Journal of Ethnopharmacology and Toxicology
Research Article

Inhibitory activity of propolis and nano-propolis against isolates of *Staphylococcus aureus* and *Escherichia coli*

Mohammed A. Aboktifa[1*], Alaa Abdelkadhim Jawad[2], Dhama Al-Sallami[3]

[1]Department of physiology, biochemistry and pharmacology, College of Veterinary Medicine, Al-Qasim Green University, Babylon 51013 Iraq.
[2]Department of microbiology and parasitology/ College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.
[3]Department of Physiology, Pharmacology and Biochemistry/ College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq.

[*]**Correspondence**
Mohammed A. Aboktifa
mohammed.aboktifa@vet.uoqasim.edu.iq

Volume: 4, Issue: 2, Pages: 22-30
DOI: https://doi.org/10.37446/jet/rsa/4.2.2026.22-30
Received: 29 May 2025 / Accepted: 21 July 2025 / Published: 25 August 2026

Background: Propolis, a plant-derived material, is widely used to enhance antibiotic activity against common pathogenic bacteria which causes significant global economic losses. This research was designed to detect the role of propolis and nano-propolis as natural antibiotic.

Methods: Fifteen isolates of *S. aureus* and 15 of *E. coli* were cultured on plates of Nutrient Agar, incubated at 37 °C, then stored at -20 °C. Bacterial sensitivity of the 15 isolates to different important antibiotics were performed separately for each type of bacteria. Also, susceptibility test by Kirby Bauer disc diffusion method was utilized with three concentrations (125,250,375 µg/ml) of propolis and nano-propolis. Zones diameters of bacterial growth inhibition for the tested isolates were measured after 24 h of incubation. In the same time MIC and MBC for both materials was determined.

Results: Results of bacterial sensitivity test show that 13 bacterial isolates of *S. aureus* were highly resistant to Benzylpenicillin (86.6%) and 12 isolates of S. aureus were resistant to Oxacillin (80%). While, 13 isolates of *S. aureus* were sensitive to Linezolid and Teicoplanin (86.66%). In addition, *E. coli* showed that 13 isolates were resistant to ampicillin (86.66) and 10 out of 15 isolates were sensitive to Piperacillin (66.66%). Furthermore, the inhibition zone of *S. aureus* was higher at 375 µg/ml as 18.36 mm and 11.87 mm with nano-propolis and propolis respectively. Moreover, *E.* coli cultures showed higher inhibition zone at 375 µg/ml as 11.78 mm with propolis and 12.56 mm with nano-propolis.

Conclusion: This study indicates that propolis and nano-propolis inhibited bacterial growth and may potentiate antibiotic effect against gram positive and gram-negative bacteria. Suggesting a good strategy against pathogens. However, nano-propolis is a promising candidate as plant derived material to decrease MDR microbes.

Keywords: *Propolis, nano propolis, antibiotic, MDR, S. aureus and E. coli*

Introduction

Multi-drug resistance (MDR) in bacteria has become a real threat to public and animal life by causing high number of deaths yearly. The excessive use of different antibiotics in medication and horizontal transfer of antibiotic resistance genes among bacteria are the main causes behind that (Marek et al., 2022; Davies & Davies, 2010). Also, the environment has shown a big role in spreading the antibiotic resistance among the microbes by transforming the resistance genes particularly through hospital waste water and manure. In addition, it is necessary to mention that waste water from Slaughterhouses is considered as a dangerous source of MDR bacteria (Alsultan et al., 2023; Al-Ubaidy & Alsultan, 2023). Microorganisms are continuously changing and improving their ways to resist antibiotics. One important way that used by pathogens is elimination of the drug outside their body by efflux pump proteins (Sharma et al., 2019; Livermore,

2003). Both Gram positive and Gram-negative bacteria have different groups of efflux pumps. NorA in *S. aureus* and AcrAB in *E. coli* are known examples to the efflux pump systems in bacteria (Blanco et al., 2016; Alsultan & Alsallami, 2022). Importantly, *E. coli* and *Staphylococcus* spp. are considered as well-known foodborne pathogens in the natural environment and have shown high MDR ratio (Marek et al., 2022, Yaseen et al.,2023). Raw milk products are considered as potential source of different pathogens which may increase risk of the infection with these bacteria (Moosavy et al., 2018; Sugrue et al., 2019; Khudeir et al., 2024). However, the high challenges, there are several strategies can be followed to control spreading of drug resistance among microorganisms as limitation of antibiotic use, using of prebiotics, dependence of new vaccine generations and bacterial sub products (Ruvalcaba-Gómez et al., 2022; Alawadi et al., 2024). Moreover, using of plant extracts can potentiate the activity of antibiotics by inhibition of efflux pump mechanism. Theses extracts called efflux pump inhibitors (EPIs) due to their ability to keep the antibiotic inside the bacterial cell (Rao et al., 2018; Mosolygó et al., 2019). Propolis is one of the plant extracts that increase antibiotic activity toward bacteria. Propolis has enhanced antibacterial effect of ciprofloxacin, erythromycin, and gentamycin against *S. aureus*. Furthermore, propolis and nano-propolis also have shown increase in the antimicrobial activity against *E. coli*, *S. aureus* and *S. typhi* (Salama et al., 2024). In conclusion, the contribution of this work is to determine effect of propolis and nano-propolis against two common pathogens. The search includes three concentrations of propolis and nano-propolis with 15 isolates of *E. coli* and *S. aureus* were clinically isolated from urine in UTI dogs.

Materials and methods

Propolis collection and extraction

The raw propolis materials that used in this study was collected from various locations in Al-Diwaniya city on April and May/2025. Ten grams of propolis powder was placed in Soxhlet extractor thimble in the extraction apparatus. Ethanol 80% w/v was measured into a 500 ml conical flask. A heating mantle was used to reflux the mixture for various time points between 1 and 4 h. After the extraction time has been reached, the extract solution was allowed to cool to room temperature. Then, filtered through a cone of filter paper (Whatman No.1) and concentrated to dryness using a rotary evaporator. Then, the extraction yield was kept in 4 °C until use (Alara et al.,2021).

Preparation of nano-propolis

The nano-propolis was prepared by sonication process. The solution was converted to nano-sized by applying ultrasonic waves using a UP200ht Hielscher device at the central laboratory/veterinary college/ University of Al-Qadisiyah. A fluid containing propolis particles was produced when a 50-watt energy wave was released for 30 minutes.

Characterization of nano propolis

The physicochemical properties of nano-propolis prepared by sonicator have been examined by employing the characterization techniques including SEM and UV–Vis spectrophotometer. The optical characteristics of the synthesized product were analyzed by utilizing UV–Vis spectrophotometer (Model: Specord 205, Analytic Zena, Germany) in the range of 280–450 nm. The FESEM was used to record the NP's morphology (Model: JSM-7610F, JEOL, USA), following their combination with acetone and drying on a glass slide to form thin coating.

Antibacterial activity

The study investigates the antibacterial activity of ethanolic propolis extract and synthesized nano-propolis against 15 different isolates of both *S. aureus* and *E. coli* collected from urine of UTI dogs. To create pure cultures, the isolated bacteria were regularly sub-cultured on Nutrient Agar (NA, Hi-media, India), then incubated at 37 °C, then stored at −20 °C. The selected isolates were identified by Vitek 2 apparatus. Prior to the experiment, all of the glassware and associated items were autoclaved at 121 °C and pressure of 115 lb. The modified Kirby Bauer disc diffusion method following the Clinical and Laboratory Institute (CLSI) guideline was utilized to assess the antibacterial susceptibility test. Concentration of 10 % DMSO was served as a negative control. Propolis and nano-propolis (125,250,375µg/ml) were dissolved in 1.0 ml of 10 % DMSO. 100 µl of the prepared samples solution were added to the wells that made in agar culture. The plates were subsequently kept at 4 °C for 3 h to allow nanoparticles to enter and diffuse across the well, after that incubation at 37 °C was performed. Clear zones were discovered surrounding the wells after 24 h of incubation, and a slide calliper was used to measure these zones' diameters.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC)

The broth micro dilution method was employed to examine the MIC and MBC following the CLSI guideline. Ten different concentrations of both propolis extract and nano-propolis in 10 % DMSO (2,4,8,16,32,64,125,250,500,1000 µg/ml) were investigated against the bacterial concentration of 5×10^5 CFU/ml. Bacterial suspension (50 µl) was added to each sample except the negative control (10 % DMSO). Both propolis and nano-propolis were incubated for 24 h at 37 ± 2 °C. After incubation period, MIC and MBC were determined by visual inspection.

Statistical analysis

Data analysis using SPSS version 28, p-value 0.05 for significant differences, mean± SE and least significant differences (LSD) post hoc test if ANOVA p-value is less than 0.05 (Ab Rahman,2015).

Results and discussion

UV-visible spectrophotometer and SEM

Nano-sized materials, which are materials with distinctive dimensions less than 100 nm, offer special advantages that have drawn interest from both the scientific community and the general public. Biomedicine is one of the biggest application areas (Adul-Rasool et al.,2024). The absorption spectra of the nano-propolis nanoparticles dispersed in ethanol alcohol is shown in Figure 2. The absorption (SPR) peak is obtained in the range at 280 nm.

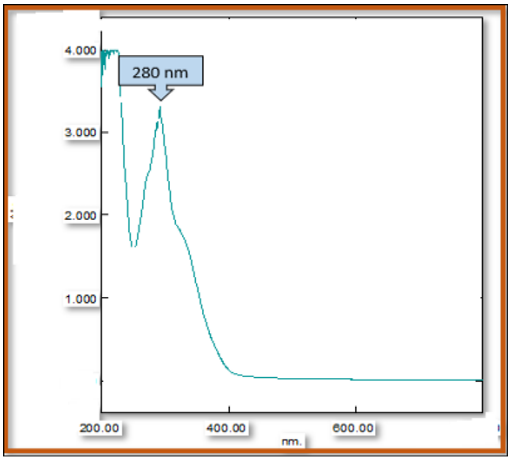

Figure 1. UV-visible spectroscopy of nano-propolis

The SEM image of the prepared colloidal solution of prepared nano-propolis nanoparticles is shown in Figure 2. The formed nanoparticles are spherical in shape with smooth surface morphology. The diameter of the nanoparticles is found to be approximately 148.82 ± 3.16 nm at range between 128.35 nm to 218.12 nm for 100 examined particles.

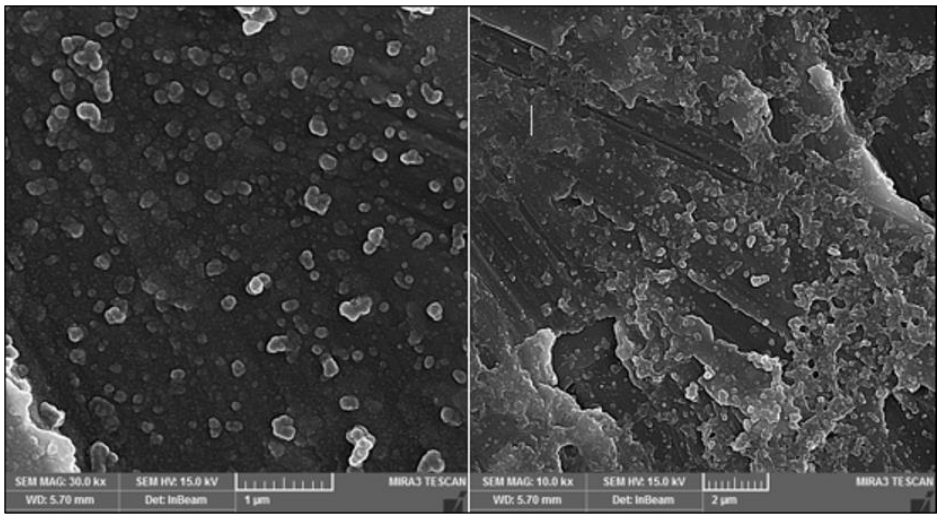

Figure 2. Scanning electron microscope examination of nano-propolis

Sensitivity of *S. aureus* isolates to 15 different commercial antibiotics

S. aureus is a common pathogenic bacterium in human and mammalians that causes infections in skin, lung, heart and also bacteraemia in some cases. This is mostly due to its resistant to different antibiotics especially methicillin (Humphreys, 2012; Guo et al., 2020; Salama et al., 2024). Many *Staphylococci* spp. is haemolytic, do not have flagella or spores, create golden colour and break down the mannitol. Methicillin-resistant *Staphylococcus aureus* (MRSA) is widely spread in the environment. It was emerged since 1961 due to its highly infection incidence in human and animals in addition to the excessive exposure to the antibiotic as treatment strategy. In this study, activity of fifteen commercial antibiotics was tested against 15 isolates of *S. aureus*. The results have shown that 13 out of 15 bacterial isolates were highly resistant to Benzylpenicillin (86.6%) and 12 isolates of S. aureus were resistant to Oxacillin (80%), 11 were resistant to Erythromycin and Fucidin (73.33%). Also, nine isolates were resistant to Vancomycin, Tetracycline and Rifampicin (60%) (Table 1). On the other hand, 13 *S. aureus* isolates were sensitive to Linezolid and Teicoplanin (86.66%), 11 were sensitive to Gentamycin (73.33), 10 are sensitive to trimeth-sulfa (66.66) and 9 are sensitive to Clindamycin (60%).

Table 1. Antibiotic sensitivity of *S. aureus* isolates to 15 different antibiotics

Type of antibiotics	*S. aureus* (No. and %)		
	R	I	S
Benzyl-penicillin	13(86.66)	0(0)	2(13.33)
Oxacillin	12(80)	0(0)	3(20)
Gentamycin	4(26.66)	0(0)	11(73.33)
Ciprofloxacin	8(53.33)	0(0)	7(46.66)
Moxifloxacin	7(46.66)	1(6.66)	7(46.66)
Erythromycin	11(73.33)	0(0)	4(26.66)
Clindamycin	6(40)	0(0)	9(60)
Linezolid	2(13.33)	0(0)	13(86.66)
Teicoplanin	2(13.33)	0(0)	13(86.66)
Vancomycin	9(60)	0(0)	6(40)
Tetracycline	9(60)	0(0)	6(40)
Tigecycline	7(46.66)	0(0)	8(53.33)
Fucidin	11(73.33)	0(0)	4(26.66)
Rifampin	9(60)	0(0)	6(40)
Trimethoprim/sulfamethoxazole	5(33.33)	0(0)	10(66.66)
X^2		57.72	
P value		0.001*	

* Highly significant difference at P<0.01

Sensitivity of *E. coli* isolates to 15 different antibiotics

E. coli is the most discovered bacterium and considered as a model to study other microorganisms. It is a common bacterium that colonised inside the digestive system and can cause gut infections. *E. coli* considered as more dangerous bacterium may threaten human health including twelve bacterial families.

Table 2. Antibiotic sensitivity of *E. coli* isolates to 15 different antibiotics

Type of antibiotics	*E. coli* (No. and %)		
	R	I	S
Ampicillin	13(86.67)	1(6.66)	1(6.66)
Piperacillin	4(26.66)	1(6.66)	10(66.66)
Cefazolin	7(46.67)	0(0)	8(53.33)
Cefoxitin	7(46.66)	0(0)	8(53.33)
Ceftazidime	10(66.66)	0(0)	5(33.33)
Ceftriaxone	9(60)	0(0)	6(40)
Cefepime	12(80)	0(0)	3(20)
Ertapenem	10(66.66)	0(0)	5(33.33)
Imipenem	12(80)	0(0)	3(20)

Amikacin	8(53.33)	0(0)	7(46.66)
Gentamicin	6(40)	0(0)	9(60)
Ciprofloxacin	7(46.66)	0(0)	8(53.33)
Tigecycline	10(66.66)	0(0)	5(33.33)
Nitrofurantoin	8(53.33)	0(0)	7(46.66)
Trimethoprim/sulfamethoxazole	10(66.66)	0(0)	5(33.33)
X^2		37.003	
P value		0.119*	

* No significant difference at P<0.05

Resistance of *E. coli* to vary antibiotics is well- documented in many researches (Hong et al., 2016; Al-Mutairi et al., 2018). *E. coli* is commensal and easily spread through the oral-faecal way among human, animals and the environment (Lopes & Sourjik, 2018; Mirsepasi-Lauridsen et al., 2019; Kwong et al., 2020; Puvača & de Llanos Frutos, 2021). In this work, table 2 shows the results of 15 *E. coli* isolates sensitivity test to fifteen different commercial antibiotics that commonly used. There are 13 isolates resistant to ampicillin (86.67), 12 isolates were resistant to cefepime and Imipenem (80%), 10 were resistant to Ceftazidime, Ertapenem, Tigecycline and Trimethoprim (66.66%). While, ten isolates were sensitive to Piperacillin (66.66%), 9 were sensitive to Gentamicin (60%) and 8 were sensitive to Cefazolin, Cefoxitin and Ciprofloxacin (53.33%) equally.

Activity of propolis and nano-propolis against the studied bacterial isolates

Propolis is a bee glue and resinous substance collected by bees from plant source. It is used by bees in their combs repair. It has several biological effects as antibacterial, antifungal and anti-inflammatory (Agarwal et al., 2012; de Souza Ferreira et al., 2014; Shabbir et al., 2016). Its natural characteristics give this compound antimicrobial activity as broad-spectrum antibiotic. Also, combination of propolis with the antibiotics helps in strengthen its activity as antibacterial (Almuhayawi, 2020). Different propolis extract activity against bacteria like *S. aureus* and *E. coli* was tested broadly However, underlying information about nano propolis concerning its antibacterial potential in comparison to the original bulk propolis has not been widely available (Zhang et al., 2022). In culture media, activity of ethanolic propolis extract and synthesized nano-propolis were examined in this work. Three distinct concentrations (125, 250 and 375 µg/ml) of propolis and nano-propolis were used against 15 isolates of both *S. aureus* and *E. coli* (Table 3). The results reveal that nano-propolis has recorded higher bacterial inhibition zone diameter (mm) than propolis in different concentrations that used with *S. aureus* culture. At concentration 125 µg/ml, the result was 12.28 mm with nano-propolis and 7.6 mm with propolis. Also, at 250 µg/ml, the inhibition zone diameter value was 15.05 mm with nano propolis and 9.12 mm with propolis. In addition, 18.36 mm with nano-propolis and 11.87 mm with propolis were the values of *S. aureus* inhibition zone diameter at 375 µg/ml concentrations. Propolis has been shown to have a broad range of antibacterial activity when tested against a variety of pathogens, including *Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus, Micrococcus luteus*, and *Escherichia coli* (Nandre et al.,2021). The increase in the inhibition zone is clear in the three concentrations of nano propolis than the bulk ethanolic propolis extract which indicates increasing propolis activity after nanosized its particles. Reducing the drug's size is thought to offer a major therapeutic benefit when employing nanomaterials. The impact of a particle's small size on the antibacterial drugs' therapeutic advantages has been confirmed by certain studies (Chandrakala et al.,2022). Moreover, *E.* coli cultures showed 8.02 mm, 9.32 mm and 11.78 mm of inhibition zone diameter at 125, 250, 375 µg/ml of propolis respectively. While, 7.9 mm, 10.02 mm, 12.56 mm were the inhibition zone diameter values with nano propolis at 125, 250 and 375 µg/ml respectively (Table 3). From the mentioned results, it is clear that the inhibitory effect of nano propolis is much more effective than ethanolic propolis extract in particular, with *S. aureus* cultures. This differences between nano-propolis effect with *E. coli* and *S. aureus* cultures may result from the wide variations between Gram negative and Gram-positive bacteria. Gram-positive and gram-negative bacteria have different cell wall structures, which cause them to react differently to antimicrobials. (Varghese & Balachandran,2021)

Table 3. Propolis and nano-propolis activity against the studied bacteria in culture media.

Concentration (µg/ml)	Type of bacteria			
	S. aureus		***E. coli***	
	Propolis	**Nano-propolis**	**Propolis**	**Nano-propolis**
125	7.6±0.18	12.28±1.12	8.02±0.55	7.9±0.45
250	9.12±0.52	15.05±0.56	9.32±0.21	10.02±0.34
375	11.87±0.63	18.36±1.14	11.78±0.74	12.56±1.02
DMSO(NC)	0±0	0±0	0±0	0±0
LSD	0.941		0.568	

Values represent mean of inhibition zone diameter in (mm)

Furthermore, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of *S. aureus* and *E. coli* isolates with ten different concentrations of propolis extract and nano-propolis particles were investigated against the bacterial concentration of 5×10^5 CFU/ml. As have been shown in table 4, values of MIC and MBC of *S. aureus* with propolis were 32 and 500 µg/ml, while, they were 16 and 125 µg/ml with nano propolis for MIC and MBC respectively. This suggests that MIC value decreased to the half with nano propolis, MBC was also much less it with than propolis. On the other hand, MIC and MBC values for *E. coli* were 64 µg/ml and 500 µg/ml with propolis; 16 µg/ml MIC and 250 µg/ml MBC with nano propolis (Table 4). These values indicate the potential role of nano propolis as antibacterial agent.

Table 4. MIC and MBC values of propolis extract and nano-propolis

Material	***S. aureus*** **MIC and MBC value (µg/ml)**		***E coli*** **MIC and MBC value (µg/ml)**	
	MIC	MBC	MIC	MBC
Propolis	32	500	64	500
Nano-propolis	16	125	16	250

Documented in table 4, *E. coli* is more resistant to propolis than *S. aureus*. This result because that different bacteria have different resistance mechanism. Also, each of *E. coli* and *S. aureus* has different efflux pump groups that eject the antibiotic out of the bacterial cell. In addition, The MIC of *S. aureus* with nano propolis (16 µg/ml) is half of the MIC with propolis (32 µg/ml). While *E coli* shows 64 µg/ml with propolis and 16 µg/ml with nano propolis. Results of this study are agreed with Zhang et al.,2022 findings about activity of propolis as downregulated to gene that responsible for biofilm, cell wall, and virulence factors in *S. aureus* MRSA (Zhang et al., 2022). However, its mechanism as antimicrobial is not fully understood. Propolis contains polyphenol and as many researches proposed that antibacterial activity of propolis related to this compound (Bankova et al., 2014). These compounds have an effect against *S. aureus* through breaking bacterial cell wall and making changes in its structure (Darwish et al., 2010; Zhang et al., 2022).

Conclusion

It can be concluded that using of propolis in a nanocomposite enhanced its antibacterial effects. Also, it's rich with pinobanksin, pinobanksin-3-acetate, and chrysin which are exist in Jordanian propolis.

Acknowledgment

The authors thank the College of Veterinary medicine in Al Qasim Green University.

Author contribution

This work was carried out in collaboration among all authors. Authors MAM and AAJ conceived and designed the research. Authors AAJ and DA performed the data analysis and interpretation. Authors MAA and AAJ contributed to writing the article. All authors read and approved the final manuscript.

Funding

The project was funded from the authors' personal resources.

Conflict of interest

There are no competing interests.

AI tool declaration

The authors have not used AI and it's related to tools to write this manuscript. The authors declare that no AI and related tools are used to write the scientific content of this manuscript.

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