

Original Research Article

Antimicrobial effect of Propolis From different Geographic Origins in Lebanon

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ABSTRACT

Keywords

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The efficacy of the ethanolic extract of propolis (EEP) from different geographic origins of Lebanon (Saffareh, Klayleh and Bsaba) was assayed for antimicrobial properties against multi-drug resistant bacteria (MDR) and yeast namely: ESBL *Klebsiella pneumoniae*, methicillin resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* using disk diffusion method. The average inhibition zone diameters for different propolis extracts in the order of Saffareh>Klayleh>Bsaba 25, 20 and 15 mm respectively against MRSA. The Saffareh and Klayleh types showed average inhibition zone diameters of 25 and 18 mm respectively against *C. albicans* while the organism was resistant to Bsaba's propolis. On the other hand, ESBL *Klebsiella pneumoniae* was resistant to Klayleh and Bsaba types and showed an inhibition zone diameter of 17 mm against Saffareh's propolis. Saffareh had a significant antimicrobial effect ($P \leq 0.05$) on microorganisms under study. Saffareh's EEP had bacteriostatic effect on both ESBL *Klebsiella pneumoniae* and MRSA, with an MIC index > 4 while it had a fungicidal effect on *Candida albicans* with an MIC index < 4 . Upon treatment of ESBL- *Klebsiella pneumoniae* and MRSA with Saffareh's EEP the extract was precipitated and adsorbed on the cell surface leading to growth inhibition. In the case of *Candida albicans* breaking down of the cell wall, degradation of cell organelles, and changes in cell permeability have been noted to occur as the most significant cellular alterations after exposure to the selected EEP.

Introduction

Infectious diseases have been reaping the lives of thousands throughout history. They are the second leading cause of death despite the introduction of many antimicrobial agents in the 20th century (Monzote et al., 2012). Over the past decade, the increase in

antibiotic resistance has generated considerable medical and economic problems. Generations of antimicrobial resistant strains have spread throughout the biosphere as a result of human's exploitation to antimicrobials (Davies & Davies, 2010)

in addition to the remarkable genetic plasticity of the microorganism (Sibanda & Okoh, 2007). In some situations resistance can be achieved without any genetic alteration. This is called phenotypic resistance (Corona & Martinez, 2013). In a time where there is a race in academic and corporate laboratories to overcome the ever growing antimicrobial resistance, the discovery of active components in ancient remedies is much needed to enrich the arsenal of antimicrobials used in medicine (Aminov, 2010).

In this context, the following study will be shedding light on a widely used natural extract in folk medicine namely: propolis and its efficacy against some multi-drug resistant microorganisms. Propolis is a resinous substance collected by *Apis mellifera* bee from plant juices and is used to disinfect and seal openings in hives (Mirzoeva et al., 1997). It is a sticky dark-colored material also known as (bee glue) once collected it is enriched with salivary and enzymatic secretions (Possamai et al., 2012).

Materials and Methods

Microorganisms

Two different bacterial isolates were used throughout the current work, one gram negative extended spectrum β -lactamase *Klebsiella* (ESBL-*Klebsiella pneumoniae*) and the other is a gram positive methicillin resistant *Staphylococcus aureus* (MRSA) and one yeast strain namely: *Candida albicans*.

All bacterial strains used throughout the present investigation were maintained on nutrient agar slants while fungal isolates were maintained on Sabouraud dextrose agar. The cultures were stored at 4°C with regular transfer at monthly intervals.

Raw materials

Three types of propolis were collected from different geographic areas in Lebanon namely: Al Klayleh-Tyre-South of Lebanon, Bsaba-Chouf and Saffareh-Jezzine throughout April, August and September respectively. The samples were hand collected and kept in sterile plastic containers in the dark before its use.

Extraction of Propolis

Propolis was cut into small pieces and extracted with 70% ethanol (1:10 w/v) for 24 hours under shaking conditions at 25°C. The extracts were filtered using 0.2 μ m bacterial filter to ensure their sterility. Sterile extracts were evaporated to dryness by means of a rotary evaporator at 40°C. The pure extract was stored at 4°C in amber vials in the dark to prevent photoisomerization (Kujumgiev et al., 1999).

Screening for antimicrobial activity

An inoculum 1.5 \times 10⁸ CFU/ml equivalent to 0.5 McFarland was prepared, and 25 μ l were swabbed over the surface of Müller-Hinton agar plate. Antimicrobial activity was carried out using a disc-diffusion method (Murray et al., 2005). After incubation for 24 hrs. at 37°C, all plates were examined for zones of inhibition. The diameters of the zones were measured in millimeters (Mahon et al., 1998).

Determination of minimal inhibitory concentration (MIC)

The most promising extract was tested for MIC by dilution method. This test was performed in sterile 96-well micro-titer plates (Ellof, 1998). The cultures were diluted in Müller-Hinton broth at a density adjusted to a 0.5 McFarland turbidity. The

final inoculum concentration was 1.5×10^8 CFU/ml of bacterial cultures. The wells were filled with 80 μ l of sterile broth, 20 μ l sterile tween 80 and 100 μ l of the extract were added to the wells by serial two fold dilution. Each well was inoculated with 100 μ l of 0.5 McFarland standard bacterial suspension so that each well got 1.5×10^8 CFU/ml. The 96- well micro-titer plates were covered, placed in plastic bags and incubated at 37°C for 24 hours. The MIC was the lowest concentration of extract that resulted in a clear well

Determination of minimal bactericidal concentration (MBC) and minimal fungicidal concentration (MFC)

The highest extract dilution not exhibiting bacterial growth was taken as the MIC. After determining the MICs, 20 μ l aliquots from each well were plated onto Müller-Hinton Agar and incubated at 37°C for 18 hrs. (Rota et al., 2008). Following incubation, the highest dilution not exhibiting bacterial growth was recorded as the minimal bactericidal concentration (MBC). The minimal fungicidal concentration MFC was determined by inoculating the contents from the MIC plates onto SDA plates. The lowest concentration of the extract that was able to kill the microorganisms was considered as the minimum fungicidal concentration (Brown, 2007).

MIC index

The MIC index (MBC or MFC / MIC) was calculated for the antimicrobial agent to determine whether the agent is bactericidal or fungicidal (MBC or MFC/MIC <4) bacteriostatic or fungistatic (MBC or MFC / MIC >4) against the growth of fungi or bacteria (Kone et al., 2004 and Chattopadhyay et al., 2007). The range of

MIC index values greater than 4 and less than 32 are considered to be bacteriostatic or fungistatic (Cutler et al., 1994).

Determination of bacterial time-kill curve

The selected extract that showed a bactericidal or fungicidal effect against the most promising bacterium under test was used, and time-kill curve was plotted. A 16-hrs culture was harvested by centrifugation. The suspension was adjusted using the McFarland standard and was then further diluted in saline 0.85% to achieve approximately 1.5×10^8 CFU/ml. The selected filter sterilized Lebanese propolis extract was added to aliquots of 1ml Müller Hinton broth in amounts that would achieve the bactericidal concentrations for the selected bacteria followed by the addition of 1ml of the inoculum. Further samples were taken from each tube to monitor growth by measuring the absorbance (optical density) at 600 nm wavelength at time intervals (0, 2, 4, 6,8,12, and 24 hrs.) (Yin et al., 2002).

Screening for the antimicrobial activity of the promising propolis against ESBL-*Klebsiella pneumoniae* and MRSA versus commonly used antibiotics

Nine different antibiotics were selected for the present study. One was used for both gram-negative and gram-positive bacteria, namely: ceftriaxone (CRO). Two antibiotic discs specific for gram-negative bacteria were used: aztreonam (ATM) and cefotaxime (CTX). The discs used for gram-positive bacteria were ceftioxin (FOX), oxacillin, and vancomycin (VA) (Abd-El Aal et al., 2007). Three antibiotics that interfere with protein synthesis were also used namely: clindamycin (DA), gentamicin (CN) and tetracycline (TE) (Fernandes Junior et al., 2005). 1.5×10^8 CFU/ml equivalent to 0.5 McFarland standard

bacterial suspension was swabbed on the top of the solidified Muller-Hinton agar plates and allowed to dry for 10 min. The antibiotics were loaded with propolis sterile extract and placed on the surface of the inoculated agar by pressing slightly. The plates were placed at 4°C for 1 hr. for compound diffusion and then incubated for 24 hrs. at 37°C (Abd El Aal et al., 2007), then the zones of inhibition were recorded in millimeters and the experiment was repeated three times.

Screening for the antifungal activity of the promising propolis against *Candida albicans*, versus commonly used fungicides

The antifungal activity of promising propolis and one common fungicide (Nystatin) was evaluated against the cells of *Candida albicans* using disc-diffusion method. An inoculum of fungal suspension (3×10^4 - 3×10^5 CFU/ml) equivalent to 0.5 McFarland was prepared, and 25µl were swabbed over the surface of Sabouraud-Dextrose agar plate. The discs combined with propolis were placed on the inoculated agar by pressing slightly. Plates were placed at 4°C for 1 hr. for compound diffusion and then incubated for 24 hrs. at 37°C (Abd el Aal et al., 2007). Zones of inhibition were recorded in millimeters and the experiment was repeated three times.

Chemical analysis (Qualitative method) (Sibi et al., 2013)

Test for Flavonoids (Ammonia test)

One (1) ml of the extract was taken in the test tube and ammonia solution was added (1:5 V/V) followed by the addition of conc. sulphuric acid. Appearance of yellow color and its disappearance on standing indicates the positive test for flavonoids.

Test for Glycosides (Keller Kiliani test)

Five (5) ml of each extract was added to 2 ml of glacial acetic acid, followed by the addition of few drops of ferric chloride solution and 1 ml of conc. Sulphuric acid. Formation of brown ring at interface confirms the presence of glycosides.

Test for Phenols (Ferric chloride test)

Half (0.5) ml of the extract was added with few drops of neutral ferric chloride (0.5%) solution. Formation of dark green color indicates the presence of the phenolic compounds.

Test for Saponins (Froth test)

One (1) ml of the extract was taken in a test tube followed by the addition of 2ml distilled water. The test tube was then kept in boiling water bath for boiling and was shaken vigorously. Existence of froth formation during warming confirms the presence of saponins

Test for Steroids (Libermann - Burchard's test)

Two (2) ml of acetic anhydride was added to 0.5ml of the extract and then added 2 ml of conc. sulphuric acid slowly along the sides of the test tube. Change of colour from violet to blue or green indicates the presence of steroids.

Test for Tannins (Ferric chloride test)

One (1) ml of the extract was added with 5 ml of distilled water and kept for boiling in hot water bath. After boiling, the samples were cooled down and 0.1% ferric chloride solution was added to each sample. Appearance of brownish green or blue black coloration confirms the presence of tannins.

Test for Terpenoids (Salkowski test)

Five (5) ml of extract was taken in a test tube then; 2 ml of chloroform was added to it followed by the addition of 3ml of conc. sulfuric acid. Formation of reddish brown layer at the junction of two solutions confirms the presence of terpenoids.

Transmission electron Microscopy (TEM)

On the basis of MIC, MBC, MFC values and time-kill curve data, methicillin – resistant *Staphylococcus aureus*, was treated with Lebanese propolis extract (6.25mg/ml), *Klebsiella pneumonia* was treated with Lebanese propolis extract (12.5mg/ml), and *Candida albicans* was treated with Lebanese propolis extract (12.5 mg/ml). Freshly taken samples were fixed using a universal electron microscope fixative as described by McDowell and Trump (1967). Series dehydration steps were followed using ethyl alcohol and propylene oxide. The samples was then embedded in labeled beam capsules and polymerized. Thin sections of cells exposed to oils were cut using LKB 2209-180 ultra-microtome and stained with a saturated solution of uranyl acetate for half hour and lead acetate for 2 min (McDowell and Trump, 1967). The procedure was applied to extract-exposed cells. Electron Micrographs were taken using a Transmission Electron Microscope (JEM-100 CX Joel), at the Electron Microscope Unit, Faculty of Science, Alexandria University, Egypt.

Statistical analysis

Quantitative traits such as diameters of inhibition zones were analyzed using analysis of variance (ANOVA) and their means were separated using Duncan's Multiple Range Test (DMRT) ($\alpha=0.05$) performed using SPSS 21 for Windows

(Statistical Product and Services Solutions, Inc, Chicago, IL, USA).

Results and Discussion

Data presented in table 1 showed average inhibition zone diameters for different propolis extracts. All extracts exhibited antimicrobial activity with varying potential. Analysis of variance indicated a significant effect exerted by both Bsaba and Saffareh however Saffareh extract was the most promising one that showed a significant antibacterial effect against all microorganisms under study with 25, 17 and 25 mm average inhibition zones against MRSA, ESBL- *Klebsiella pneumoniae* and *Candida albicans* respectively. This comes in agreement with Kilic et al. (2005) who assessed the antimicrobial activity of three Turkish propolis samples (one from Mamak and two from Kemaliye). They found that propolis can be used against MRSA infections as an alternative therapy. Darwish et al. (2010) revealed that different types of Jordanian propolis had effect against MRSA with average inhibition zones ranging from 14-17 mm. Salomãõ et al. (2007) assessed the antimicrobial activity of eleven Brazilian samples to show that Brazilian propolis present a higher activity against Gram positive bacteria than Gram-negative ones as observed in the case of *K. pneumoniae*. Al Waili et al. (2012) assessed the antimicrobial activity of Saudi and Egyptian propolis to find that both inhibited the growth of *Candida albicans*. The effect of propolis against gram positive bacteria and yeasts is more prominent than against gram negative bacteria (Boyanova et al.,2005).

MIC determination by broth dilution methods is mainly used for determining the potency of EEP. From the disk-diffusion method, the most promising extract (Saffareh) was selected on the basis of

statistical analysis where it was highly significant compared to other extracts. Saffareh type had MIC values of 6.25, 12.5, and 12.5 mg/ml against MRSA, ESBL-*Klebsiella pneumoniae*, and *Candida albicans*, respectively (table 2). On the contrary, Kilic et al. (2005) reported a higher MIC concentration against MRSA ranging from 7.8-31.2 µg/ml. The tested EEP in the present study showed equal efficacy towards gram negative ESBL-*Klebsiella pneumoniae*, and *Candida albicans* with MIC value of 12.5 mg/ml. Sforcin et al. (2001) compared propolis collected during the four seasons by its *in vitro* antimicrobial activity against yeast pathogens isolated from human infections. He concluded that *Candida tropicalis* and *Candida albicans* were susceptible to low concentrations of propolis, the latter showing a higher susceptibility. On the other hand, Monzote et al. (2012) reported that Cuban propolis showed no activity against *Candida albicans* even at the highest concentration (64µg/ml).

MBC determination was based on MIC, where the most promising EEP under test (Saffareh) showed a bacteriostatic effect against MDR gram positive MRSA and gram negative ESBL-*Klebsiella pneumoniae* with MBC values of 50 mg/ml and 100 mg/ml respectively and an MIC index >4 (table 2 and plate 1). In accordance with the data obtained by Drago et al. (2000) who proved that propolis showed a bacteriostatic activity against *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Candida albicans* and can be bactericidal at high concentrations. On the contrary, LU et al. (2005) showed that Taiwan propolis has a bactericidal effect rather than bacteriostatic mode of action against *S. aureus*. Moreover,

Saffareh EEP had a fungicidal mode of action against *Candida albicans* with an MBC of 25 mg/ml and an MIC index <4. These results are in accordance with D'auria et al. (2003) results who reported that propolis significantly inhibited the *C. albicans* strains tested, showing a rapid (between 30 seconds and 15 minutes), dose-dependent cytotoxic activity and an inhibitory effect at a concentration of 0.22 mg/ml. Propolis produced by *Apis mellifera* has both bacteriostatic and bactericidal activity when tested *in vitro* (Boyanova et al., 2005; Lu et al., 2005; and Mirzoeva et al., 1997).

The Saffareh EEP used in the present study appears particularly effective with respect to the time needed to exert lethal effect on the microbial growth. Time –Kill curve analysis showed that MRSA treated with the selected propolis were susceptible more rapidly than ESBL-*Klebsiella pneumoniae* and *Candida albicans*. The MBC of EEP was successful in killing viable cells within 24, 30 and 36 hrs against MRSA, ESBL- *Klebsiella pneumoniae* and *Candida albicans* respectively. Concerning the negative control, MRSA, ESBL- *Klebsiella pneumoniae* and *Candida albicans* showed an increase in absorbance with time, and it was noticed that the logarithmic phase of microorganisms under test extended till the end of 24 hours, and then the bacterial growth entered the decline phase (figures 1, 2, and 3).

The combined action of two antimicrobial agents may be synergistic when the combined action is significantly greater than the sum of both effects or it may be antagonistic when the combined action is less than that of the more effective agent when used alone. In other cases the combined action is not greater than the sum of both and that is called indifference.

Addition is when the combined action is equivalent to the sum of the actions of each drug when used alone. Screening experiments revealed that there was a significant increase in the inhibition zone diameters against MRSA when mixed with extract compared to the inhibition zones of antibiotics when used alone with 30, 27, and 25 mm with FOX, VA, and CRO respectively when mixed with extract compared to the inhibition zones of antibiotics when used alone 13, 20 and 15 mm. The increase in the inhibition zone was only of statistical significance and not sufficient to be referred to as either synergistic or additive (table 3). In the case of ESBL-*Klebsiella pneumoniae* only (ATM-30µg) showed a significant increase in the inhibition zone of antibiotic mixed with Saffareh extract (20mm) compared to the resistance towards the antibiotic when used alone (10mm). No record of synergistic effect was investigated while antagonism was documented in the case of (CRO-30µg) (DA-2µg), (CN-10µg) and (TE-30µg) (Table4). The antifungal agent used for *Candida albicans* namely: Nystatin (NS-100u) showed no significant effect with combination since no significant increase in the inhibition zone diameters of the antifungal mixed with Saffareh extract (14mm) was detected compared with inhibition zone of antifungal alone (17mm)

(Table 5). On the contrary, Stepanovic et al. (2003) documented synergism was between EEP and different classes of synthetic antimicrobial against MDR *S.aureus*, *K. pneumoniae* and *C. albicans*. Also Fernandes et al. (2005) investigated a synergistic effect of EEP and five drugs chloramphenicol 30 mg, gentamicin 10 mg, netlimicin 30 mg, tetracycline 30mg and clindamycin against 25 strains of *S. aureus*.

Qualitative analysis of the most promising EEP (Saffareh) showed that it contained alkaloids, flavonoids, phenols, saponins, steroids, tanins, and terpenoids. Data of the present investigation are in accordance with what is commonly found in propolis from temperate regions (Bankova et al., 1999). It also agrees with the expected compounds to be obtained upon the use of ethanol as a solvent (Fokt et al., 2010). The chemical composition of propolis is also highly variable and in distinct geographic regions the antibacterial compounds in bee glue are different. For example, flavonoids and cinnamic acid derivatives are found in European samples, while diterpenic acids and prenylated coumaric acids are found in Brazilian ones. For this reason, the complete characterization of antibacterial activity of propolis has to involve qualitative and quantitative analysis (Popova et al., 2005).

Table.1 The antibacterial effect of different types of Lebanese propolis against the growth of multi-drug resistant microorganisms

Microorganism under study	Average Inhibition zone (mm)		
	Propolis types		
	Klayleh	Saffareh	Bsaba
MRSA	20.00 ^b	25.00 ^a	15.00 ^a
ESBL <i>Klebsiella pneumoniae</i>	*R	17.00 ^a	R
<i>Candida albicans</i>	18.00 ^b	25.00 ^a	R

*R: resistant colonies

Means followed by the same letter in the same column are not significantly different at p≤ 0.05

Table.2 Minimum inhibitory concentration (MIC) and minimum bactericidal /fungicidal concentration (MBC&MFC) of selected propolis against ESBL-Klebsiella pneumoniae, MRSA, and Candida albicans

Propolis sample used	Minimum Inhibitory Concentration (mg/ml)			Minimum bactericidal/Fungicidal concentration (mg/ml)		
	MRSA	ESBL- <i>Klebsiella pneumoniae</i>	<i>Candida albicans</i>	MRSA	ESBL- <i>Klebsiella pneumoniae</i>	<i>Candida albicans</i>
Saffareh	6.25	12.50	12.50	50.00	100.00	25

Table.3 The combined effect of extract and antibiotics against MRSA

Antibiotic used	Average Inhibition zone (mm)			Combined action
	Antibiotic alone	Extract alone	Antibiotic + Saffareh	
CRO	13.00 ^g	25.00 ^d	25.00 ^{cd}	Indifference
FOX	12.00 ^g	25.00	30.00 ^a	Indifference
OX	12.00 ^{gh}	25.00	20.00 ^e	Antagonism
VA	19.00 ^f	25.00	27.00 ^{bc}	Indifference
DA	29.00 ^{ab}	25.00	21.00 ^e	Antagonism
CN	20.00 ^e	25.00	21.40 ^e	Antagonism
TE	30.00 ^a	25.00	27.00 ^{bc}	Indifference

Means followed by the same letter in the same column are not significantly different at $p \leq 0.05$

Table.4 The combined effect of extract and antibiotics against ESBL-Klebsiella pneumonia

Antibiotic used	Average Inhibition zone (mm)			Combined action
	Antibiotic alone	Extract alone	Antibiotic + saffareh	
CRO	*R	17.50 ^b	R	Antagonism
ATM	10.00 ^e	17.50	20.00 ^a	Indifference
CTX	10.00 ^e	17.50	11.00 ^e	Antagonism
DA	R	17.50	R	Antagonism
CN	R	17.50	10.20 ^e	Antagonism
TE	10.00 ^e	17.50	13.30 ^c	Antagonism

Means followed by the same letter in the same column are not significantly different at $p \leq 0.05$ R: resistant colonies

Table.5 The combined effect of extract and antifungal against Candida albicans

Antifungal used	Inhibition zone (mm)			Combined action
	Antibiotic alone	Extract alone	Antibiotic + saffareh	
NS	17.00 ^b	25.00 ^a	14.00 ^c	Antagonism

Means followed by the same letter in the same column are not significantly different at $p \leq 0.05$

Figure.1 Time kill curve of MRSA treated with EEP

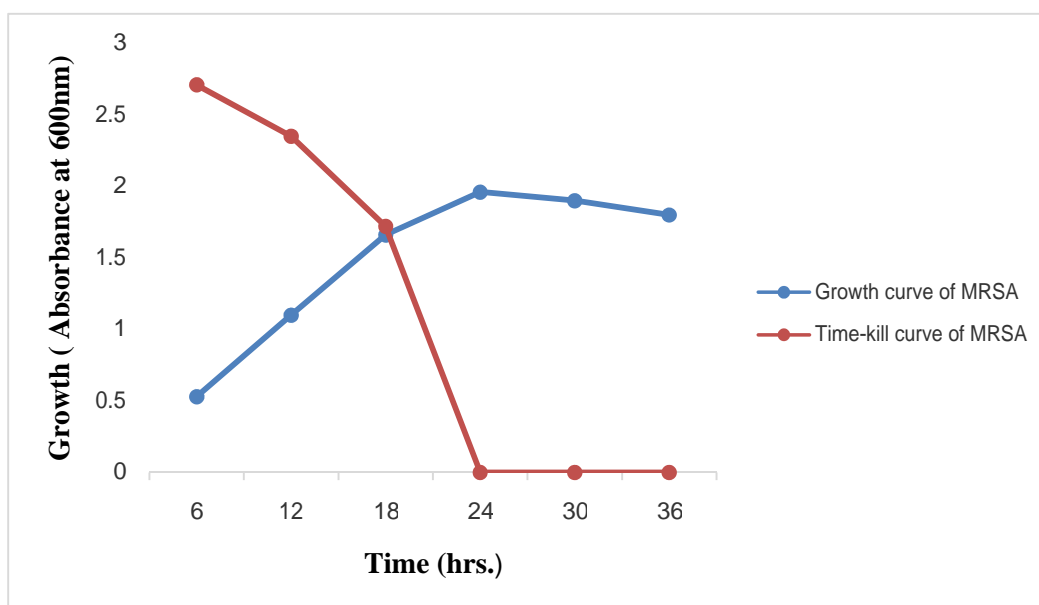


Plate.1 Minimum bactericidal /fungicidal concentrations of propolis against MRSA (a), ESBL-Klebsiella pneumoniae (b), and *Candida albicans* (c)

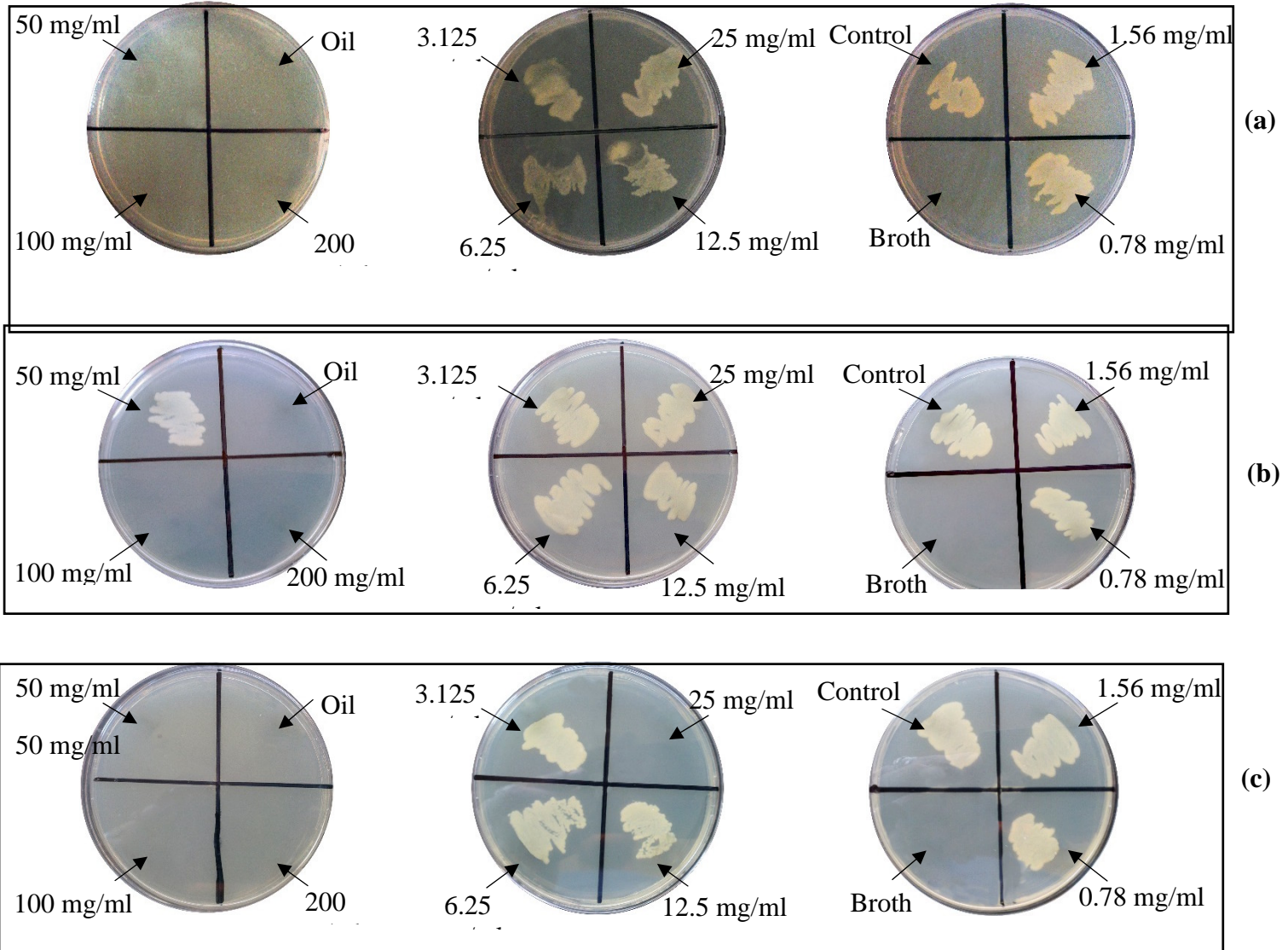


Figure.2 Time kill curve of ESBL-Klebsiella pneumonia treated with EEP

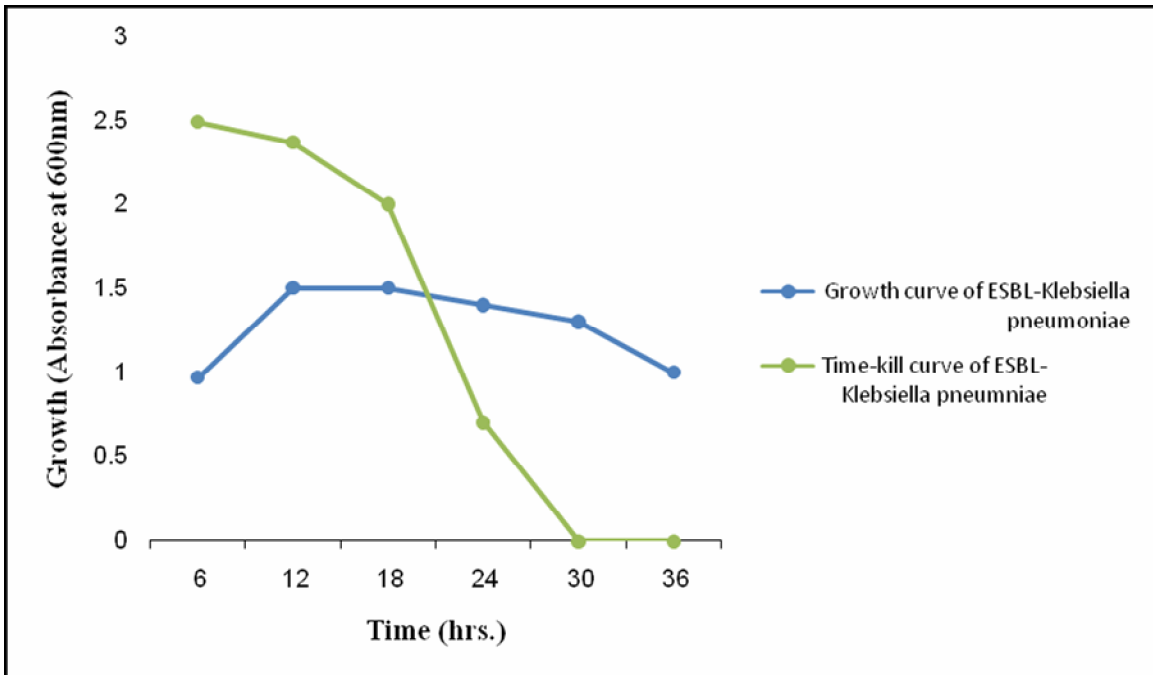


Figure.3 Time-kill curve of Candida albicans treated with EEP

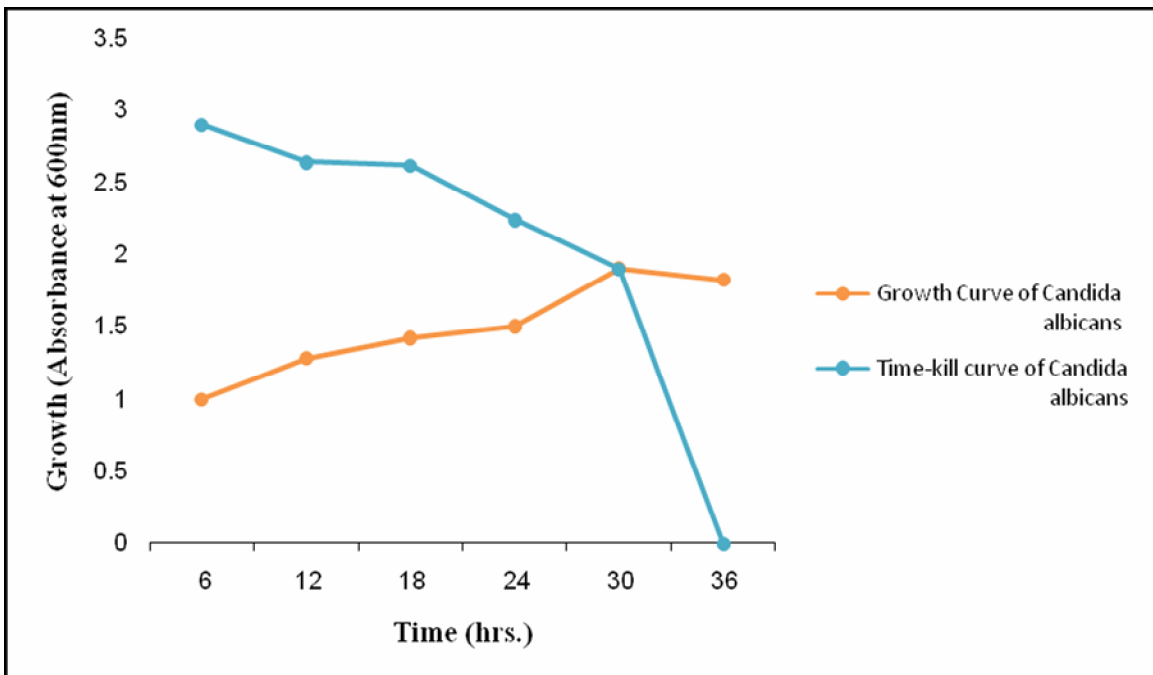


Figure.4 TEM micrograph of ESBL-Klebsiella pneumoniae with EEP treatment (12.5 mg/ml)



Figure.5 TEM micrograph of MRSA with EEP treatment (6.25 mg/ml)

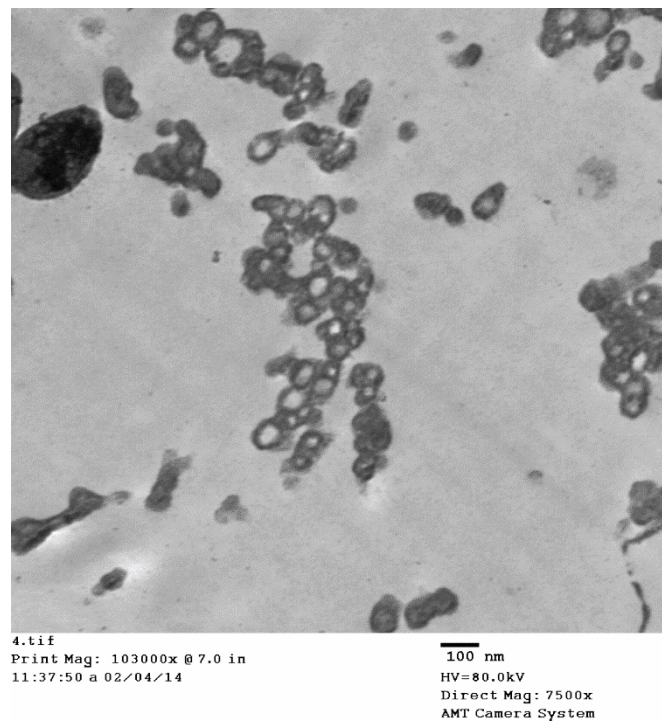
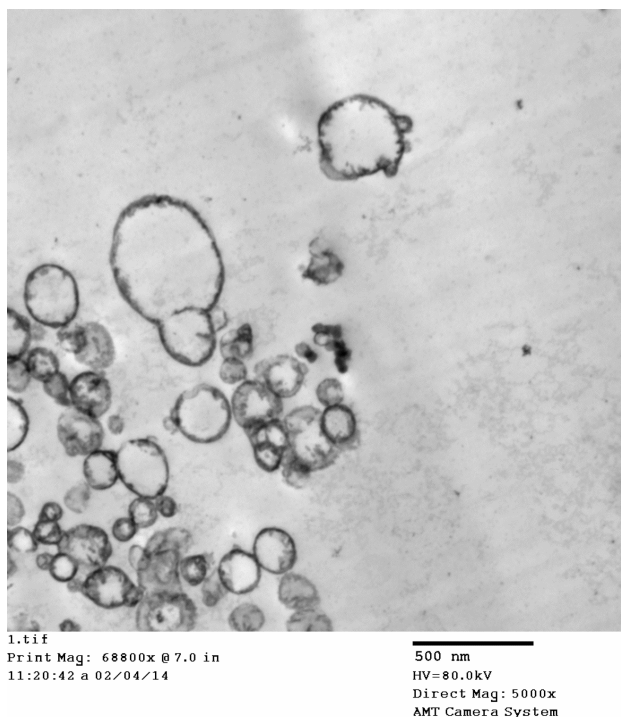


Figure.6 TEM micrograph of *Candida albicans* with EEP treatment (12.5 mg/ml)



The cytotoxic effect of Saffareh extract against the tested pathogens was carried out using transmission electron microscopy. Bacterial growth was inhibited due to the adsorption of the extract on the cell wall surface (figures 4 and 5). The cytotoxic effect of the propolis against *Candida albicans* revealed a breakdown of the cell wall, degradation of cell organelles, and changes in cell permeability have been noted (figure 6). The mechanism of propolis antimicrobial activity seems to be related to some of its components. The potent bacteriostatic and bactericidal effects of propolis can be associated with their combined action manifested by inhibition of protein synthesis and bacterial growth by preventing cell division. As for the effect of propolis on yeast strains this could be due to the induced expression of apoptotic and necrotic factors by propolis alongside the

formation of reactive oxygen species (Castro et al., 2011).

The Lebanese propolis ethanolic extract particularly Saffareh type showed antimicrobial effect against human pathogens which suggests its possible application as a potential antimicrobial agent in the pharmaceutical pipelines for the treatment of infectious diseases caused by MDRB and *Candida albicans*. Further investigations must be done for the cytotoxic effect of the extract to be safely used in medicine

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