonoids are the active components that can block the chemical signaling process. It could be possible that in the PSH, PTBL, and PTAK extracts, the strong inhibition of violacein formation and the anti-quorum inhibition observed results from the presence of caffeic acid. These findings are in agreement with the results of Sorucu & Ceylan, (2021) who reported a great QSI activity of PEE which has a high content of caffeic acid. Moreover, chrysin was identified in all studied samples except in PAZ, thus, QSI could be attributed to chrysin since this compound was previously reported by Kharsany *et al*. (2019) to possess a strong violacein inhibition capacity (78.82%) at a concentration of 1.25 mg/ml (Nayaka *et al*., 2014). It is noteworthy that quercetin and naringenin were detected in our propolis samples, these compounds were reported to reduce violacin production (Asfour, 2018). Furthermore, Gopu *et al.* (2015) evidenced that quercetin efficiently inhibited QS by suppressing violacein production in CV026 in a concentration-dependent manner. Many flavonoids proved their feasibility as QS inhibitors such as rutin and ellagic acid. In this context, Truchado *et al*. (2012) documented that rutin and ellagic acid effectively interfere with the QS system. Furthermore, Skogman *et al*. (2016) tested the capacity of flavonoids to inhibit QS and found that most of the tested flavonoid compounds show QSI activity. There are three different mechanisms for QS inhibition, (i) stopping the synthesis of signaling molecules, (ii) degradation of signaling molecules such as AHL, and (ii) inhibition of signaling receptors (John & Ramesh, 2020).

9 Anti-urease activity

Urease (urea amidohydrolase: EC 3.5.1.5) is the enzyme that catalysis the hydrolysis of urea to generate ammonia. This enzyme is present in many organisms, such as bacteria, which is known as one of the factors contributing to different pathologies such as UTI. In the case of UTI, the ammonia resulting from urease activity causes an increase in urine pH. Furthermore, inorganic ions contained within it, such as magnesium and calcium phosphate crystals, precipitate, these aggregates accumulate in the urine and provide an optimal home for bacterial growth and persistence.

A large body of clinical evidence supports the classification of uropathogenic urease as a virulence factor. Indeed, ureolytic activity plays an important role in the colonization and persistence of various uropathogens (Mora & Arioli, 2014). As a result, urease inhibitors are being investigated as potential treatments to attenuate virulence bacteria. Herein, the urease inhibitory activity of different propolis extracts was performed using the microplate method and the results are presented in figure 20.

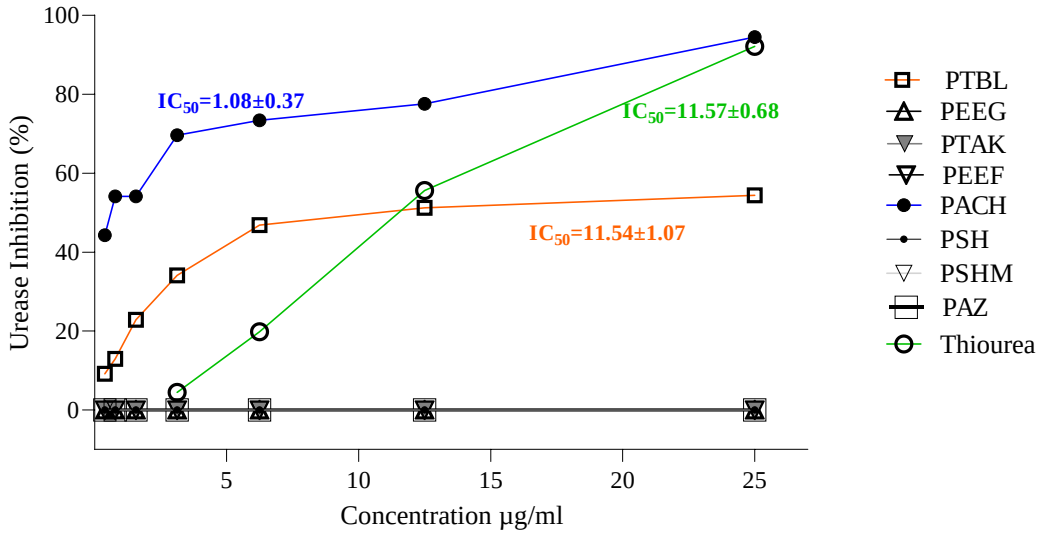

Figure 20. Inhibitory effect of propolis ethanolic extract against urease enzyme at different concentrations. The percentages of inhibition were reported as mean±SD (n=3).

Among all the tested extracts only PACH and PTBL extracts exhibited substantial inhibitory effects against the virulence enzyme urease, while none of the remaining extracts showed anti-urease activity. The highest activity was recorded within PACH extract with an IC_{50} of 1.08±0.37µg/ml which was more strongly active compared to the employed standards Thiourea (11.57±0.68µg/ml). On the other hand, PTBL showed less activity (IC_{50}=11.54 1.07µg/ml) but approximately similar inhibition was recorded when compared to the standards Thiourea. In a previous study conducted on 11 extracts of Turkish propolis, significant activity was recorded within all extracts with an IC_{50} value ranging from 1.11 to 5.87mg/ml *(*Can *et al.*, 2022). Another study was conducted on 15 extracts of Turkish propolis and the results showed good inhibition with an IC_{50} in the range of 0.26 to 1.52 mg/ml (Baltas *et al.*, 2016). This inhibition effect is related to the chemical content of propolis, the reported activity may be the result of interaction between components in the complex natural matrix of propolis. As a result, certain samples may be more active than others. Overall, these findings indicate that propolis can play a significant role in the elimination of urease harmful effects.

10 Anti-inflammatory activity of propolis ethanolic extracts

The anti-inflammatory activity of propolis extracts was evaluated to examine its ability to inhibit heat-induced BSA protein denaturation. As presented in table 13, all propolis extracts harvested from El-Taref region exerted a potent preventive effect on thermally albumin denaturation in a dose-dependent manner. Among the tested extracts, PTBL showed the highest inhibitory activity within the range of 94.73± 2.19% to 50.92± 2.25% at the

concentration range of 62.5 to 500 µg/ml. Relevantly, PTBL demonstrated a good IC_{50} value of 1.00± 0.14µg/ml compared to the commercial anti-inflammatory diclofenac (63.5 ± 0.02 µg/ml), employed as a drug reference. Similarly, PTAK sample presented a powerful inhibitory effect at the high concentration of 500µg/ml within the range of 72.84±7.74%. On the other hand, at 500µg/ml PSH extract showed moderate anti-inflammatory activity lower than the employed standard. Unexpectedly, the remaining extracts show no protective effect against BSA protein.

Table 13. Anti-inflammatory activity of propolis ethanolic extracts.

Extracts	MAX inhibition (%)	IC_{50} (µg/ml)
PTBL	94.73± 2.19	1.00±0.14
PTAK	72.84±7.74	2.65±0.27
PSH	66.94 2.30	14.52±3.21
PSHM	-	-
PACH	-	-
PEEG	-	-
PEEF	-	-
PAZ	-	-
Diclofenac	98.22±2.09	63.5 ± 0.02

PTBL: Ethanolic extract of propolis from Besbes-Lakhdara, **PTAK:** Ethanolic extract of propolis from Ain-Khiar, **PSH:** Ethanolic extract of propolis from Ouillen, **PSHM:** Ethanolic extract of propolis from Ouled-Moumen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PEEF:** Ethanolic extract of propolis from Ain-Fakroun, **PACH:** Ethanolic extract of propolis from Ain-Chadjra, **PAZ:** Ethanolic extract of Propolis from Ain-Zitoun.

Inflammation response is one of the body's defense strategies against external and internal attacks, and it results in the onset of several pathological diseases. Given that, one of the hallmarks of inflammation is protein denaturation. Most denaturized proteins lose their biological roles, resulting in the generation of autoantigens and the induction of many autoimmune dysfunctions. Indeed, tissue protein denaturation is a well-known cause of inflammation (Mouffouk *et al.*, 2018). Therefore, natural substances having anti-protein denaturation effects are of tremendous interest for the development of anti-inflammatory drugs.

Based on the results of the *in vitro* anti-inflammatory activity we can affirm that propolis extracts possess a potent protective capacity since it maintains albumin three-dimensional structure. These findings are in agreement with the results obtained by Mendez-Encinas *et al*. (2023) who demonstrated that Sonoran propolis inhibited protein denaturation with a percentage of 79% to 100%, showing high anti-inflammatory activity. Similarly, Portuguese propolis revealed good anti-inflammatory activity, and the *in vitro* assay showed good potential in preventing BSA denaturation (Araújo *et al*., 2022).

Algerian propolis is characterized by the abundance of a variety of bioactive compounds known to possess a bunch of pharmacological properties. It is logical to correlate the potent anti-inflammatory activity detected in the studied samples harvested from El-Taref region to propolis polyphenolics content since much literature proved that polyphenolics are potent anti-inflammatory agents. Among the flavonoids present in our propolis extracts, apigenin, ellagic acid, and rutin have been reported as anti-inflammatory agents (Hussain *et al*., 2016). In another study, the infusion of quercetin suppressed inflammatory response in a rat model (Hu *et al*., 2019).

Propolis has been shown to have anti-inflammatory effects by regulating key inflammatory mediators, enhancing anti-inflammatory cytokines, reducing the generation of pro-inflammatory cytokines, and inhibiting the activation of nuclear factor (NF-)B *(*Campos *et al*., 2015). However, due to the lack of human studies on propolis supplementation to manage inflammation in rUTI patients, clinical trials are mandatory to confirm its efficacy.

11 Cytotoxicity

In this study, the cytotoxicity of four selected propolis extracts was tested using brine shrimp lethality assay, the results showing mortality percentages and LC_{50} are presented in table 14. The results demonstrate that propolis extracts exhibited a dose-dependent effect after 24h of exposure. All extracts showed mortality lower than 50% against brine shrimp nauplii at a concentration of 4000µg/ml. while no mortality was recorded under the concentration of 1000µg/ml. Furthermore, all extracts showed variable median lethal concentrations with a value ranging from 84.14±11.36 µg/ml to 118.01±3.44µg/ml, showing weak toxicity in comparison to the positive standard potassium dichromate ($K_2Cr_2O_7$) which was extremely toxic presenting an LC_{50} of 21.11±3.47µg/ml.

Table 14. Toxic effect of propolis extracts against brine shrimp larvae.

Concentrations	PSH	PTBL	PACH	PEEG	$K_2Cr_2O_7$ % of Mortality
500	0.00± 0.00	0± 0.00	0± 0.00	0±0.00	0± 0.00
1000	10.00±0.00	10.00± 0.00	16.68±5.77	0±0.00	50± 0.00
2000	30.00± 0.00	30.00± 0.00	33.43±5.77	10.00±0.00	80± 0.00
4000	40.00± 0.00	43.33± 5.77	46.67±5.77	32.33±4.70	100± 0.00
5000	53.33±5.77	66.67± 5.77	73.33±4.70	53.33±5.77	100± 0.00
LC_{50} (µg/ml)	105.02± 14.92	91.32± 9.81	84.14±11.36	118.01±3.44	21.11±3.47

PTBL: Ethanolic extract of propolis from Besbes-Lakhdara, **PSH:** Ethanolic extract of propolis from Soug-Ahras Ouillen, **PEEG:** Ethanolic extract of propolis from Tamlouka, **PACH:** Ethanolic extract of propolis from Ain-Chadjra. $K_2Cr_2O_7$: Potassium dichromate.

The brine shrimp lethality approach is a rapid preliminary assay that is frequently used to determine the general cytotoxicity of compounds (Benouchenne *et al*., 2022). According to Clarkson's toxicity classification, three main categories can be employed to determine extract

toxicity: extracts with LC_{50} above 1000 µg/mL are non-toxic, LC_{50} of 500 - 1000 µg/mL are low toxic, extracts with LC_{50} of 100 - 500 µg/mL are medium toxic, while extracts with LC50 of 0 - 100 µg/mL are highly toxic (Clarkson *et al*., 2004). The LC_{50} values found in the present study were within the range of 100 - 500 µg/ml for PSH and PEEG extracts thus indicating moderate toxic properties, while PTBL and PACH showed a toxicity under 100µg/ml underlying high toxicity effect. The results recorded within PSH and PEEG are in line with a study conducted on Malaysian propolis which showed a moderate level of toxicity (Yusop *et al*., 2019). Whereas the results recorded within PTBL and PACH are in agreement with many reports disclosing the potential cytotoxic effect of propolis (Tanvir *et al*., 2018; Ngassapa *et al*., 2022). Indeed, these findings is an indicative of cytotoxicity, antibacterial activities, and various pharmacologic actions which suggest the notable clinical importance of these extracts against bacteria…etc. Furthermore, the brine shrimp assay is considered as a convenient probe for a preliminary assessment of toxicity, and detection of fungal toxins, bactericidal, and other pharmacological actions (Meyer *et al*., 1982).

According to many researches, propolis toxicity degree differs according to its chemical profile, being the first factor responsible for the occurrence of toxicity. For instance, a study conducted on twenty-eight samples of Tanzanian propolis showed a significant difference between the degree of toxicity among the studied samples, as it was recorded, fourteen samples showed varying degrees of toxicity, while the remaining samples showed no toxic effect (Ngassapa *et al*., 2022). On the other hand, a great number of studies have demonstrated the safety of propolis supplementation for human consumption. Indeed, clinical trials in mice and humans showed that propolis is generally well tolerated and non-toxic until provided in extremely high doses (Braakhuis, 2019). However, more clinical trials are required.

Conclusion

The global wide-spread of bacterial resistance among uropathogens is of major concern since it increases the severity of infection among UTI patients and thus it hinders the healing process. Recently, the big hurdle behind MDR uropathogens is the incapacity to impair its virulence with common antibiotics. Therefore, targeting bacterial virulence factors with natural therapeutic agents may be a suitable approach. The golden bee product "Propolis" harvested from eight different eastern Algerian regions was chosen to investigate the chemical profile and to report its efficiency in suppressing MDR uropathogens isolated from women with rUTI, including different related virulence factors (biofilm, quorum sensing, urease). The toxicity and biological effects against free radicals and inflammation were also screened.

The rich chemical profile and the potential biological efficacy of propolis extracts have been positively reported.

The different propolis ethanolic extracts exhibited high and variable polyphenolic profiles reflecting the importance of the harvested samples and underlying the great impact of the different factors responsible of this variation such as the phytogeographical conditions.

Based on the HPLC-DAD analyses the results demonstrated a richness of the different studies extracts in phenolic compounds. Overall 19 compounds were detected and variable quali-quantitative contents were observed among propolis extracts. These compounds are extremely known to possess valuable pharmacological importance. As a first detection, cynarin was the newly identified compound in all propolis extracts of Algerian propolis.

The antioxidant capacity of propolis extracts using different antioxidant approaches namely; DPPH, ABTS, CUPPRAK, GOR, reducing power, and phenanthroline revealed that all samples exhibited powerful antioxidant activity, demonstrating propolis's potency in eliminating the harmful effect of free radicals.

Interestingly, the antimicrobial activity of PEE revealed a significant ability to eradicate several MDR uropathogens and different related virulence factors such as biofilm and quorum sensing, proving its great efficiency in managing MDR uropthogens responsible of rUTI. Furthermore, the results of the anti-urease activity showed that both extracts collected from El-Taref (PTBL) and Ain-chadjra (PACH) have very strong inhibitory activity against the target enzyme.

On the other hand, the anti-inflammatory activity showed that propolis from El-Taref (PTBL and PTAK) and Soug-Ahras (PSH) exerted a potent preventive effect on thermally

albumin denaturation in a dose-dependent manner. This activity is of tremendous interest for the development of anti-inflammatory drugs without negative side effects.

The cytotoxic effect of propolis against brain shrimp larvae showed variable toxic effects among the different selected extracts, no mortality was recorded under the concentration of 1000µg/ml.

According to the above positive results, we can affirm that propolis can be regarded as an important pharmaceutical agent capable of countering MDR uropathogens and could be used as an alternate or supplement remedy for the management of rUTI caused by MDR uropathogens, prevention of inflammation, and free radical's harmful effects.

Further bio-guided methods are recommended to provide additional insights to describe the highly active ingredients responsible for the studied biological activities and to determine the proper mechanism. Furthermore, due to the lack of human studies on propolis supplementation to manage rUTI caused by MDR uropathogens, clinical trials are mandatory to confirm its efficacy.

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Appendix 1

Diffrent HPLC-DAD analysis standars :

Fumaric acid, Gallic acid, P-benzoquinone, Protocatechuic acid, Theobromine, Theophylline,Catechin, 4-hydroxybenzoic acid, 6,7-dihydroxycoumarin, methyl-1,4 benzoquinone, Vanillic acid,Caffeic acid, Vanillin, Chlorogenic acid, P-coumaric acid, Ferrulic acid, Cynarin, Coumarin,Prophylgallate, Rutin, Trans-cinnamic acid, Ellagic acid, Myricetin, Fisetin, Kersetin, Trans-cinnamik acid, Luteolin, Rosmarinic acid, Kaempferol, Apigenin, Chrysin, 4 hydroxylresorkinol,1,4-dichlorobenzene, Pyrocatechol, 4-hydroxy benzaldehid, Epicatechin, 2,4-dihydroxybenzaldehide, Hesperedin, Oleuropein, Naringenin, Hesperetin, Genistein, and Curcumin.

Appendix 2

Recurrent UTI patient questionnaire :

Recurrent UTI Patient Questionnaire

Name : …… Age: …….. Gender: ……

1. When did you start first urine infections?

- ☐ Less than six months ago
- ☐ 6-12 months ago
- ☐ 1 - 2 years ago
- ☐ 2 - 5 years ago
- ☐ More than 5 years ago
- ☐ Since childhood (age of first infection)

2. How many infections have you had in the last:

6 month……...
12 months …….

3. Do you think that anything in particular 'triggers' your urinary infections? If so, please describe below:

_____

_____

4. What symptoms do you get with a urine infection? (Tick all thatapply)

- ☐ Burning and/or stinging when passing urine
- ☐ Burning and/or stinging after passing urine
- ☐ Passing urine frequently
- ☐ Rushing to the toilet
- ☐ Pains in abdomen/tummy
- ☐ Pains in flank/side
- ☐ Pains in back
- ☐ Fever
- ☐ Not listed above (please describe below)

_____

_____

5. How soon after antibiotics finish does the infection return?

- ☐ Less than a week
- ☐ More than a week

6. Have you ever passed blood in your urine? YES / NO

7. If you have any urinary symptoms when you don't have an Infection, please tick the relevant boxes below:

- ☐ Passing urine frequently
- ☐ Rushing to the toilet
- ☐ Abdominal pain
- ☐ Straining to pass water
- ☐ Poor flow of urine or slow stream
- ☐ Feeling of incomplete bladder emptying
- ☐ Burning or stinging
- ☐ Leakage of urine
- ☐ Fever

8. Do you use feminine hygiene products e.g. perfumed sprays or Vagisil? YES / NO

7. Do you have or have you had any of the following?

- ☐ Diabetes
- ☐ Kidney stones
- ☐ Operations on kidney/bladder
- ☐ Multiple sclerosis/other neurological disease
- ☐ Urinary catheter
- ☐ Long-term steroids
- ☐ Are you pregnant?
- ☐ Urinary tract infections as a child

8. Is there anything else you would like to tell us about your urinary infections? Please describe below

____

____

Please list your current regular medication below:

_ _

Appendix 3

Identification of bacterial strains

1. **Macroscopic and Microscopic examination**

The macroscopic investigation is the first step of identification it is generally based on the visual observation of the characters of the colonies such as the size, shape, contour, consistency, transparency, and color.

The microscopic identification allows the morphological characterization of bacterial cells: shape, grouping mode, mobility, and GRAM.

Employed medium:

Hektoen, EMB, Cetrimide, Chapman, Chrom-agar orientation.

2. **Catalase test for GRAM (+)**

A droplet of H_2O_2 (3%) was deposit on a slide previously containing a bacterial colony. The appearance of air bubbles indicates positive results (Berkowitz and Jerris, 2016).

3. **Oxidase test for GRAM (-)**

Moisten the oxidase-impregnated discs with sterile distilled water before placing them on the suspected colonies, then leave for about 20-30 minutes at room temperature. The appearance of purple color indicates positive results

4. **Coagulase test for *Staphyloccocus sp***

A 2ml of human plasma was placed in a glass tube with bacterial colony and incubated for 24h in 37C°. The coagulase-positive *S. aureus*, causes the plasma to form a clot in the tube (Subhash, 2012).

5. **API system**

API 20E and API 20 staph system were used to confirm bacterial isolates. This system consists of 20 diagnostic tests arranged in a strip containing several wells, each wells bearing the name of a specific test (Shell et al., 2017).

Interpretation

Identification of bacterial strains by API system biochemical tests

- ***API 20E:***

TABLEAU DE LECTURE DE LA GALERIE MINIATURISÉE API 20E

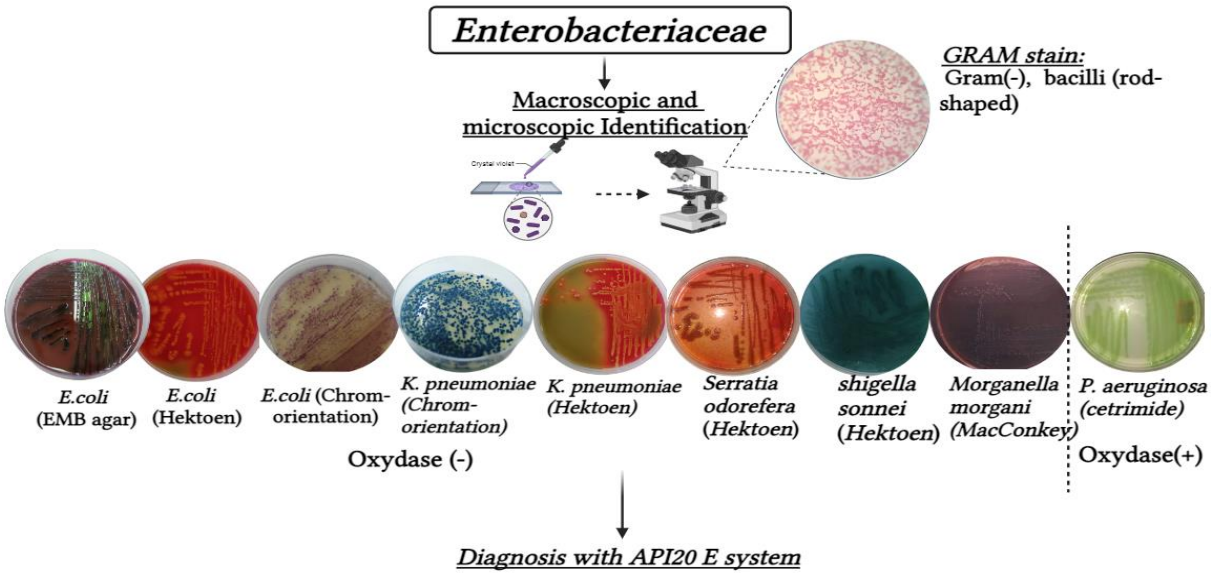
Microtube	Substrat :	Caractère recherché :	Révélateur	Lecture directe ou indirecte Test (si nécessaire)	Résultat -	Résultat +
ONPG	ONPG = Ortho-Nitro-Phényl-Galactoside	Beta galactosidase		Lecture directe		
ADH LDC ODC	Arginine Lysine Ornithine	Arginine Dihydrolase Lysine Décarboxylase Ornithine Décarboxylase	Rouge de Phénol	Lecture directe		
CIT	Citrate	Utilisation du citrate	BBT	Lecture directe		
H_2S	Thiosulfate de sodium	Production d'H_2S		Lecture directe		
URÉ	Urée	Uréase	Rouge de Phénol	Lecture directe		
TDA	Tryptophane	Tryptophane désaminase		**Lecture indirecte** Ajouter une goutte de réactif chlorure de fer III		
IND	Tryptophane	Tryptophanase ou production d'indole		**Lecture indirecte** Ajouter une goutte de réactif Kovacs		
VP	Pyruvate de sodium	production d'acétoïne (3-hydroxybutanone		**Lecture indirecte** Ajouter 1 goutte de VP1 et VP2 Attendre 10 minutes		
GEL	Gélatine	gélatinase		Lecture directe		
GLU à ARA = zymogramme	Substrat carboné (glucide)	Utilisation de substrats carbonés (glucides)	BBT	Lecture directe		
NO_2^-/N_2	Nitrates (NO_3^-)	Nitrate réductase		**Lecture indirecte** Ajouter 1 goutte de NIT1 et NIT2 et zinc éventuellement		

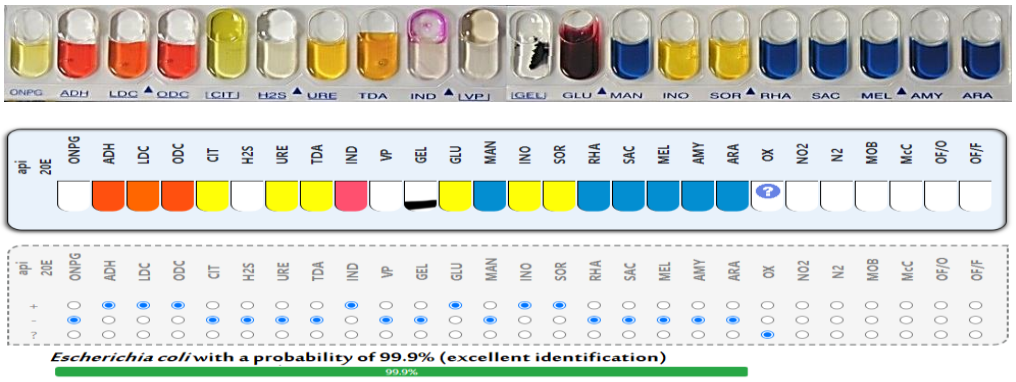
- ***API 20 STAPH:***

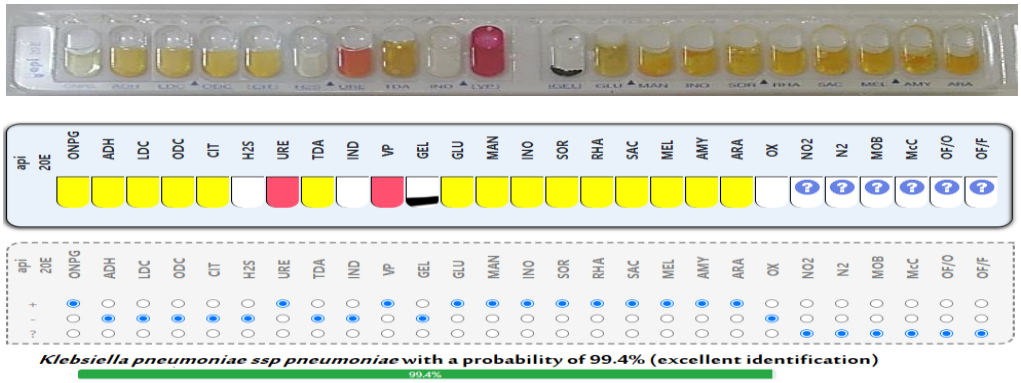
TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS / ENZYMES	RESULT	
				NEGATIVE	POSITIVE
0	No substrate		Negative control	red	—
GLU	D-glucose	1.56	(Positive control) (D-GLUcose)	red *	yellow
FRU	D-fructose	1.4	acidification (D-FRUctose)		
MNE	D-mannose	1.4	acidification (D-ManNosE)		
MAL	D-maltose	1.4	acidification (MALtose)		
LAC	D-lactose (bovine origin)	1.4	acidification (LACtose)		
TRE	D-trehalose	1.32	acidification (D-TREhalose)		
MAN	D-mannitol	1.36	acidification (D-MANnitol)		
XLT	xylitol	1.4	acidification (XyLiTol)		
MEL	D-melibiose	1.32	acidification (D-MELibiose)		
NIT	potassium nitrate	0.08	Reduction of NITrates to nitrites	NIT 1 + NIT 2 / 10 min colorless-light pink	red
PAL	ß-naphthyl phosphate	0.0244	ALkaline Phosphatase	ZYM A + ZYM B / 10 min yellow	violet
VP	sodium pyruvate	1.904	Acetyl-methyl-carbinol production (Voges Proskauer)	VP 1 + VP 2 / 10 min colorless-light pink	violet-pink
RAF	D-raffinose	1.56	acidification (RAFfinose)	red	yellow
XYL	D-xylose	1.4	acidification (XYLose)		
SAC	D-saccharose (sucrose)	1.32	acidification (SACcharose)		
MDG	methyl-αD-glucopyranoside	1.28	acidification (Methyl-αD-Glucopyranoside)		
NAG	N-acetyl-glucosamine	1.28	acidification (N-Acetyl-Glucosamine)		
ADH	L-arginine	1.904	Arginine DiHydrolase	yellow	orange-red
URE	urea	0.76	UREase	yellow	red-violet

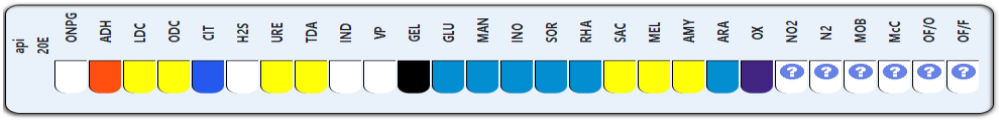
Appendix 4

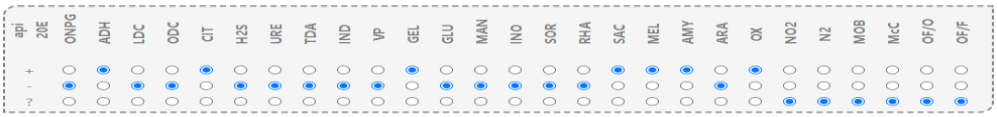
Identification of Enterobacteriaceae:



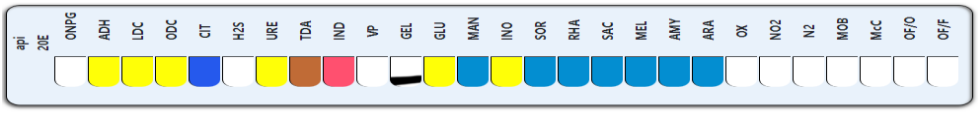


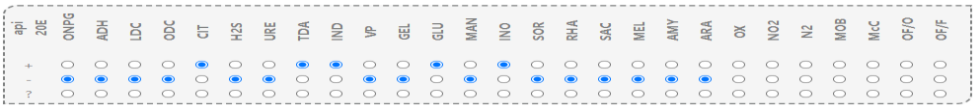




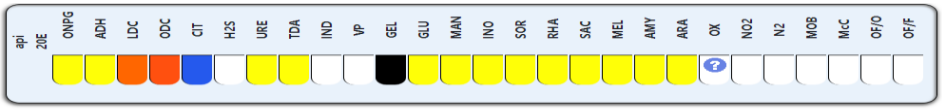


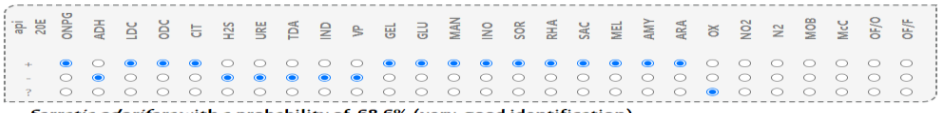
Pseudomonas aeruginosa **with a probability of 100% (excelent identification)**



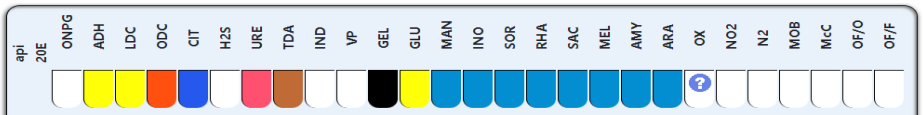


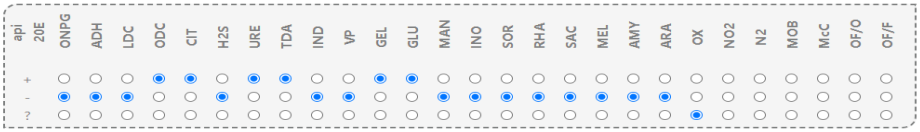
Providencia stuartii **with a probability of 97.8% (excellent identification)**



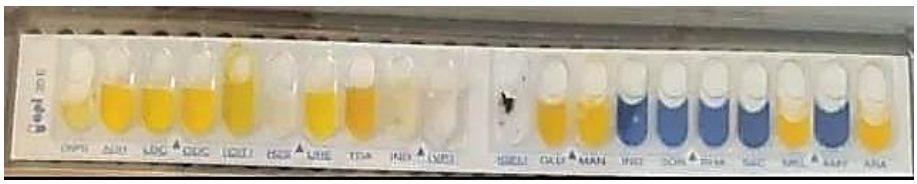


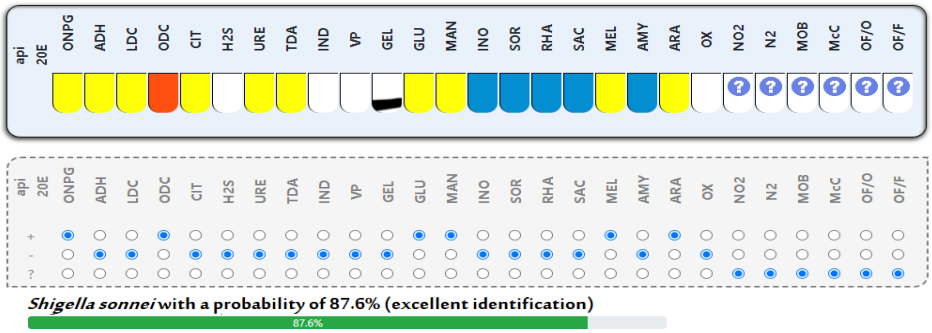
Serratia odorifera **with a probability of 68.6% (very good identification)**



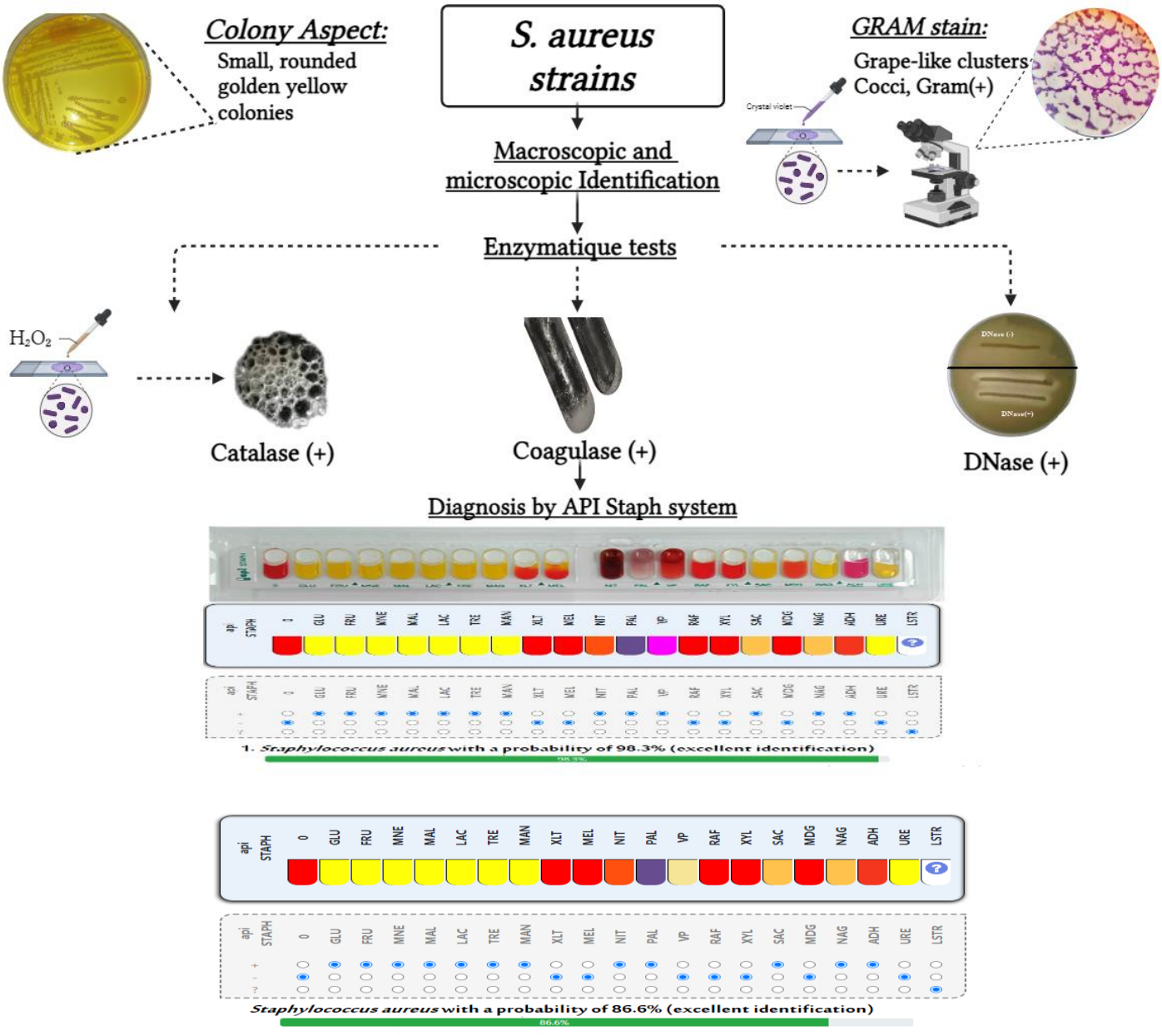


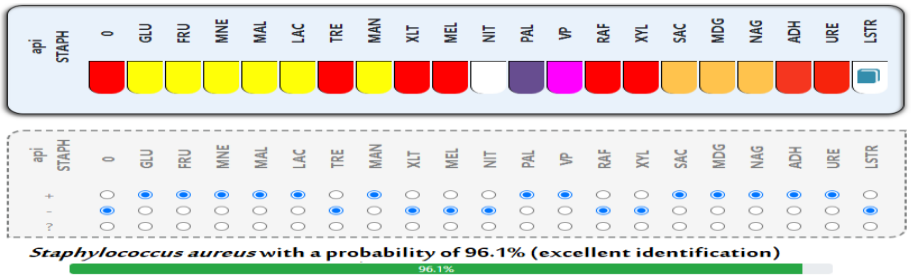
Morganella morganii **with a probability of 99.6% (excellent identification)**





Identification of *S. aureus* :

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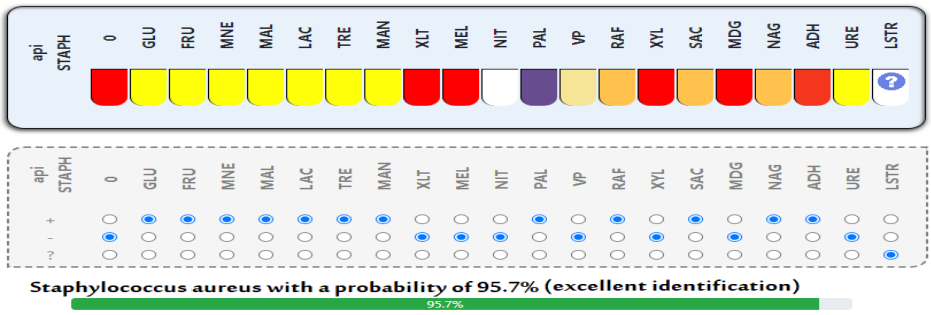
+
-
?
Staphylococcus aureus with a probability of 96.1% (excellent identification)
96.1%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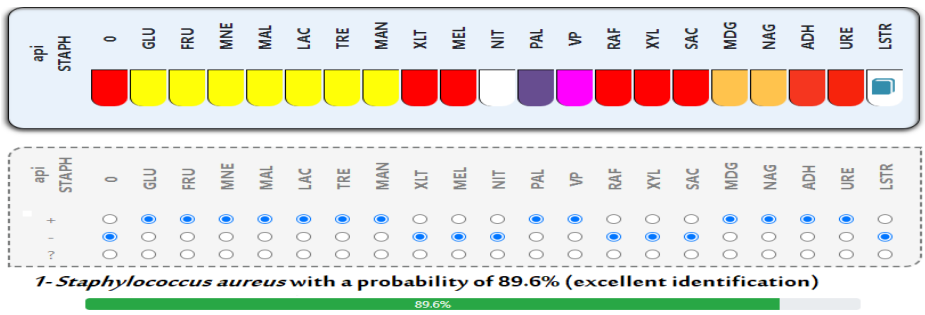
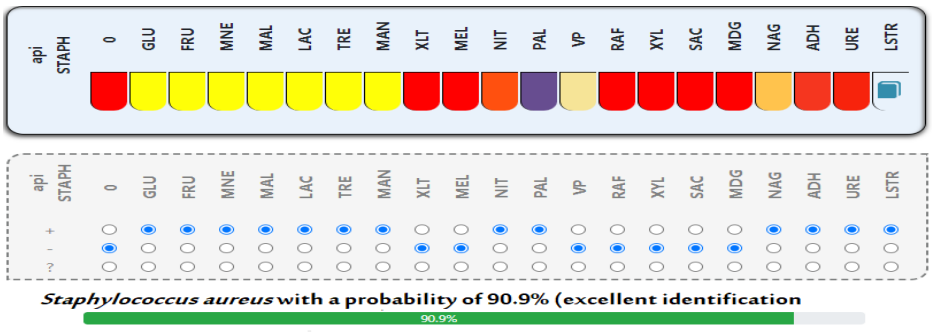
Staphylococcus aureus with a probability of 95.7% (excellent identification)
95.7%


api STAPH
0 GLU FRU MNE MAL LAC TRE MAN XLT MEL NIT PAL VP RAF XYL SAC MDG NAG ADH URE LSTR
1- Staphylococcus aureus with a probability of 89.6% (excellent identification)
89.6%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0 GLU FRU MNE MAL LAC TRE MAN XLT MEL NIT PAL VP RAF XYL SAC MDG NAG ADH URE LSTR
Staphylococcus aureus with a probability of 90.9% (excellent identification
90.9%

Appendix 5

Anti-Qs activity of different propolis extracts against CV026 (Left) and CV12475 (Right):