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Egyptian bee (*Apis Mellifera*) propolis: A promising antibacterial agent for combating antibiotic resistance and biofilm formation of multidrug-resistant *Staphylococcus aureus*

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Abstract: Bacterial biofilm is a major factor in the development of multidrug-resistant phenotypes and the progression of chronic infections, as biofilm forms robust shields around bacterial cells enabling them to escape from antibiotic's destructive effect. One solution is multi-targeting antimicrobial natural products that combat biofilms and re-sensitize resistant bacteria to unworkable antibiotics. Two Egyptian bee propolis samples were collected from different regions and ethanolic extracts were prepared. Anti-staphylococcal and anti-biofilm activities against MDR *Staphylococcus aureus* revealed that propolis extracts differed in their potency and exhibited average MICs of (362 ± 0.19 and 432 ± 0.063µg/mL) for West-Nile and Upper-Egypt propolis samples respectively. Propolis extracts revealed a potent inhibitory effect on staphylococcal biofilm formation (from 49.5% to 29%) at their MICs. Both propolis extracts eradicated successfully the preformed staphylococcal biofilm within two hours of treatment (from 97.4% to 25%). Moreover, the anti-adherence activity of both extracts at different concentrations (1X MIC, 1/4, and 1/8 MIC) was similar (from 63.6% to 56.5%) after two hours of exposure to propolis. The effect of combination with antibiotics was assayed by the Kirby-Bauer test which revealed noticeable antibiotic synergistic effects that were more obvious with Upper-Egypt propolis extract. Synergistic effects with different classes of antibiotics were expressed as a percentage with; Amikacin (56.3%), Penicillin G (53%), Ampicillin (52%), Clindamycin (39.5%) then Ciprofloxacin (32.5%). Egyptian propolis was proved to be a promising antibacterial, anti-biofilm, and antibiotic-saving natural product.

Keywords: Biofilm, Multidrug Resistance, Propolis, Synergism, *Staphylococcus aureus*

1. INTRODUCTION

Staphylococcus aureus can be classified as Gram-positive cocci that exhibits pathogenicity to humans and causes various serious systemic infections [1]. It is responsible for widespread severe clinical infections in hospital locations; from acute bacteremia [2] to pneumonia, endocarditis, and biofilm-associated chronic infections in prosthetic devices [3]. However; it is guilty of much severe community-acquired infections [4]. The abuse of antibiotics has commanded bacterial resistance evolution, creating a collection of "superbugs" including *Staphylococcus aureus* which depicts Methicillin resistance and is known as MRSA. It is a mutant subtype that is resistant to beta-lactam antibiotics, including Penicillin, Amoxicillin, Oxacillin, and Methicillin [5]. They are characterized by a transformed penicillin-binding protein PBP2a with nil affinity to Methicillin [6]. MRSA is normally categorized as hospital-acquired, healthcare-associated, and community-initiated; besides community-acquired infections[7,8]. Infections triggered by MRSA strains, which are identified as community-acquired, have been guilty with the

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progression of rational lethal infections and worse clinical consequences in comparison to infections related to healthcare-associated MRSA and community-related MSSA strains [9]. In Egypt, the major bacterial species that cause wound and surgical site infections were *Staphylococcus aureus* [10]. Now it is accepted to find that once MRSA recorded 50–82% among hospitalized patients [11]. On one occasion, MRSA recorded deaths reached 11,000 each year [7]. Methicillin-resistant strains of *Staphylococcus aureus* were blamed for most global Staphylococcus *aureus* bacteremia cases that have been recorded inside intensive care units, various infections related to prosthetic devices, and indwelling catheters [12] incomparable to Methicillin-sensitive *Staphylococcus aureus* [13] causing major morbidity besides mortality [14]. Additionally, hospital-acquired MRSA transmission to home, health-care, and household contacts has been communal and happens in about 20% of house-hold contacts [15]. *Staphylococcus aureus* can produce a multilayered biofilm which is entrenched within a glycocalyx [17]. A vital virulence factor is biofilm which works as an effective shelter of bacterial communities from environmental stress factors such as antibiotics [16]. Biofilms represent predominant reasons for bacterial resistance progression to antibiotics that appears in the clinically isolated *Staphylococcus aureus* strains [18]. The creation of bacterial biofilms initiates the expansion of chronic infections, as biofilms are remarkably resisting to the destructive effect of antibiotics [17]. As soon as enclosed in biofilm surrounding substance, shielded cells of bacteria become host immunity evader and persistent to the lethal effects of many antibacterial agents, which necessitate increasing the antibiotic concentration required to cause bacterial death by 1000x greater [19–21]. Normally, biofilms surround *Staphylococcus aureus* cells are uncontrollable and cause deteriorating of antimicrobial potency in therapy, and hence trigger many recurrent infections [22]. Furthermore, complex cell constellations, rooted in extracellular polysaccharide shield, which enables microorganism's adherence, are the head frame of biofilm which hinder antibiotic penetration [23]. Innovative approaches for eradication and inhibition the early formation of biofilms will become necessary, particularly with the fading accessibility to new antibacterial agents. Propolis can be encountered among the supreme effective natural anti-biofilm products that can be obtained from beehives [8, 24]. Bees put on propolis in a thin layer on the interior walls of their hive to seal off holes and cracks to become un-penetrable [25]. It is composed of resins (50%), wax (30%), essential oils (10%), pollen (5%), and trace elements (5%), including organic remains, but this composition differs in consistence with the bees plant feeding source [26]. Propolis is made by bees to protect hives by preserving them from many microbial intrusion [27] and this can be attributed to its enriched content with flavonoids as key components besides other phenolic compounds, aromatic acids, esters, aldehydes, ketones, fatty acids, and several other compounds in trace amounts [28]. Propolis consists of a diverse combination of chemical compounds contributing to its antimicrobial potency. The main collection of chemical components discovered in propolis composition is polyphenols, together with flavonoids, other than various phenolic acids [29]. The propolis chemical profile is dissimilar among different samples at both qualitative and quantitative level according to the region of collection [24]. Regarding the method of extraction, typically, ethanolic extraction was the furthermost convenient method for obtaining extracts with the maximum content of compounds that possess biological activities [30]. In vitro, propolis synergetic effect with many antibacterial agents has been explored in many studies [31–33] in a trial to overcome the rapidly evolving and non-stop expiration of antibiotics potency arise from microbial resistance. In our work, we investigated the antibacterial, anti-biofilm potency of propolis against MRSA clinical isolates, along with the combined effect with different antibiotics classes in a trial to find a solution to overcome bacterial resistance triggered by biofilm formation, to restore the potency of unworkable antibiotics, and to keep the last resort of them effective.

2. METHODS

2.1. *Staphylococcus aureus* Isolation from Clinical Specimens

Clinical isolates identified as *Staphylococcus aureus* were recovered from different 150 specimens; blood culture, pus, sputum, urine, CSF, and pleural effusion which were collected from patients hospitalized in separate wards at Beni-Suef University hospital (Beni-Suef City, Egypt).

All specimens were cultured on tryptone soy agar (TSA; Oxoid, UK) and 5% sheep blood agar then sub-cultured on mannitol salt agar plates (MSA; Oxoid, UK). Yellow pigmented colonies on mannitol salt agar plates were further identified as *Staphylococcus aureus* by Gram stain as gram-positive cocci arranged in clusters, and confirmed by biochemical tests such as catalase, tube coagulase, and DNase tests (Oxoid, UK). Bacterial isolates were stored at -20^{o} C in glycerol broth until further steps.

2.2. Detection of *Staphylococcus aureus* Resistance to Methicillin

All isolates previously identified as *Staphylococcus aureus* were further screened for Methicillin resistance using the Cefoxitin disc diffusion method. Fresh overnight well-isolated and pure bacterial colonies were suspended in a sterile 0.9% saline to achieve 0.5 McFarland visual turbidity and swapped over Muller Hinton agar plates (MHA; Oxoid, UK). Cefoxitin disc (30µg, Oxoid, UK) was aseptically placed on previously loaded agar surface then incubated overnight at 37° C for 18 hours. The zones of inhibition were measured, and according to CLSI guidelines [34]; those < 21mm in diameter were considered as Methicillin-resistant strains. *Staphylococcus aureus* standard strains MSSA ATCC 25923 and MRSA ATCC 43300 were used as negative and positive controls, respectively.

2.3. Antimicrobial Sensitivity Testing

Staphylococcus aureus clinical isolates were subjected to antimicrobial sensitivity testing by Kirby Bauer disc diffusion method referring to the CLSI guidelines [34], using the following antibiotic discs (Oxoid, UK): Penicillin G (P, 10IU), Ampicillin (AMP, 30µg), Clindamycin (DA, 2µg), Ceftazidime (CTZ, 30µg), Ciprofloxacin (CIP, 5µg), Gentamycin (CN, 10µg), Linezolid (LZD, 10µg), and Amikacin (AK, 30µg). The diameters of inhibition zones were measured in mm. Isolates were sorted as susceptible, intermediate, or resistant referring to CLSI guidelines [34].

2.4. Ethanolic Extract Preparation of Egyptian Propolis

Two *Apis mellifera* propolis crude samples were obtained from apiaries located at two different provinces in Egypt; West-Nile (Delta, Egypt) and Upper-Egypt (Beni-Suef, Egypt) during April 2016. Briefly, crude samples were firstly frozen at -20º C then crushed in a miller to bring the powder-like form to avoid the sticky nature of propolis then weighed 10 g directly while frozen and dissolved in 100 mL of 70% ethanol in a 1:10 (w/v) ratio. Samples were kept in a well-stoppered opaque glass container in a dark room with occasional shaking at 37°C for two weeks.

The obtained extracts were filtered using gravity filtration on a Whatmann No.4 filter (Millipore, USA) and left overnight in dark at 4° C without shaking then centrifuged (3900xg/10 min) to eliminate remnant wax deposits on the bottom and left in dark in a semi-closed glass container till complete dryness followed by storage at 4° C [35].

Both samples were extracted by the same method. Each yield of the extraction process was expressed in percentage. The yield percentage was calculated following Equation:

$$\text{Yield} = (\text{extract lyophilisate weight}) / (\text{crude propolis weight}) \times 100$$

2.5. Assessment of Antibacterial Potency of Ethanolic Extract of Egyptian Propolis

Minimum inhibitory concentration was estimated by broth micro-dilution method in a 96-well microtiter plate [36]. *Staphylococcus aureus* isolates (n=7) that were multidrug-resistant (exhibited the highest resistant profile) and two reference strains (MRSA ATCC 43300 and MSSA ATCC 25923) were used.

Bacterial culture was grown on Muller-Hinton agar for 24 hours at 37° C. Inoculum of two to three pure isolated colonies was suspended in a sterile 0.9% saline solution to achieve 0.5 McFarland visual turbidity followed by dilution in Muller-Hinton broth (MHB; Oxoid, UK) at a ratio of 1:100 (v/v).

A hundred microliters of ethanolic extract of propolis were serially diluted in a bi-fold manner using MHB medium. The bi-fold dilutions of the propolis were prepared with final concentrations of 6250, 3125, 1562, 780, 390, 195, 97.5, and 48.75µg/mL (considered after addition of inoculum). An aliquot of 100µL of previously adjusted inoculum was dispensed into the wells of columns 1–10. Microtiter plates then were incubated for 24 hours at 37º C. The final concentration of the cells in all wells was approximately 5 x 10^5 CFU /mL. Column 11 contained 200 µL of inoculum as the positive control, column 12 contained 200µL of the MHB broth only as a sterility control, and MHB with the previously mentioned propolis extract concentrations as visual turbidity negative control to avoid existence of turbidity to any reason other than the bacterial growth. Ethanol as 70% concentration was included as a solvent control to exclude any inhibitory effect except that for the investigated extract. The assay was performed in triplicates. Minimum inhibitory concentration was interpreted as the lowest concentration in which no bacterial growth is visible.

2.6. Combination Effect between Ethanolic Extract of Propolis and Antibiotics

A concentration equal to 1/4 MIC of ethanolic extract of propolis was considered as a sub-inhibitory concentration. The assay was carried out on both MDR MRSA clinical isolates (n=4) and two standard strains (MRSA ATCC 43300 and MSSA ATCC 25923) according to Kirby Bauer method.

The evaluated antibiotics (Oxoid, UK) were as follow, Penicillin G (P, 10IU), Ampicillin (AMP, 30µg), Clindamycin (DA, 2µg), Cefoxitin (FOX, 30µg), Ciprofloxacin (CIP, 5µg), Gentamycin (CN, 10µg), Linezolid (LZD, 10µg) and Amikacin (AK, 30µg).

By using sterile forceps, antibiotic discs were loaded onto both previously inoculated plain MHA agar plates and plates supplemented with the sub-inhibitory concentration of ethanolic extract of propolis. Each extract was tested in a separate plate, followed by incubation at 35° C for 18 hours. The assay was performed in triplicates and inhibition zones diameters (in mm) and the mean values with standard deviations were calculated.

2.7. Biofilm Formation Capacity of MDR Isolates

Biofilm formation capability for MDR MRSA isolates (n=7) which were previously subjected to antibacterial assessment of ethanolic extract of propolis and two standard strains (MRSA ATCC 43300 and MSSA ATCC 25923) was assessed using tissue culture plate (TCP) method [37].

Planktonic bacterial cells and bacterial biofilm viability were quantitatively measured using crystal violet assay (CV) conducted by Benaducci *et al.* [38]. In this assay, bacterial culture was suspended in tryptone soy broth (TSB, Oxoid, UK) supplemented with 1% glucose and 1% sodium chloride to enhance biofilm formation and incubated at 37° C for 24 hours.

Aliquot of 200µL of 1.0 $\times 10^6$ CFU/mL bacterial inoculum was loaded into each well of flat bottom microtiter plate then incubated for 48 hours at 37º C. After incubation time, the plate was emptied then washed gently with sterile phosphate buffer solution (PH=7.4) three times to remove non-adherent cells. Then the plate was subjected to heat fixation at 60º C for 1 hour to fix the adhered cells.

Following this, each well was stained with 200µl of 1% previously filtered crystal violet dye and incubated for 15 min at 25º C. The plates were rinsed three times with water that was previously distilled, then air-dried at a laminar airflow cabinet for 15 min. A volume of 200µl of a de-staining solution (33% acetic acid) was then added and transferred to a new plate.

The absorbance was determined at 590nm using a microtiter plate reader (Tecan®, Sunrise). All tests were performed independently in triplicates. Non-biofilm producer *Staphylococcus epidermidis* standard strain ATCC 12228 was utilized as the negative control, while MSSA ATCC 25923 was considered as the positive control.

Then tested isolates were sorted as non-producing, weak, moderate, or strong-producing; regarding the cut-off value (ODc). It is equal to three times the value of the standard deviations (SD) above the mean OD of the negative control (OD_N):

$$ODc = (OD_N) + (3 \times SD \text{ of negative control})$$

The final OD value of a tested strain (OD_T) is expressed as the mean OD value of the strain (OD_{Av}) reduced by ODc value as follow:

$$(OD_T = OD_{Av} - ODc)$$

After ODc value was calculated for each microtiter plate individually, negative values obtained were interpreted as zero, while any positive value indicated biofilm production. Then: if $OD_T \leq ODc$ = biofilm production not exist; $ODc < OD_T \leq 2\times ODc$ = biofilm production is weak; $2\times ODc < OD \leq 4\times ODc$ = biofilm production is moderate; $4\times ODc < OD$ = biofilm production is strong [39].

2.8. Inhibition of *S. aureus* Biofilm Formation By Ethanolic Propolis Extracts

To inspect the effect of propolis extracts on biofilm formation [40], previously determined MICs of propolis extracts were used against MDR MRSA isolates (n=7) and standard strains (MRSA ATCC 43300 and MSSA ATCC 25923). This concentration was chosen to be considered in treatment and compared with common antibiotics whose MIC needs to be doubled several times to be effective as anti-biofilm agents.

Bacterial inoculum containing 1×10^6 CFU/ml was prepared as previously mentioned and then 100µl was loaded into each well of the polystyrene microtiter plate. Ethanolic propolis extract was diluted in tryptone soy broth (TSB) to achieve 1X MIC (considered as the final concentration after adding to the previously adjusted bacterial inoculum).

Aliquot of 100µl was pipetted into each well; afterward, plates were incubated at 37° C for 24 hours. Inoculated wells without propolis treatment were considered as the negative control.

Biofilm was measured as described previously in test and control wells. All tests were performed independently in triplicates. The inhibition of biofilm was measured as a percentage [41] using the calculation formula of:

$$(100-\{(OD590 \text{ isolate} / OD590 \text{ unprocessed control}) \times 100\})$$

2.9. Egyptian Propolis Ethanolic Extracts Eradication Potency of Preformed Biofilms of *Staphylococcus aureus*

The biofilms of MDR MRSA isolates (n=7) and standard strains (MRSA ATCC 43300 and MSSA

ATCC 25923) were allowed firstly to be formed within 48 hours before the addition of propolis extract.

Propolis extracts were diluted in TSB to prepare concentrations of 1/4 and 1/8 MIC. These low concentrations were chosen to consider the sticky nature of propolis when used topically for eradication of biofilms on topical surfaces, to consider economic burden, and to allow the diluted form to penetrate well within biofilm matrix.

Biofilms of tested cultures were formed in polystyrene 96-well microtiter plates [37], as described above. Plates were then incubated for a short time at 37° C (2 hours). Afterward, the wells were washed a couple of times with sterile PBS to get rid of planktonic and deceased cells.

Biofilms were exposed to C.V assay as previously mentioned. Results were compared to bacterial biofilms without propolis treatment that are considered as control and the influence on the biofilm disruption was established as a percentage [40, 42]. All the assays were performed in triplicates.

2.10. Adherence Inhibition Assay

Since adherence is the predisposing factor in biofilm formation and in sticking to abiotic surfaces, so it was valuable to test anti-adherence ability of propolis. Anti-adhesion assays were conducted on MDR MRSA isolates (n=7) and standard strains (MRSA ATCC 43300 and MSSA ATCC 25923) according to Adrião *et al.*[43] with some modifications.

A quantity of 200µL of an overnight culture was centrifuged and the pellets were re-suspended in TSB supplemented with propolis extract at 1X MIC, 1/4 MIC, and 1/8 MIC and without propolis extract as the positive control.

A quantity of 200µL of prepared culture was inoculated into each well of a polystyrene flat-bottom micro-plate and left to adhere for 1 hour at 25° C in an air-flow cabinet. After incubation, non-adherent cells and residues of media component were disposed by emptying the media then wells were gently washed using sterile PBS. Then wells were dried in an airflow cabinet for 15 min.

The adherent cells were heat-fixed at 60° C for 30 min then the adhered cells count was measured using the C.V method as mentioned firstly. Wells with propolis alone in tryptone soy broth with was considered as a turbidity control to ensure exclusion of staining of any particles of propolis that may be precipitated by the diluting broth. Results calculated as follow [44]:

Adherence inhibition % = [OD590 of adhered cells/ (OD590 of adhered cells+OD590 of supernatant cells)].

3. RESULTS

3.1. *Staphylococcus aureus* Isolation from Clinical Specimens

Sixty eight *Staphylococcus aureus* isolates were recovered from different 150 clinical specimens (45% positive isolation) ; blood culture (n= 36/68, 53%), pus (n=14/68, 20%), sputum (n=4/68, 6%), urine specimens (n=8/68, 12%), CSF (n=4/68, 6%) and pleural effusion (n=2/68, 3%) from patients who were hospitalized in different wards at Beni-Suef university hospital Table (1).

Twenty-three strains were isolated from the male patients (34%) and 45 from the female patients (66%). Patients whose ages were from 40 years old and older (n=31/68, 45%) constituted the highest proportion of isolated *Staphylococcus aureus*, while patients who were 12 till less than 40 years old represented 28% (n=19/68) and 21% were neonates who ranged in age from one week to 12 months (n=14/68). The least proportion was detected among children from one year in age to 12 years old and represented 6% (n=4/68).

3.2. Detection of *Staphylococcus aureus* Resistance to Methicillin

According to susceptibility to Cefoxitin, 90% of isolates (n= 61/68) were resistant and therefore considered as MRSA, whereas 10% of isolates (n=7/68) were Cefoxitin sensitive and considered as MSSA, as seen in Table (2).

3.3. Antimicrobial Sensitivity Testing

Isolates of *Staphylococcus aureus* (n=68) were screened for their sensitivity to different classes of antibiotics using disc diffusion assay by Kirby-Bauer method. The antibiotic sensitivity pattern of isolates pointed at a high resistance profile as seen in Figures (1, 2).

All isolates which were previously classified as MRSA were resistant to Ampicillin and Penicillin G (100%) followed by Ceftazidime (93%), while resistance to both Gentamycin and Clindamycin was almost similar (49% and 52% respectively). Ciprofloxacin resistance ratio among MRSA reached 34 %; however, more than 8% of isolates were resistant to Amikacin and no resistance to Linezolid was recorded.

Methicillin-sensitive *Staphylococcus aureus* (MSSA) isolates showed Penicillin G resistance by 100%, while in the case of Ampicillin, 71% of isolates were resistant. Clindamycin intermediate pattern was detected in 29% of MSSA isolates and 29% showed resistance to Ceftazidime, while no resistance was observed in the case of Ciprofloxacin, Gentamycin, Amikacin, or Linezolid.

3.3.1. Multi-Drug Resistance among *Staphylococcus aureus* Isolates (MDR)

Resistance to several antibiotics families was recognized among the collection of tested MRSA clinical isolates. Multi-Drug Resistance phenotypes that were resistant to three different antibiotic classes were noticed in 52% (n=32/61), whereas 35% (n=22/61) showed resistance to four antibiotic classes, and 8% (n=5/61) exhibited resistance to five antibiotic classes. None of the isolates depicted resistance to linezolid.

3.4. Ethanolic Extract Preparation of Egyptian Propolis

Propolis extraction yielded 20% pure material from its crude form by 70% ethanolic extraction for two weeks.

Yield = (2 g pure matter) / (10 g crude material) ×100 = 20%

3.5. Assessment of Antibacterial Potency of Ethanolic Extract of Egyptian Propolis

The Microdilution method was done for investigating propolis antibacterial activity against MDR MRSA isolates (n=7) and standard strains (MRSA ATCC 43300 and MSSA ATCC 25923). Prominent antibacterial activity was noticed and MIC values ranged from 97µg/mL to 781µg/mL, Table (3).

Mean MIC was calculated for clinical isolates with standard deviation (362±0.19 and 432 ±0.063µg/mL for West Nile and Upper Egypt propolis ethanolic extracts respectively). West Nile ethanolic extract of propolis showed a lower mean MIC value, Figure (3).

3.6. Combination Effect between Ethanolic Extract of Propolis and Antibiotics

All eight antibacterial drugs' efficacy (FOX; Cefoxitin, DA; Clindamycin, CN; Gentamycin, CIP; Ciprofloxacin, LZD; Linezolid, PG; Penicillin G, AMP; Ampicillin and AK; Amikacin) was increased prominently when combined with propolis, Table (S1). More potent antibacterial effect was noticed against *Staphylococcus aureus* standard strains (MRSA ATCC 43300 and MSSA ATCC 25923) rather than MDR clinical isolates (n=4). This assay revealed a prominent synergism between propolis ethanolic extract and antibiotics against MDR MRSA strains; Figures (4 and 5).

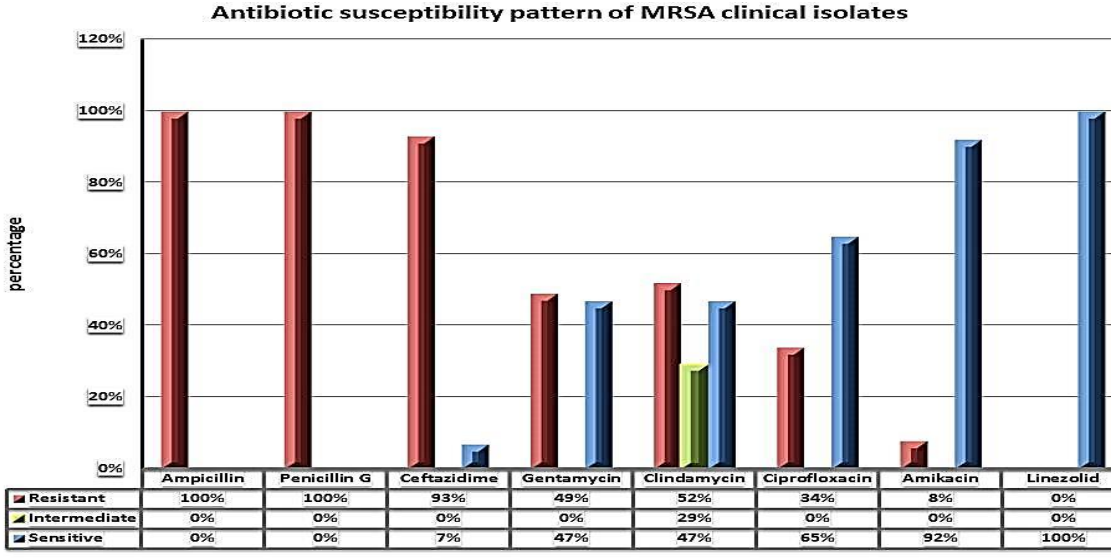

Figure 1. Antibiotic susceptibility profile of MRSA clinical isolates (n=61) towards different classes' antibiotics.

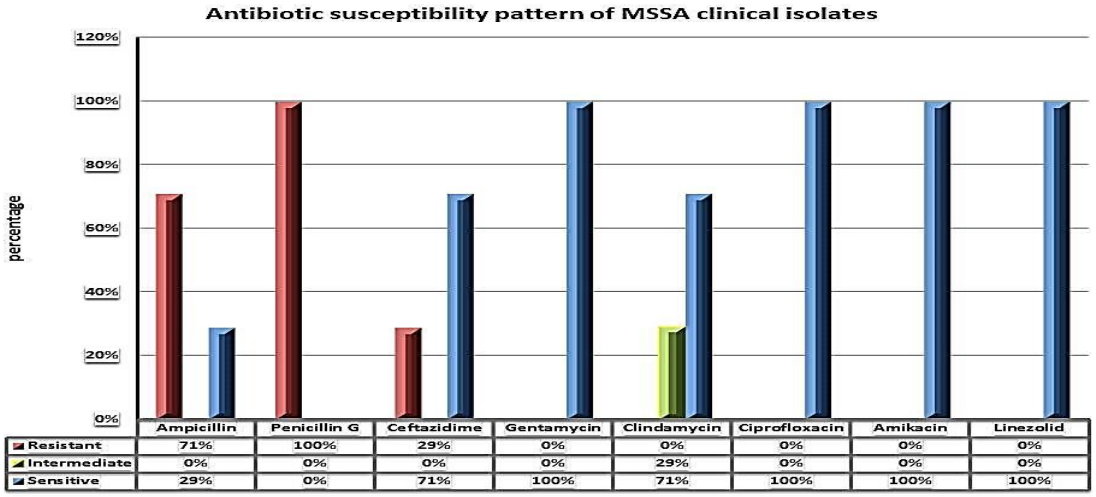

Figure 2. Antibiotic susceptibility profile of MSSA clinical isolates (n=7) towards different classes' antibiotics.

Table 1. *Staphylococcus aureus* clinical isolates number collected from each specimen from relative individual hospital ward considering sub-classification according to methicillin resistance into MRSA and MSSA

Specimen	Ward	Total	MSSA Isolate	MRSA Isolate
Blood	Internal	6	0	6
	Ped. Care	10	2	8
	Neonatology	8	2	6
	ICU	10	1	9
	Endemic	2	0	2
Pus	Surgery	8	2	6
	ICU	6	0	6
Urine	Urology	8	0	8
sputum	ICU	4	0	4
CSF	Neurology	4	0	4
Pleural effusion	Endemic	2	0	2
Total		68	7	61

*Ped. Care is an abbreviation for pediatric care ward.
* MRSA is Methicillin resistant *S. aureus* and MSSA is Methicillin sensitive *S. aureus.*

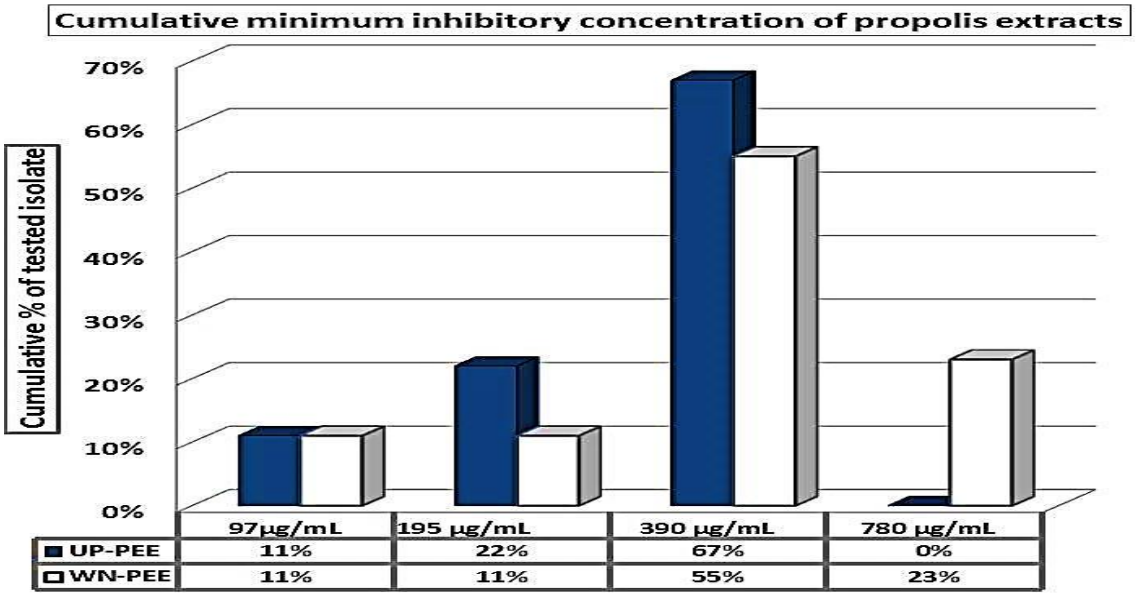

Figure 3. Cumulative minimum inhibitory concentrations (MICs) of propolis extracts expressed as a percentage of tested isolates.

Table 2. The phenotypic Cefoxitin screening test for differentiation of *S. aureus* into MSSA or MRSA.

Number of tested Isolates (n= 68).	**Cefoxitin Sensitivity (Diameter of inhibition zone)**
MRSA *S. aureus* isolates (n=61)	<22 mm
MSSA *S. aureus* isolates (n=7)	≥23 mm
MRSA ATCC 43300	21 mm
MSSA ATCC 25923	38 mm

* MRSA is Methicillin resistant *S. aureus* and MSSA is Methicillin sensitive *S. aureus.*

Table 3. Values of 1X, 1/4 and 1/8 Minimum inhibitory concentrations of propolis extracts against each MSSA and MRSA *S. aureus* clinical isolates and standard strains.

Isolate Code.	**1X MIC- UPE-PEE**	**¼ MIC- UPE-PEE**	**1/8 MIC- UPE-PEE**
***S. aureus* 17**	390 µg/mL	97.5 µg/mL	48.75 µg/mL
***S. aureus* 65**	390 µg/mL	97.5 µg/mL	48.75µg/mL
***S. aureus* 11**	390 µg/mL	97.5 µg/mL	48.75 µg/mL
***S. aureus* 15**	390 µg/mL	97.5 µg/mL	48.75µg/mL
***S. aureus* 90**	195 µg/mL	48.75µg/mL	24.4 µg/mL
***S. aureus* 31**	390 µg/mL	97.5 µg/mL	48.75 µg/mL
***S. aureus* C10**	390 µg/mL	97.5 µg/mL	48.75 µg/mL
MRSA ATCC 43300	390 µg/mL	97.5 µg/mL	48.75 µg/mL
MSSA ATCC 25923	390 µg/mL	97.5 µg/mL	48.75 µg/mL
Isolate Code.	**1X MIC- WN-PEE**	**¼ MIC- WN-PEE**	**1/8 MIC- WN-PEE**
***S. aureus* 17**	390 µg/mL	97.5 µg/mL	48.75 µg/mL
***S. aureus* 65**	195 µg/mL	48.75µg/mL	24.4 µg/mL
***S. aureus* 11**	780 µg/mL	195 µg/mL	24.4µg/mL
***S. aureus* 15**	390 µg/mL	97.5 µg/mL	48.75µg/mL
***S. aureus* 90**	97.5 µg/mL	24.4µg/mL	12. 2 µg/mL
***S. aureus* 31**	780 µg/mL	195 µg/mL	24.4 µg/mL
***S. aureus* C10**	390 µg/mL	97.5 µg/mL	48.75 µg/mL
MRSA ATCC 43300	97.5 µg/mL	24.4µg/mL	12. 2 µg/mL
MSSAATCC 25923	195 µg/mL	97. 5µg/mL	48.75 µg/mL

***** UP-PEE: Upper-Egypt propolis ethanolic extract.
*WN-PEE: West-Nile propolis ethanolic extract.

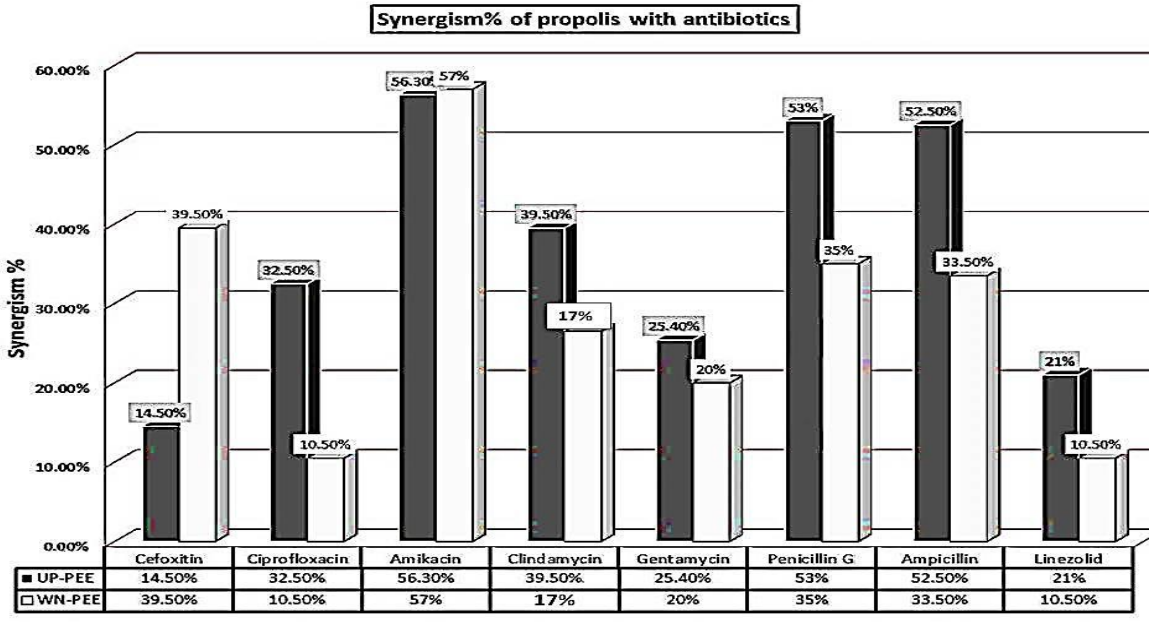

Figure 4. Synergism percentage of propolis extracts with different antibiotics considering total *S. aureus* strains (n=6).

3.5. Assessment of Antibacterial Potency of Ethanolic Extract of Egyptian Propolis

The Microdilution method was done for investigating propolis antibacterial activity against MDR MRSA isolates (n=7) and standard strains (MRSA ATCC 43300 and MSSA ATCC 25923). Prominent antibacterial activity was noticed and MIC values ranged from 97µg/mL to 781µg/mL, Table (3).

Mean MIC was calculated for clinical isolates with standard deviation (362±.19 and 432 ±0.063µg/mL for West Nile and Upper Egypt propolis ethanolic extracts respectively). West Nile ethanolic extract of propolis showed a lower mean MIC value, Figure (3).

3.7. Biofilm Formation Capacity of MDR Isolates

Seven MDR MRSA isolates which showed a high resistant profile were assessed for biofilm formation capability using tissue culture ELISA plate method. According to the strength of biofilm production, they were divided into two groups as depicted from the ELISA plate reader (OD readings); four out of seven isolates, as well as MRSA ATCC 43300 and MSSA ATCC 25923, were strong in biofilm production, and three were moderate as seen in Table (S2).

3.8. Inhibition of *S. aureus* Biofilm Formation by Ethanolic Propolis Extracts

Ethanolic extract of Egyptian Propolis exerted a noticed interference with *Staphylococcus aureus* capability to form biofilms in both standard strains (MSSA ATCC 25923 and MRSA ATCC 43300) and clinical isolates.

Biofilm formation was inhibited from 49.5% to 25% in the case of ethanolic extract of West Nile propolis and from 33% to 15% by ethanolic extract of the Upper Egypt propolis. However ethanolic extract of West Nile propolis depicted a higher potential in the prevention of biofilm formation than ethanolic extract of Upper Egypt propolis as seen in Table (S3).

The percentage of inhibition of biofilm formation for each tested Egyptian propolis extracts was calculated using the formula:

100-{(OD590 of isolates/OD590 of the unprocessed control) x 100}

Mean biofilm inhibition percentage for all tested isolates with standard deviation was calculated (36% ± 0.075 and 21% ± 0.062) for ethanolic extract of West-Nile propolis and Upper-Egypt propolis respectively (Figure 6).

3.9. Egyptian Propolis Ethanolic Extracts Eradication Potency of Preformed Biofilms of *Staphylococcus aureus*

The effect of both propolis ethanolic extracts on *Staphylococcus aureus* mature biofilms in clinical isolates and standard strains at different concentrations (1/4 and 1/8 MIC) was prominent and expressed as percentage of biofilm eradication along with OD ±SD relevant values were mentioned in Tables (S 4 and 5).

Both ethanolic extracts of propolis successfully exhibited eradication of biofilms produced by standard strains MRSA ATCC 43300 and MSSA ATCC 25923 more than clinical isolates as some biofilms were more resistant than others amongst clinical isolates; however, two clinical isolates biofilms were eradicated by a high percentage.

Upper Egypt propolis showed a higher potency in biofilms disruption for both standard strains and clinical isolates than West Nile propolis. Mean biofilm eradication ratios for all tested isolates with standard deviation are seen in Figure (6).

3.10. Adherence Inhibition Assay

By using the following formula;

Percentage of adherence inhibition = (OD590 of adhered cells/ (OD590 of adhered cells + OD590 of propolis ethanolic extract supernatant cells))

Both propolis samples showed a similar prominent anti-adherence activity against standard strains and clinical isolates. However, no significant difference ($p < 0.05$) was noticed among the two investigated propolis ethanolic extract samples when compared by each other at the same concentrations. Both propolis samples inhibited adherence (Tables S 6, 7) in a much better manner at relevant 1XMIC values than 1/4 or 1/8 MIC. Mean adherence inhibition ratio for all isolates with standard deviation are shown in Figure (6).

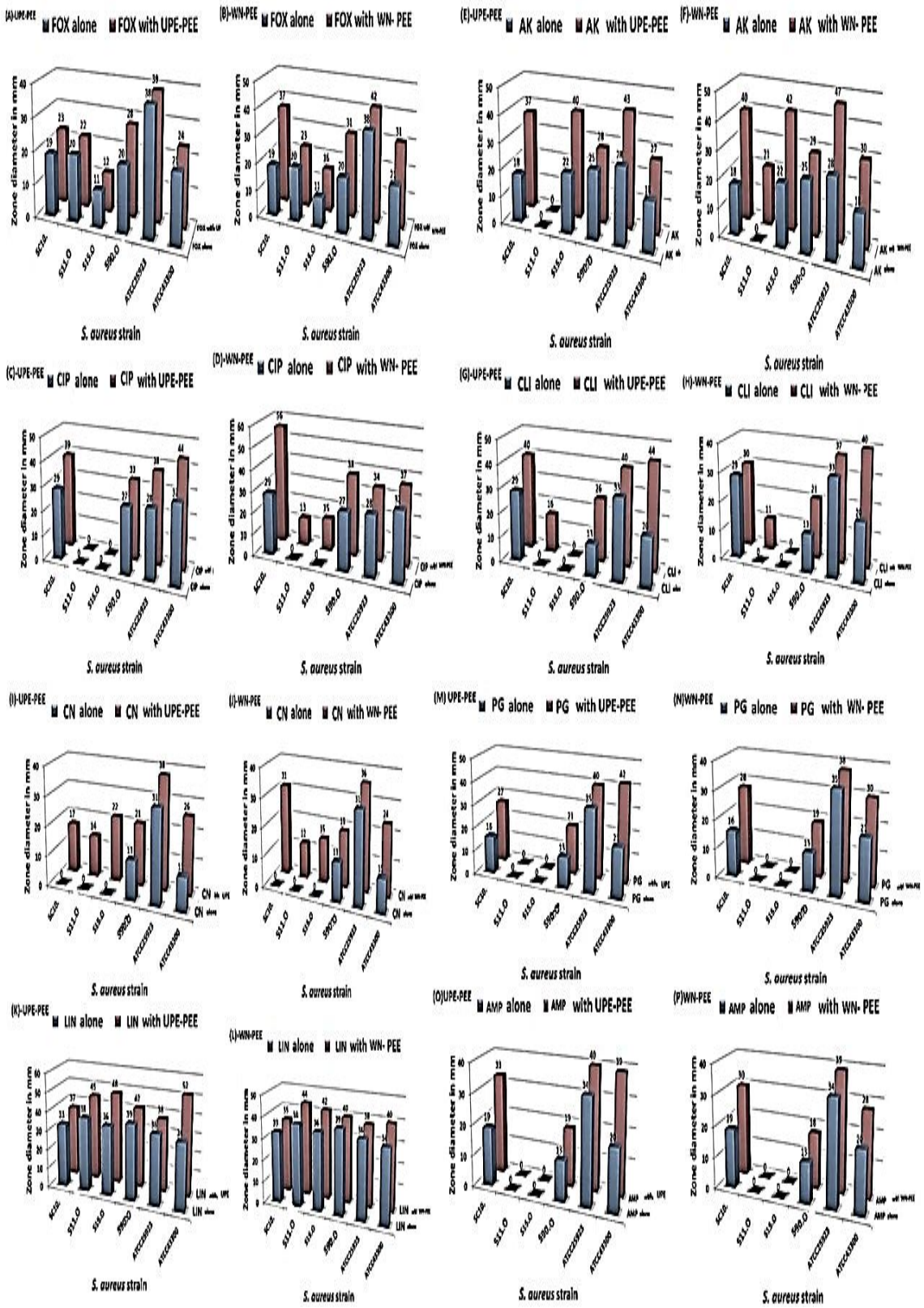

Figure 5. Combination effect between propolis extract at ¼ MIC and antibiotics acting on cell wall synthesis inhibition (Cefoxitin), antibiotics function by inhibiting a type II topoisomerase (DNA gyrase) and topoisomerase IV (Ciprofloxacin), antibiotics acting on protein synthesis (Amikacin and Clindamycin), antibiotics acting on protein synthesis (Gentamycin and Linezolid) and antibiotics acting on cell wall (Penicillin G and Ampicillin) against four *S. aureus* clinical isolates and two standard strains MRSA ATCC 43300 and MSSA ATCC 25923.

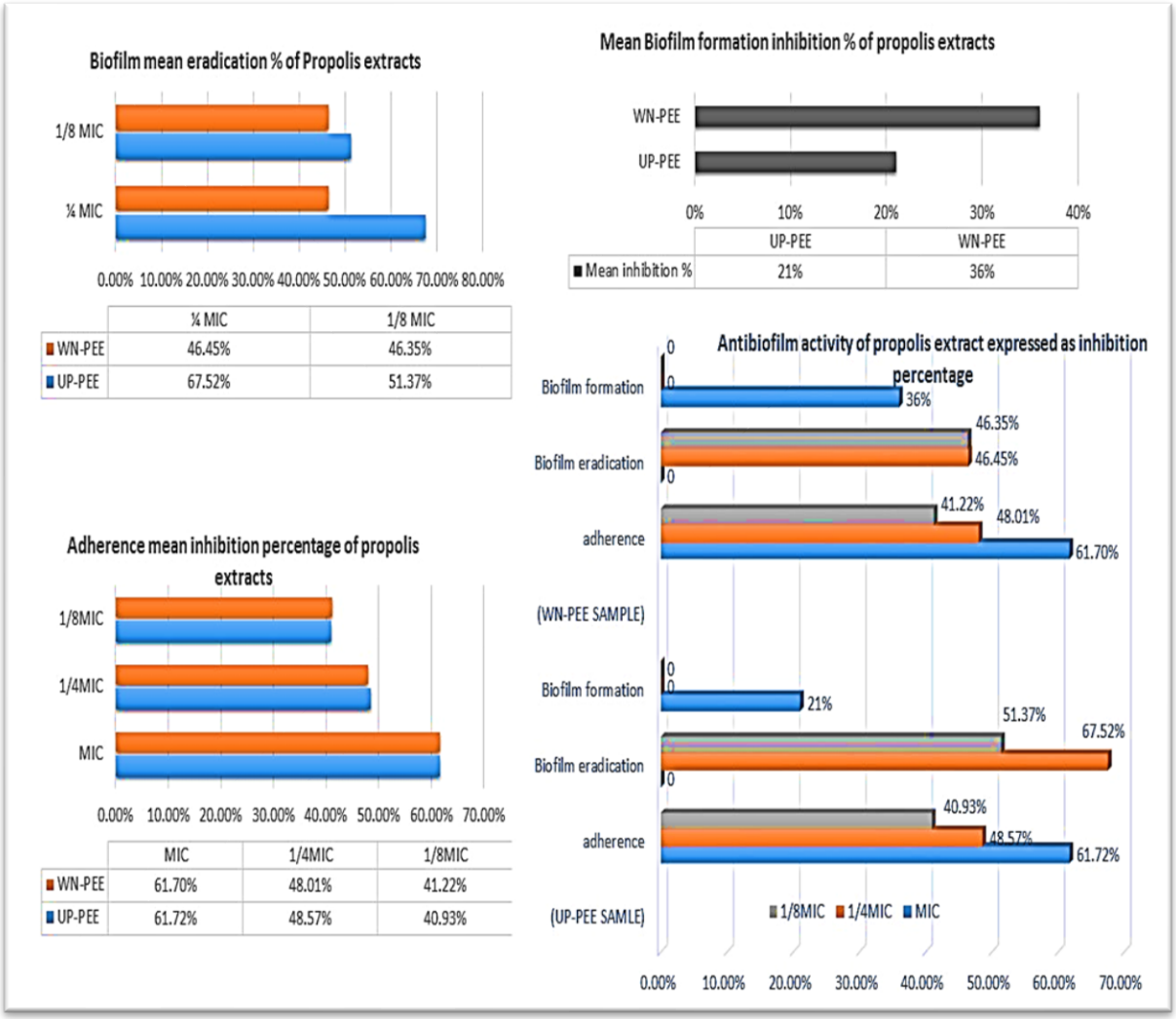

Figure 6. Mean inhibition percentage of all tested *S. aureus* biofilms when treated with each propolis extract and Collective data showing anti-biofilm activities of propolis extract on *S. aureus* biofilms (biofilm formation inhibition, eradication and anti-adherence).

4. DISCUSSION

Staphylococcus aureus now is categorized as a frequent causative agent of hospital-associated and device-related infections with an increased incidence of drug resistance [45]. Our study revealed a high Methicillin resistance pattern among *Staphylococcus aureus* clinical isolates (90%, n=61/68). In Egypt, *Staphylococcus aureus* isolates, mainly MRSA strains, are the major pathogens causing a wound and surgical site infections [10]. In recent decades, due to the evolution of bacterial resistance and the abuse of antibiotics; a high resistance profile was detected among clinical isolates. MSSA (n=7/68, 7%) and MRSA (90%, n=61/68) isolates depicted a high resistance pattern to the antibiotic panel; Penicillin G resistance was similar in MSSA and MRSA isolates (100%) which comes in agreement with Rağbetli *et al* who noted a comprehensive penicillin G resistance among isolates [46]. Also, Bhatt *et al* reported that all *Staphylococcus aureus* tested isolates were resistant to Penicillin G and 90% of them showed a sensitive pattern to Amikacin which is similar to our results (92%) [47]. MRSA showed a high resistance pattern to Clindamycin (53%), however, a higher ratio (72.3%) was recorded in a study by Kot *et al* [48]. Gentamycin and Ciprofloxacin resistance among MRSA was 49% and 35% respectively, which comes in agreement with what was recorded by Arjyal *et al* who mentioned that MRSA isolates showed high resistance patterns to Gentamicin (50%) and Ciprofloxacin (25%) [49]. In our study, 52% and 35% of clinically isolated *S. aureus* were resistant to three and four different antibiotic classes respectively, which indicates a high level of multiple antibiotic

resistance in the collection of *Staphylococcus aureus* clinical isolates. However, a relatively higher ratio was recorded in a study by Kot *et al* who stated that a large number of MRSA isolates showed resistance to Levofloxacin (83.9%), Ciprofloxacin (83%), Erythromycin (77.7%), and Clindamycin (72.3%); however, a lower ratio among MRSA showed Amikacin resistance (14.2%) [48]. This finding is higher than what reported by Ceballos *et al* who mentioned that 16.7% (18/108) depicted four or more different antibiotics families resistance (Tetracycline, Erythromycin, Clindamycin, Gentamycin), the authors also stated that resistance to Linezolid was not detected [50]. However, in a report by Abd El-Hamid *et al* [51]; multidrug resistance (MDR) was detected in 85.8% of hospital acquired-MRSA. Neopane P *et al* mentioned that there is a substantial association between the production of bacterial biofilm and the antibacterial resistance [52], in addition to Methicillin resistance [53]. These findings coincide with our results that showed an increased biofilm formation tendency among multidrug-resistant isolates. Propolis provides a bypass for opening new fields to develop new natural therapies to overcome antibiotic resistance that is especially caused by biofilm-forming bacterial species [54, 55]. From a clinical approach, the most valuable feature of propolis is its anti-staphylococcal and anti-biofilm activities [8, 24, 56]. The potency of inhibiting bacterial growth of standard strains MRSA ATCC 43300 and MSSA ATCC 25923 along with clinical isolates was prominent and this can be attributed to the unique multi-mode of action arises from the synergism among propolis components in comparison to single target agents (antibiotics) that act on a specific component in the bacterial cell and which is easy for bacteria to evolve a strategy of resistance towards it [57]. Propolis extracts showed a prominent antibacterial activity against MDR *Staphylococcus aureus* isolates with MIC values ranged from 780 µg/mL to 97.5 µg/mL. Relative results were reported by Wojtyczka *et al* in their study of polish propolis on *Staphylococcus aureus* clinical isolates, as authors mentioned varying degrees of susceptibilities with MIC ranged from 390µg/mL to 780µg/mL [58]. Samples that were collected from Europe along with American North and South regions displayed a similar anti-staphylococcal activity, with MIC values from 0.125 to 0.5 mg/mL [59]. The mechanism of propolis antimicrobial activity could be attributed to the synergistic activity between phenolic acids and other compounds [60]. Three MDR isolates were moderate biofilm producers and four were strong which explains the high resistance profiles of these isolates. Although biofilm presents a physical resistance to antimicrobials, this can be reversed by disrupting the bacterial shield [61]. Thus, acting on the early stages of biofilm formation is an interesting strategy to control biofilm [62]. It was witnessed that West Nile propolis had a better efficacy in hindering biofilm formation than Upper Egypt propolis. In a report by Dogan, propolis extracts (at 400µg/mL) from Turkey were studied, as the author mentioned that 22% biofilm inhibition was recorded against MRSA isolates and *Staphylococcus aureus* ATCC 29213 biofilms, while 19% biofilm inhibition was achieved when propolis samples were tested on *Staphylococcus aureus* ATCC 33862 biofilm [63]. However, in our investigation, Egyptian propolis samples successfully recorded a higher potency in preformed biofilm eradication at lower concentrations (195µg/mL to 24.4µg/mL). In a report by Grecka *et al*, only residual growth of *Staphylococcus aureus* ATCC 25923 was detected in the medium containing propolis ethanolic extract at concentrations higher than 128µg/mL, and only slightly lower susceptibility was observed for Staphylococcus *aureus* ATCC 29213 [33]. However, propolis samples investigated in our study recorded a higher anti-biofilm potency with MSSA ATCC 25923 which was achieved by lower concentrations (from 97.5µg/mL to 24.4µg/mL). Assessment of anti-adherence propensity of ethanolic extracts of propolis towards *Staphylococcus aureus* is important, as the virulence of the organism was noted to vary according to its capability to adhere to the surface [64]. El-Guendouz *et al* mentioned that the adherence inhibition of MRSA strains by propolis was strain-dependent [54], and in our study, all clinical MRSA isolates adherence along with standard strains were particularly affected (89.8% to 52.1% adherence inhibition) when compared with the control. The tendency of *Staphylococcus aureus* strains to adhere was impaired when treated with 1X MICs, 1/4 MICs, and 1/8 MICs concentrations of Egyptian propolis ethanolic extracts. Among the tested isolates, adherence of two clinical isolates S.17 and S.C10 biofilms was greatly inhibited by the propolis extract (89.9% and 73.5%, respectively), but it recorded a lower adherence inhibition potential against S.65 isolate (46.3%). Stan *et al* reported that the hindrance of biofilm production promoted by propolis was also detected to be reliant on the concentration used [65], while El-Guendouz *et al* noted that *Staphylococcus aureus* ATCC 6538 cells when treated with propolis extract, adherence was not affected; conversely, the diverse MRSA strains cells were repressed to form biofilm when treated with same propolis sample [54]. Also, ethanolic extracts of propolis increased *Staphylococcus aureus* sensitivity to lethal effects of antibiotics at concentrations equal to ¼ MIC. The combination effect between propolis and antibiotics in disc diffusion assay was conducted in many studies [66]. In particular, a strong combined

effect was perceived in both propolis ethanolic extracts at ¼ MICs (from 97.5µg/ml to 24.4µg/ml) with Amikacin; however, Upper Egypt propolis showed a higher synergy with all tested antibiotics but West Nile propolis exceptionally exhibited a more prominent synergetic effect with Cefoxitin. However, some studies reported a higher propolis concentration (up to 600µg/ml) that was required to obtain a significant effect [67]. Synergetic effect of propolis ethanolic extracts with different class's antibiotics makes a way to investigate mechanisms of propolis antibacterial activity observed in many studies and also in our findings. Many reports stated that; synergetic effect of propolis with different antibiotic families could help in the recognition of diverse mechanisms that propolis could act by as an antibacterial agent. Polyphenols, which are the key propolis components, have been proved to exert their antibacterial action through membrane perturbations which is joined with the influence of β-lactams on the transpeptidation of the bacterial cell membrane and hence could bring about the augmented antimicrobial effect [68]. Protein translation inhibition (by acting on different targets) could explains the noticeable synergism with different antibiotics acting on protein synthesis at different stages which also was covered by our investigated antibiotic panel (Gentamycin, Clindamycin, and Linezolid). Synergism of propolis with antibiotics that interfere with bacterial protein synthesis has been mentioned by Fernandez Junior [69]. Fernandes and his colleagues mentioned that the synergism of propolis ethanolic extract with drugs that act by inhibiting protein synthesis was observed. Additionally, RNA-polymerase inhibition by propolis compounds was long-established by Takaisi-Kikuni & Schilcher which supports this conclusion [70]. Propolis showed a good synergism with antibiotics acting on protein syntheses like Gentamycin, Amikacin, and linezolid [71]; however, propolis showed weak synergism with Ciprofloxacin [72]. Our study showed a good synergy between Clindamycin and propolis which comes following a report by Boisard *et al* who mentioned that propolis depicted a good synergy with Clindamycin [73].

5. CONCLUSION

Our study revealed prominent anti-staphylococcal and anti-biofilm potency of Egyptian propolis ethanolic extracts; however, origin of propolis samples affects their potency as revealed in our work. Propolis gives a choice for saving antibiotic efficacy against MDR *S. aureus* to overcome rapid and continuous bacterial resistance evolution to many antibiotics and hence provide a chance to re-sensitize resistant bacteria to the unworkable antibiotics and keep the last resort of them effective.

Ethical approval: This study was approved by Scientific Research Ethics Committee of Faculty of Pharmacy-Girls, Al-Azhar University.

Conflicts of Interest: The authors announce that they have no conflicts of interest.

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Interpretation of results and data analysis: MEO, NMKl, AFA and FAH
Statistical analysis of data: FAH
Manuscript writing and revision: All authors.

REFERENCES

1. Buenaventura-Alcazaren FA, Dela Tonga A, Ong-Lim A, Destura RV. Prevalence and molecular characteristics of MRSA nasal carriage among hospital care workers in a tertiary hospital in the Philippines. J Microbiol Immunol Infect. 2020 Oct; 53(5):739-745. DOI:10.1016/j.jmii.2018.12.016. Epub 2019 Jul 17. PMID: 31375429.
Available from: https://pubmed.ncbi.nlm.nih.gov/31375429/

2. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, *et al*. Antimicrobial-Resistant Pathogens Associated with Healthcare-Associated Infections: Summary of Data Reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011-2014. Infect Control HospEpidemiol. 2016 Nov 1; 37(11):1288–301.
Available from: https://pubmed.ncbi.nlm.nih.gov/27573805/

3. Otto M. MRSA virulence and spread. Cell Microbiol. 2012 Oct; 14(10):1513.
Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3443268/

4. Algammal AM, Hetta HF, Elkelish A, Alkhalifah DHH, Hozzein WN, Batiha GES, *et al*. Methicillin-resistant *Staphylococcus aureus* (MRSA): One health perspective approach to the bacterium epidemiology, virulence factors, antibiotic resistance, and zoonotic impact. Infect Drug Resist. 2020; 13(7519829):3255–65. Available from: /pmc/articles/PMC7519829/

5. Active Bacterial Core Surveillance (ABCs) 2013 MRSA Report | CDC. Available from:https://www.cdc.gov/abcs/reports-findings/survreports/mrsa13.html

6. Łęski TA, Tomasz A. Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of *Staphylococcus aureus*: Evidence for the cooperative functioning of PBP2, PBP4, and PBP2A. J Bacteriol. 2005 Mar; 187(5):1815–24.
Available from: /pmc/articles/PMC1064008/

7. Sutton JP, Steiner CA. Hospital-, Health Care-, and Community-Acquired MRSA: Estimates from California Hospitals, 2013: Statistical Brief #212. Healthcare Cost and Utilization Project (HCUP) Statistical Briefs. Agency for Healthcare Research and Quality (US); 2006.
Available from: http://www.ncbi.nlm.nih.gov/pubmed/27875041

8. Doganli GA. Phenolic Content and Antibiofilm Activity of Propolis against Clinical MSSA Strains. Vol. 10(5): 617 - 627, Records of Natural Products. 2016. Available from: www.acgpubs.org/RNP

9. Francis JS, Doherty MC, Lopatin U, Johnston CP, Sinha G, Ross T, et al. Severe community-onset pneumonia in healthy adults caused by methicillin-resistant *Staphylococcus aureus* carrying the Panton-Valentine leukocidin genes. Clin Infect Dis. 2005 Jan 1; 40(1):100–7.
Available from: https://pubmed.ncbi.nlm.nih.gov/15614698/

10. Ahmed EF, Gad GFM, Abdalla AM, Hasaneen AM, Abdelwahab SF. Prevalence of Methicillin-Resistant *Staphylococcus aureus* among Egyptian Patients after Surgical Interventions. Surg Infect (Larchmt). 2014 Aug 1; 15(4):404–11. Available from: https://www.liebertpub.com/doi/10.1089/sur.2013.212

11. El-Gendy MMAA, El-Bondkly AMA, Keera AA, Ali AM. Incidence of Methicillin-Resistant *Staphylococcus aureus* (MRSA) in Microbial Community of Cancer Patients and Evaluation of Their Resistant Pattern. Arab J SciEng. 2018 Jan 1; 43(1):83–92.
Available from: https://doi.org/10.1007/s13369-017-2670-4

12. Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG. *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. ClinMicrobiol Rev. 2015; 28(3):603–61.
Available from: https://doi.org/10.1128/CMR.00134-14

13. Hassoun A, Linden PK, Friedman B. Incidence, prevalence, and management of MRSA bacteremia across patient populations-a review of recent developments in MRSA management and treatment. Vol. 21, Critical care (London, England). Crit Care; 2017. p. 211.
Available from: https://pubmed.ncbi.nlm.nih.gov/28807042/

14. Nickerson EK, Hongsuwan M, Limmathurotsakul D, Wuthiekanun V, Shah KR, Srisomang P, *et al*. *Staphylococcus aureus* bacteremia in a tropical setting: Patient outcome and impact of antibiotic resistance. PLoS One. 2009 Jan 30; 4(1). Available from: /pmc/articles/PMC2628727/?report=abstract

15. Lucet JC, Paoletti X, Demontpion C, Degrave M, Vanjak D, Vincent C, *et al*. Carriage of methicillin-resistant *Staphylococcus aureus* in home care settings: Prevalence, duration, and transmission to household members. Arch Intern Med. 2009 Aug 10; 169(15):1372–8.
Available from: https://pubmed.ncbi.nlm.nih.gov/19667299/

16. Mack D, Riedewald J, Rohde H, Magnus T, Feucht HH, Elsner HA, *et al*. Essential functional role of the polysaccharide intercellular adhesin of *Staphylococcus epidermidis* in hemagglutination. Infect Immun. 1999; 67(2):1004–8.
Available from:https://pubmed.ncbi.nlm.nih.gov/9916125/

17. Otto M. Staphylococcal biofilms. Vol. 322, Current Topics in Microbiology and Immunology. Curr Top MicrobiolImmunol; 2008. p. 207–28.
Available from: https://pubmed.ncbi.nlm.nih.gov/18453278/

18. Manandhar S, Singh A, Varma A, Pandey S, Shrivastava N. Biofilm producing clinical *Staphylococcus aureus* isolates augmented

prevalence of antibiotic resistant cases in tertiary care hospitals of Nepal. Front Microbiol. 2018 Nov 27; 2018; 9: 2749. Available from: /pmc/articles/PMC6277500/

19. Walters MC, Roe F, Bugnicourt A, Franklin MJ, Stewart PS. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob Agents Chemother. 2003 Jan 1; 47(1):317–23.
Available from: https://pubmed.ncbi.nlm.nih.gov/12499208/

20. Fux CA, Wilson S, Stoodley P. Detachment characteristics and Oxacillin resistance of *Staphyloccocus aureus* biofilm emboli in an in vitro catheter infection model. J Bacteriol. 2004 Jul; 186(14):4486–91.
Available from: https://pubmed.ncbi.nlm.nih.gov/15231780/

21. Hall-Stoodley L, Stoodley P. Evolving concepts in biofilm infections. Vol. 11, Cellular Microbiology. Cell Microbiol; 2009. p. 1034–43.
Available from: https://pubmed.ncbi.nlm.nih.gov/19374653/

22. Jones SM, Morgan M, Humphrey TJ, Lappin-Scott H. Effect of vancomycin and rifampicin on meticillin-resistant *Staphylococcus aureus* biofilms. Lancet. 2001 Jan 6; 357(9249):40–1.
Available from: https://pubmed.ncbi.nlm.nih.gov/11197363/

23. Donlan RM, Costerton JW. Biofilms: Survival mechanisms of clinically relevant microorganisms. Vol. 15, Clinical Microbiology Reviews. ClinMicrobiol Rev; 2002. p. 167–93.
Available from: https://pubmed.ncbi.nlm.nih.gov/11932229/

24. Stefania De Marco, Miranda Piccioni, Rita Pagiotti, Donatella Pietrella, "Antibiofilm and Antioxidant Activity of Propolis and Bud Poplar Resins versus *Pseudomonas aeruginosa*", Evidence-Based Complementary and Alternative Medicine, vol. 2017, Article ID 5163575, 11 pages, 2017.
Available from: https://doi.org/10.1155/2017/5163575 25.

25. Bankova VS, de Castro SL, Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie. 2000 Jan 1 14]; 31(1):3–15.
Available from: http://www.edpsciences.org/10.1051/apido:2000102

26. Cushnie TPT, Lamb AJ. Recent advances in understanding the antibacterial properties of flavonoids. Vol. 38, International Journal of Antimicrobial Agents. Elsevier B.V.; 2011. p. 99–107.
Available from: https://pubmed.ncbi.nlm.nih.gov/21514796/

27. De Groot AC. Propolis: A review of properties, applications, chemical composition, contact allergy, and other adverse effects. Vol. 24, Dermatitis. Dermatitis; 2013. p. 263–82.
Available from: https://pubmed.ncbi.nlm.nih.gov/24201459/

28. Farnesi AP, Aquino-Ferreira R, De Jong D, Bastos JK, Soares AEE. Effects of stingless bee and honey bee propolis on four species of bacteria. Genet Mol Res. 2009; 8(2):635–40.
Available from: https://pubmed.ncbi.nlm.nih.gov/19554760/

29. Milena P. Popova, Vassya S. Bankova, Stefan Bogdanov, Iva Tsvetkova, Christo Naydenski, *et al*.. Chemical characteristics of poplar type propolis of different geographic origin. Apidologie, Springer Verlag, 2007, 38 (3), pp.306-306.
Available from: https://hal.archives-ouvertes.fr/hal-00892262/document

30. Machado BAS, Silva RPD, Barreto GDA, Costa SS, Da Silva DF, Brandão HN, et al. Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil. PLoS One. 2016 Jan 8; 11(1):e0145954.
Available from: //journals.plos.org/plosone/article?id=10.1371/journal.pone.0145954

31. Probst IS, Sforcin JM, Rall VLM, Fernandes AAH, Fernandes JA. Antimicrobial activity of propolis and essential oils and synergism between these natural products. J Venom Anim Toxins Incl Trop Dis. 2011; 17(2):159–67.
Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S1678\

32. Fernandes A, Balestrin EC, Betoni JEC, De Oliveira Orsi R, De Souza Da Cunha MDLR, Montelli AC. Propolis: Anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. Mem Inst Oswaldo Cruz. 2005 Aug 31; 100(5):563–6.

Available from:
http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-

33. Grecka K, Kuś PM, Okińczyc P, Worobo RW, Walkusz J, Szweda P. The anti-staphylococcal potential of ethanolic Polish propolis extracts. Molecules. 2019; 24(9). Available from: https://pubmed.ncbi.nlm.nih.gov/31058881/

34. Weinstein MP. M02 - Performance Standards for Antimicrobial Disk Susceptibility Tests, 13th Edition. 2018; 92.
Available from:
https://clsi.org/standards/products/microbiology/documents/m02/.

35. Kateete DP, Kimani CN, Katabazi FA, Okeng A, Okee MS, Nanteza A, *et al*. Identification of *Staphylococcus aureus*: DNase and Mannitol salt agar improve the efficiency of the tube coagulase test. Ann Clin. Microbiol. Antimicrob. 2010 Aug 13; 9(1):23. Available from:
http://ann-linmicrob.biomedcentral.com/articles/10.1186/1476-0711-9-23

36. Antimicrobial Susceptibility Testing Protocols. Antimicrobial Susceptibility Testing Protocols. CRC Press; 2007. Available from: https://www.taylorfrancis.com/books/antimicrobial-susceptibility-testing-protocols-richard-schwalbe-lynn-steele-moore-avery-goodwin/10.1201/9781420014495

37. Nostro A, Roccaro AS, Bisignano G, Marino A, Cannatelli MA, Pizzimenti FC, *et al*. Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Med Microbiol. 2007 Apr; 56(4):519–23. Available from: https://pubmed.ncbi.nlm.nih.gov/17374894/

38. Benaducci T, Sardi J de CO, Lourencetti NMS, Scorzoni L, Gullo FP, Rossi SA, et al. Virulence of Cryptococcus sp. Biofilms In Vitro and In Vivo using *Galleria mellonella* as an Alternative Model. Front Microbiol. 2016 Mar 9; 7(MAR):290.
Available from:
http://journal.frontiersin.org/Article/10.3389/fmicb.2016.00290/abstract

39. Stepanović S, Vuković D, Hola V, Bonaventura G DI, Djukić S, Ćirković I, *et al*. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS. 2007 Aug 1; 115(8):891–9.
Available from:
https://pubmed.ncbi.nlm.nih.gov/17696944/

40. Merino N, Toledo-Arana A, Vergara-Irigaray M, Valle J, Solano C, Calvo E, et al. Protein A-mediated multicellular behavior in *Staphylococcus aureus*. J Bacteriol. 2009 Feb; 191(3):832–43.
Available from:
/pmc/articles/PMC2632097/?report=abstract

41. Lopes LAA, dos Santos Rodrigues JB, Magnani M, de Souza EL, de Siqueira-Júnior JP. Inhibitory effects of flavonoids on biofilm formation by *Staphylococcus aureus* that overexpresses efflux protein genes. MicrobPathog. 2017 Jun 1; 107:193–7.
Available from:
https://pubmed.ncbi.nlm.nih.gov/28365326/

42. Sardi J de CO, Polaquini CR, Freires IA, Galvão LC de C, Lazarini JG, Torrezan GS, *et al*. Antibacterial activity of diacetylcurcumin against *Staphylococcus aureus* results in decreased biofilm and cellular adhesion. J Med Microbiol. 2017 Jun 1; 66(6):816–24.
Available from:
https://www.microbiologyresearch.org/content/journal/jmm/10.1099/jmm.0.000494

43. Adrião A, Vieira M, Fernandes I, Barbosa M, Sol M, Tenreiro RP, *et al*. Marked intra-strain variation in response of Listeria monocytogenes dairy isolates to acid or salt stress and the effect of acid or salt adaptation on adherence to abiotic surfaces. Int J Food Microbiol. 2008 Mar 31; 123(1–2):142–50.
Available from:
https://pubmed.ncbi.nlm.nih.gov/18258322/

44. Wang S, Wang C, Gao L, Cai H, Zhou Y, Yang Y, *et al*. Rutin Inhibits Streptococcus suis Biofilm Formation by Affecting CPS Biosynthesis. Front Pharmacol. 2017 Jun 16; 8(JUN):379.
Available from:
http://journal.frontiersin.org/article/10.3389/fphar.2017.00379/full

45. Guo Y, Song G, Sun M, Wang J, Wang Y. Prevalence and Therapies of Antibiotic-Resistance in *Staphylococcus aureus*. Vol. 10, Frontiers in Cellular and Infection Microbiology. Frontiers Media S.A.; 2020. p. 107.
Available from:
/pmc/articles/PMC7089872/?report=abstract

46. Rağbetli C, Parlak M, Bayram Y, Guducuoglu H, Ceylan N. Evaluation of Antimicrobial Resistance

in *Staphylococcus aureus* Isolates by Years. InterdiscipPerspect Infect Dis. 2016; 2016:4 pages. Available from: https://www.hindawi.com/journals/ipid/2016/9171395/

47. Bhatt, Cp&Karki, Bms&Baral, Bikram &Gautam, S & Shah, A & Chaudhary, A. (2014). Antibiotic susceptibility pattern of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* in a tertiary care hospital. Journal of Pathology of Nepal. 4. 10.3126/jpn.v4i7.10297.48. Available from: https://www.researchgate.net/publication/273679657_Antibiotic_susceptibility_pattern_of_*Staphylococcus_aureus*_and_methicillin resistant_*Staphylococcus_aureus*_in_a_tertiary_care_hospital/citation/download

48. Kot B, Wierzchowska K, Piechota M, Grużewska A. Antimicrobial Resistance Patterns in Methicillin-Resistant *Staphylococcus aureus* from Patients Hospitalized during 2015–2017 in Hospitals in Poland. Med PrincPract. 2020 Jan 1; 29(1):61–8. Available from: https://www.karger.com/Article/FullText/501788

49. Arjyal C, Kc J, Neupane S. Prevalence of Methicillin-Resistant *Staphylococcus aureus* in Shrines. Int J Microbiol. 2020; 2020:10 pages.

Available from: https://www.hindawi.com/journals/ijmicro/2020/7981648/

50. Ceballos S, Aspiroz C, Ruiz-Ripa L, Zarazaga M, Torres C. Antimicrobial resistance phenotypes and genotypes of methicillin-resistant *Staphylococcus aureus* CC398 isolates from Spanish hospitals. Int J Antimicrob Agents. 2020 Apri1; 55(4):105907. Available from: https://pubmed.ncbi.nlm.nih.gov/31991217

51. Abdel-Maksoud M, El-Shokry M, Ismail G, Hafez S, El-Kholy A, Attia E, *et al*. Methicillin-Resistant *Staphylococcus aureus* Recovered from Healthcare- and Community-Associated Infections in Egypt .Int J Bacteriol. 2016; 2016:1–5. Available from: https://pubmed.ncbi.nlm.nih.gov/27433480/

52. Abd El-Hamid MI, Y. El-Naenaeey E, M kandeel T, Hegazy WAH, Mosbah RA, Nassar MS, et al. Promising Antibiofilm Agents: Recent Breakthrough against Biofilm Producing Methicillin-Resistant *Staphylococcus aureus*. Antibiotics. 2020 Oct 3; 9(10):667. Available from: https://www.mdpi.com/2079-6382/9/10/667

53. Neopane P, Nepal HP, Shrestha R, Uehara O, Abiko Y. In vitro biofilm formation by *Staphylococcus aureus* isolated from wounds of hospital-admitted patients and their association with antimicrobial resistance. Int J Gen Med. 2018; 11:25–32. Available from: /pmc/articles/PMC5779313/

54. El-Guendouz S, Aazza S, Lyoussi B, Bankova V, Popova M, Neto L, *et al*. Moroccan Propolis: A Natural Antioxidant, Antibacterial, and Antibiofilm against *Staphylococcus aureus* with No Induction of Resistance after Continuous Exposure. Evidence-based Complement Altern Med. 2018; 2018 (November):19 pages. Available from: https://www.hindawi.com/journals/ecam/2018/9759240/

55. Meto A, Colombari B, Meto A, Boaretto G, Pinetti D, Marchetti L, *et al*. Propolis Affects Pseudomonas aeruginosa Growth, Biofilm Formation, eDNA Release and Phenazine Production: Potential Involvement of Polyphenols. Microorganisms. 2020 Feb 12; 8(2). Available from: http://www.ncbi.nlm.nih.gov/pubmed/32059431

56. Laranjo M, Andrade N, Queiroga C. Antibiofilm activity of propolis extracts. In: Enrique Torres-Hergueta AM-V, editor. Understanding microbial pathogens : current knowledge and educational Edited by. 1st Editio. formatexMicrobiology Series N°7; 2018. p. 1–8. Available from: https://dspace.uevora.pt/rdpc/handle/10174/23826

57. Pobiega K, Kraśniewska K, Derewiaka D, Gniewosz M. Comparison of the antimicrobial activity of propolis extracts obtained by means of various extraction methods. J Food SciTechnol. 2019 Dec 1; 56(12):5386–95. Available from: https://doi.org/10.1007/s13197-019-04009-9

58. Wojtyczka RD, Dziedzic A, Idzik D, Kepa M, Kubina R, Kabała-Dzik A, *et al*. Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs. Molecules. 2013 Aug; 18(8):9623–40. Available from: /pmc/articles/PMC6269688/?report=abstract

59. Seidel V, Peyfoon E, Watson DG, Fearnley J. Comparative study of the antibacterial activity of propolis from different geographical and climatic zones. Phyther Res. 2008 Sep 1; 22(9):1256–63. Available from: http://doi.wiley.com/10.1002/ptr.2480

60. Sánchez-Maldonado AF, Schieber A, Gänzle MG. Structure-function relationships of the antibacterial activity of phenolic acids and their metabolism by lactic acid bacteria. J ApplMicrobiol. 2011 Nov 1; 111(5):1176–84. Available from: http://doi.wiley.com/10.1111/j.1365-2672.2011.05141.x

61. Bryan J, Redden P, Traba C. The mechanism of action of Russian propolis ethanol extracts against two antibiotic-resistant biofilm-forming bacteria. Lett ApplMicrobiol. 2016 Feb 1; 62(2):192–8. Available from: http://doi.wiley.com/10.1111/lam.12532

62. Burdock GA. Review of the biological properties and toxicity of bee propolis (propolis). Food ChemToxicol. 1998 Apr 6; 36(4):347–63. Available from: https://europepmc.org/article/med/9651052

63. Dogan N. Antibiofilm Effect of Two Propolis Samples from Turkey. J ApplBiol Sci. 2014; 8(2):27–31. Available from: http://www.jabsonline.org/index.php/jabs/article/view/389

64. Baddour LM, Christensen GD, Hester MG, Bisno AL. Production of experimental endocarditis by coagulase-negative staphylococci: Variability in species virulence. J Infect Dis. 1984; 150(5):721–7. Available from: https://pubmed.ncbi.nlm.nih.gov/6491379/

65. Stan T, Măruţescu L, Chifiriuc MC, Lazăr V. Anti-Pathogenic Effect of Propolis Extracts from Different Romanian Regions on *Staphylococcus* Sp. Clinical Strains. Vol. 21(1): 11166-11175, Romanian Biotechnological Letters. 2016. Available from:https://e-repository.org/rbl/vol.21/iss.1/11.pdf

66. Tiza N uni, Thato M, Raymond D, Jeremy K, Burtram CF. Additive antibacterial activity of naringenin and antibiotic combinations against multidrug resistant *Staphylococcus aureus*. African J Microbiol Res. 2015 Jun 10; 9(23):1513–8. Available from: https://repository.uwc.ac.za/handle/10566/2294

67. Wojtyczka RD, Dziedzic A, Idzik D, Kepa M, Kubina R, Kabała-Dzik A, *et al*. Susceptibility of *Staphylococcus aureus* clinical isolates to propolis extract alone or in combination with antimicrobial drugs. Molecules. 2013 Aug; 18(8):9623–40. Available from: https://pubmed.ncbi.nlm.nih.gov/23941882/

68. Esimone CO, Iroha IR, Ibezim EC, Okeh CO, Okpana EM. In vitro evaluation of the interaction between tea extracts and penicillin G against *Staphylococcus aureus*. African J Biotechnol. 2006 Jun 2 [cited 2021 Feb 26]; 5(11):1082–6. Available from: http://www.academicjournals.org/AJB

69. Fernandes A, Balestrin EC, Betoni JEC, De Oliveira Orsi R, De Souza Da Cunha MDLR, Montelli AC. Propolis: Anti-*Staphylococcus aureus* activity and synergism with antimicrobial drugs. Mem Inst Oswaldo Cruz. 2005 Aug 31; 100(5):563–6. Available from: https://pubmed.ncbi.nlm.nih.gov/16184236/

70. Fernandes A, Balestrin EC, Betoni JEC, De Oliveira Orsi R, De Souza Da Cunha MDLR, Montelli AC. The RNA-polymerase inhibition by propolis compounds was verified. Mem Inst Oswaldo Cruz. 2005 Aug 31; 100(5):563–6. Available from: http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762005000500018&lng=en&nrm=iso&tlng=en

71. Wojtyczka RD, Kępa M, Idzik D, Kubina R, Kabała-Dzik A, Dziedzic A, *et al*. In vitro antimicrobial activity of ethanolic extract of polish propolis against biofilm forming *Staphylococcus epidermidis* strains. Evidence-based Complement Altern Med. 2013; 2013: 590703. Available from: https://www.hindawi.com/journals/ecam/2013/590703/

72. Shrivastava R, Rai VK, Kumar A, Sinha S, Tripathi P, Gupta K, *et al*. An in vitro Comparison of Endodontic Medicaments Propolis and Calcium Hydroxide alone and in Combination with Ciprofloxacin and Moxifloxacin against *Enterococcus Faecalis*. J Contemp Dent Pract . 2015 May 1; 16 (5):394–9. Available from: https://pubmed.ncbi.nlm.nih.gov/26162260/

73. Boisard S, Le Ray AM, Landreau A, Kempf M, Cassisa V, Flurin C, *et al*. Antifungal and antibacterial metabolites from a French poplar type propolis. Evidence-based Complement Altern Med. 2015; 2015: 319240. Available from: https://www.hindawi.com/journals/ecam/2015/319240/