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Antibacterial and Anti-biofilm Activities of Iraqi Propolis Extracts against Some Antibiotic-Resistant Pathogenic Bacteria

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Abstract: The prevalence of antibiotic-resistant bacterial infections is still a significant problem in the world, a leading cause of high morbidity and death, particularly in the elderly and individuals with medical problems. As a result of the difficult-to-treat diseases caused by these antibiotic-resistant bacteria, has demonstrated a need to develop and use alternative antimicrobial agents to control these bacteria. There has been a growing interest in natural products and extracts for the discovery of new natural therapeutic alternatives. Therefore, this current study aimed to know the antibacterial activity of aqueous and alcoholic extracts of the Iraqi propolis against multidrug-resistant clinical bacterial isolates (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and the effect of these extracts on biofilm production as an important virulence factor. The study results showed that the aqueous and alcoholic extracts are effective as antibacterial and anti-biofilm against the studied multidrug-resistant bacterial isolates at all tested concentrations with significant differences. The study also showed that the alcoholic extract is more effective as an anti-bacterial and anti-biofilm than the aqueous extract of the propolis.

Keywords: Antibacterial activity, Anti-biofilm activity, Antibiotic-resistant bacteria, Iraqi propolis.

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Introduction

Propolis (bee glue) is the generic name for the resinous substance collected by honey bees (*Apis mellifera* L.) from various plant sources (substances exuded from wounds in plants: lipophilic materials on leaves and leaf buds, gums, resins, latices, etc.); it is used to seal holes in the honeycombs and smooth out the internal walls. Propolis is also used to protect the entrance against intruders; moreover, it contains antimicrobial agents active against a variety of pathogens (1, 2). A variety of phenolic compounds, proteins, amino acids, carbohydrates, lipids and fatty acids, enzymes, coenzymes, vitamins and bio-elements are almost all present in bee pollen and propolis (2, 3). They are regarded as foods that improve health because they contain biologically active substances like polyphenols, carotenoids, proteins, lipids, vitamins, and minerals. These compounds have therapeutic properties like being antibacterial, antifungal, antioxidant, hepatoprotective, and anti-inflammatory (3).

The management of bacterially infected individuals has grown to be a substantial concern for medical personnel. This is due to the fact that since bacteria proliferate quickly,

chronic drug or antimicrobial therapy make bacterial species resistant to these treatments (4).

According to the National Institute of Health, the production of biofilm, which is responsible for more than 80% of infections, is the primary causative factor that causes pathogenic infections in people (5, 6). Biofilms are frequently present in chronic wounds, kidney infections, cystic fibrosis, severe gum infections, endocarditis, meningitis, infections linked to medical devices, etc. (4, 7).

In nature, most bacterial genera, among which *S. aureus* and *P. aeruginosa*, is likely to form biofilm attached to biotic and abiotic surfaces as a survival strategy (1). Biofilms are of considerable medical importance because of their involvement in persistent infections (8). Sessile bacteria show enhanced resistance to conventional antibiotics and host defenses. Within a biofilm matrix, bacteria are able to resist antibiotics at concentrations up to 1000–1500 times higher than that conventionally used (9, 10). Many antibiofilm compounds against this bacterium have been identified from diverse natural sources (6).

Because microbes are becoming increasingly resistant to commercially accessible medications, there is a rising interest in learning more about natural products and their active components (6, 11).

Natural antibacterial agents such as medicinal plants and their essential oils, isolated compounds, propolis, bee honey etc. now represent a notable source for pharmaceutical and food industry and are widely used in pharmacology and cosmetology. Natural products have been used as potentially important sources of novel antibacterial in combating pathogenic bacteria (1).

To date, limited studies have evaluated the antibacterial effects of the aqueous and alcoholic extracts of Iraqi propolis on multidrug-resistant pathogenic bacteria and its impact on bacterial biofilm formation. Thus, the present study was conducted aiming at an in-vitro assessment of the effects of propolis extracts on two isolates of MDR bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) isolated from skin infections patients.

Materials and methods

Bacterial isolate

In the present study, two multidrug-resistant bacterial isolates were selected from among 106 bacterial isolates isolated from skin infections of Iraqi patients admitted to Baghdad hospitals for the period from December 2021 to May 2022. One of them was Gram-positive (*Staphylococcus aureus*), and one was Gram-negative (*Pseudomonas aeruginosa*) which were identified by some morphological characteristics and biochemical tests, based on the diagnostic characteristics of Holt *et al*. (12), then confirmed by the Vitek-2 system.

Antibiotics susceptibility test (AST)

Antibiotics susceptibility test for two pathogenic bacterial isolates (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) to various classes of antibiotics (Ampicillin, Tetracycline, Amikacin, Ciprofloxacin, cefotaxime, Gentamicin, Imipenem, and oxacillin) was done by disk diffusion method (Kirby-Bauer method) as described by (13, 14) and Clinical Laboratory Standard Institute recommendations (15).

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Quantitative biofilm formation assay

Quantitative Biofilm Formation Assay of *S. aureus* and *P. aeruginosa* to determine a capacity to produce biofilms was assessed using a 96-well microtiter plate test based on the crystal violet staining technique according to Diriba *et al*. (16).

Preparation of aqueous and ethanolic Iraqi propolis extracts

A sample of Iraqi propolis was obtained from the apiaries of the College of Agricultural Engineering Sciences / University of Baghdad, and its diagnosis was confirmed by a specialist in the same college. The preparation of aqueous and ethanolic extracts of the Iraqi propolis were similar to that described in a study by Musa *et al*. (17) with some modifications.

Determination of total phenolic and total flavonoid content in Iraqi propolis extract

The total phenolic content of the aqueous and alcoholic extract of propolis was investigated using the Folin-Ciocalteu method according to Cottica *et al*. (18). and was expressed as Gallic acid equivalents per gram of propolis extract, and the total flavonoid content of the aqueous and alcoholic extract of propolis was investigated using the aluminum chloride colorimetric method according (19), and was expressed as Rutin equivalents per gram of propolis extract.

Antibacterial activity assay of Iraqi propolis extracts

Serial dilution concentrations (20%, 10%, 5%, and 2.5%) of propolis extracts were used and prepared according to Ibrahim *et al*. (20).

The antibacterial activity of the Iraqi propolis extracts against multi-drug resistance *S. aureus* and *P. aeruginosa* was carried out (21). 0.1 ml of standardized bacterial inoculum (1.5 $\times 10^8$ CFU/ml, 0.5 McFarland's standard) of each bacterial isolate and (0.1 ml) of each concentration prepared from the extract were transferred to a sterile test tube, then incubated at 37 °C for15 mints, and the mixture was poured onto sterile Petri dishes containing Muller-Hinton agar (MHA) and spreading it by the spreader. And all the dishes were incubated at 37 °C for 24 hours, and the antibacterial activity was observed by counting bacterial colonies and compared with the control. Three dishes (replicates) were used for each concentration to reduce the errors that result from conducting the experiment.

Anti-biofilm activity assay of propolis extracts

Anti-biofilm effect of propolis extracts was done according to Diriba *et al*. (16), with some modification: Microtiter plate containing 199µL of Mueller–Hinton broth augmented with 1% glucose was inoculated with 100µL from suspended bacterium and 100 µL of each extracts concentration, Microplates are incubated for 24 h at 37°C. Adhesive cells were rinsed twice with phosphate-buffered saline (PBS), and the wells were parched at 37°C for less than an hour at 37°C. Crystal violet was then dyed on the specimen and incubated for 15 minutes. The crystal violet-stained microplate wells were rinsed twice with (PBS. After air-drying microplate wells, 150µL of 95% ethanol re-solubilizes biofilm color. The microplate reader was measured spectrophotometrically at OD 580 nm after 5-10 min.

Statistical analysis

The Statistical Analysis System-SAS (2018) program was used to detect

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the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study (22).

Results and discussion

Antibiotics susceptibility test

Staphylococcus aureus and *Pseudomonas aeruginosa* isolates showed a multi-drug resistance (MDR) pattern in an antibiotic susceptibility test using the Kirby-Bauer technique and were resistant to all the various antibiotics used (Ampicillin, Tetracycline, Amikacin, Ciprofloxacin, cefotaxime, Gentamicin, Imipenem, and oxacillin) with the exception of the Imipenem, to which the isolates of *S. aureus* showed sensitivity. as shown in Figure (1), and the results of Vitek 2 system supported these findings and the isolate was resistant to 11 antibiotics.

This is consistent with the fact that several strains of *S. aureus* and *P. aeruginosa* have developed antibiotic resistance during the past 10 years (23,38), and one of the recently discovered strains of *S. aureus* and *P. aeruginosa* was extensively drug-resistant (XDR) and immune to all classes of antibiotics.

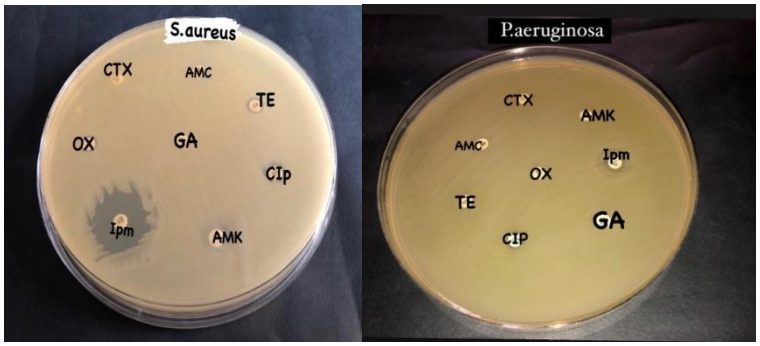

Figure (1): Results of antibiotics susceptibility testing by Kirby Bauer method

Determination of total phenolic and total flavonoid content in Iraqi propolis extract

The results as shown in Table (1) revealed that the total content of phenols and flavonoids for alcoholic extracts was higher than that of the aqueous extracts.

Table (1): Total phenolic and flavonoid content of the aqueous and alcoholic propolis extracts

The sample	TPC (mg Gallic /gm.)	TFC (mg Rutin / gm.)
Alcoholic extracts	22.58	12.66
Aqueous extracts	18.58	10.25

The total phenols content was considered the main responsible for the antibacterial, and antioxidant activity of different extracts, including propolis (24). Phenolic compounds are very important plant constituents because they exhibit antioxidant activity by inactivating lipid free radicals or preventing the decomposition of hydroperoxides into free radicals (25). Total phenolic content was expressed in three ways considering different compounds as calibration references: rutin, gallic acid, and pinocembrin/galanin, as they are indistinctly used in the reported bibliography to measure the total phenolic content in propolis (18).

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Flavonoids are key candidate compounds for evaluating the quality of propolis products. Propolis is commercialized in different parts of the world and it is recognized as an important source of compounds with pharmacological properties to contain this compound (19).

A study by Escriche and Juan-Borrás (26) find that most of the active compounds are found in the tested six kinds of propolis, including flavonoids and phenols but in different quantitative proportions. Of the 13 quantified compounds, all of them showed significant differences between samples and only one presented significant differences considering the method of extraction. This result demonstrates the influence of the kind of propolis on the quantification of this type of compound.

Antibacterial activity of Iraqi propolis extracts

The aqueous and alcoholic propolis extracts were tested for their antibacterial activity against chosen MDR bacterial isolates. The study was conducted by using serial dilutions of the aqueous and alcoholic propolis extract (2.5%, 5%, 10%, 20%) against inoculum suspension (1.5×10^8 CFU/ ml). The results showed antibacterial activity against the two tested MDR isolates compared with untreated control as listed in Tables **(**2, and 3), and Figures (2,3,4 and 5**)** while the aqueous extract showed a lower effect against tested bacterial isolates, this is maybe due to the alcoholic extract's high content of active compounds. Also, as is evident from the results, the Gram-positive bacteria were more sensitive to the extracts than the Gram-negative bacteria.

Table (2): Antibacterial activity of the alcoholic propolis extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Concentration (%)	Mean $\times 10^8$ CFU/ml	
	S. aureus	***P. aeruginosa***
Control	1.5 ±0.00	1.5 ±0.00
2.5%	28 ±2.61	83 ±8.02
5%	21 ±1.85	39 ±3.25
10%	13 ±1.29	19 ±1.84
20%	4.6 ±0.73	12 ±1.05
LSD value	17.533 *	29.865 **
*** (P≤0.05), ** (P≤0.01).**		

Figure (2): Antibacterial activity of the alcoholic propolis extract against *Pseudomonas aeruginosa.*

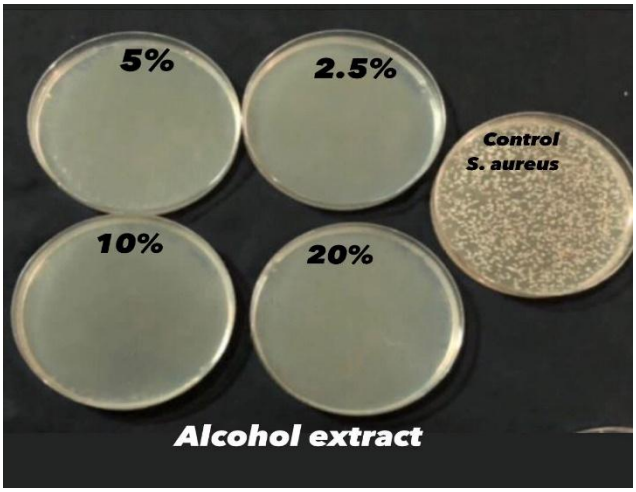

Figure (3): Antibacterial activity of the alcoholic propolis extract against *Staphylococcus aureus*.

Table (3): Antibacterial activity of the aqueous propolis extract against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Concentration (%)	Mean $\times 10^8$ CFU/ml	
	S. aureus	***P. aeruginosa***
Control	1.5 ±0.00	1.5 ±0.00
2.5%	17 ±2.57	380 ±41.64
5%	104 ±14.06	118 ±17.54
10%	16.33 ±2.38	37 ±2.79
20%	9 ±1.24	11 ±0.94
LSD value	31.372 **	56.894 **
** (P≤0.01).		

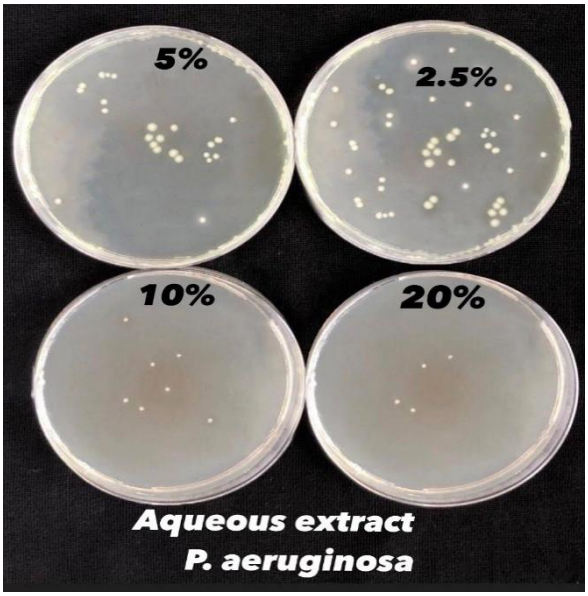

Figure (4): Antibacterial activity of the aqueous propolis extract against *Pseudomonas aeruginosa*.

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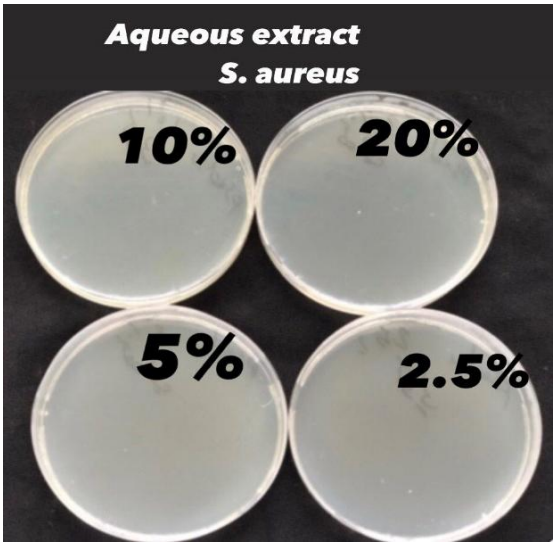

Figure (5): Antibacterial activity of the aqueous propolis extract against *Staphylococcus aureus*

The results of the current study are in agreement with the results of Kareem *et al*. (27) who provided that local Iraqi propolis has antibacterial activity against gram-positive and gram-negative bacteria, which increases as the concentration increasing of propolis extract. While the early study results revealed that *S. aureus* was highly sensitive to ethanolic extract of Iraqi propolis than other Gram-positive and Gram-negative bacteria. One study showed that the Iranian propolis was mainly active against gram-positive (28,40). As well as it is observed that the antimicrobial activity of propolis is higher in relation to Gram-positive than Gram-negative bacteria (29). This is explained by the species-specific structure of the outer membrane of the Gram-negative bacteria (30), and the production of hydrolytic enzymes which break down the active ingredient of propolis.

The composition of propolis can vary depending on the location of the bees and what trees and flowers they have access to. Also, the composition of the plant source determines the chemical composition of propolis (27). On the other hand, the antimicrobial efficiency of propolis varies greatly depending on the extraction procedure of the crude material and the solvents used in this process (31). They found that the highest extraction efficiency of flavones and flavonols (21% w/v) was obtained when 80% ethanol while water appeared to be the least efficient extraction medium, resulting in extracts containing only around 0.6%, w/v, of flavones and flavonols (32). This is attributed to the rather poor aqueous solubility of those compounds. Previous studies for antimicrobial activity of the propolis aqueous extract used higher concentrations of the extract (33).

Anti-biofilm activity of Iraqi propolis

Since biofilm is the most dangerous virulence factor in pathogenic bacteria isolated from skin infections, it was worth examining the inhibitory activity of aqueous and alcoholic propolis extracts on biofilm formation ability when using the sub-MIC (sub-minimum inhibitory concentration) of propolis extracts, which was 10% for aqueous and alcoholic extracts.

As shown in Figures (6, and 7) there is a sharp decrease in biofilm

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productivity compared to control, were both selected isolates were strongly biofilm producers. The results showed both the alcoholic and aqueous propolis extracts have an inhibitory effect on the ability of bacteria to biofilm formation with clearly significant differences (P≤0.01), As it is clear the alcoholic extract had the highest anti-biofilm effect against *P. aeruginosa* then *S. aureus*. Whereas, the aqueous extract had the least effect on biofilm production. It is obvious that the bacterial isolates were somewhat more sensitive to the alcoholic extract compared to the aqueous extract.

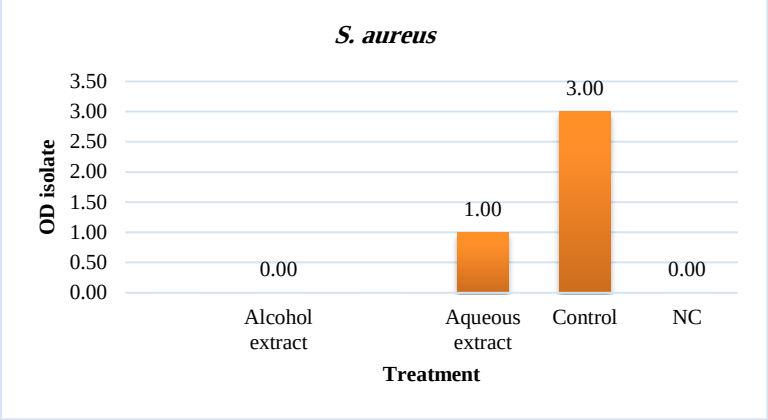

Figure (6): The anti-biofilm activity of alcoholic and aqueous propolis extracts against *S. aureus*.

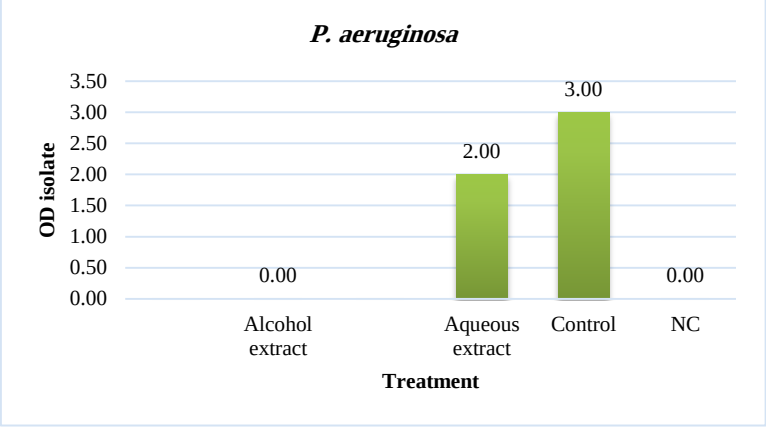

Figure (7): The anti-biofilm activity of alcoholic and aqueous propolis extracts against *P. aeruginosa.*

A previous study (34) stated that natural compounds have an anti-biofilm effect. such as phenols, terpenes, and alkaloids (35,36) as phenolics consist of a group of compounds. It has seven subclasses which include phenolic acids, quinones, flavonoids, flavones, flavonols, tannins, and coumarins, out of which tannins, specifically condensed tannins, have anti-biofilm activity. These entire compounds act on biofilm inhibition (37,19). Natural substances' success at preventing binding makes them a potentially useful tool for decreasing microbial colonization on various surfaces. A particularly intriguing strategy for preventing microbial infection seems to be the administration of anti-adhesion drugs (38).

The sugars in the biofilm are (rhamnose, mannose, galactose, and glucose), which are present in high abundance, although xylose is present

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in the polysaccharides of some bacteria, its presence is sometimes considered uncommon, these sugars play an important role in the formation of the biofilm (39).

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