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Antibacterial and Anti Biofilm activity of *Eucalyptus* Plant Extract Spp

Noor A. Al-taai [1], Nagham M. Al-Gburi [2], Nuha K. Khalil [3]
[1],[2],[3] Unite of Zoonotic Diseases, College of Veterinary Medicine, University of Baghdad, Iraq
Corresponding author: Nuha K. Khalil noha_k22@covm.uobaghdad.edu.iq

Abstract

The biofilm membrane is one of the basics for the causing of pathological infections of most types of bacteria, so destroying or stopping the formation of this membrane is the first necessary step to control the spread of bacterial diseases.

The aim of this research is to study the effect of the *Eucalyptus* plant as an antibacterial and an inhibitor of biofilm formation against a number of bacteria types taken from the zoonosis research unit (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteuse, Citrobacter, Listeria* and *Enterococcuse*) by using method of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), staining with crystal violet and measuring the optical density (OD) by ELISA , the results revealed that zone of growth inhibition of selected bacteria by *Eucalyptus* extract was ranged from 20-35 mm at concentration 20 mg/ml, minimum inhibitory concentration (MIC) ranged from (0.6 -20) mg/ml, the extract of *Eucalyptus* was highly effective against *Pseudomonase* at 0.6 mg/ml and at 20 mg/ml against *Citrobacter*, while the minimum bactericidal concentration (MBC) of *Eucalyptus* was the lowest at 0.3 mg/ml against *Pseudomonas* and *Enterococcus* and the highest at 10 mg/ml against *Staphylococcus* and *Proteus* and no effect against *E. coli* and *Citrobacter*.

Results of *Eucalyptus* extract biofilm inhibition by measuring the optical density (OD) were (28.3, 25 %) on *Pseudomonas* and *Staphylococcus aureus* at zero time and (26.6, 25 %) after 24 hr from incubation respectively.

It was concluded from result above that the antibacterial effect of *Eucalyptus* extract depending on its concentration and it was active in high concentration.

Key words: *Eucalyptus*, biofilm, antimicrobial, MBC, MIC, optical density.

Introduction

Eucalyptus is one of the most important and widely used genera and its species well known for as medicinal plant because of their therapeutic effects. The pharmacological studies revealed that *Eucalypts* possessed gastrointestinal therapy, antiinflammatory, analgesic, antidiabetic, antioxidant, anticancer, antimicrobial, antiparasitic, insecticidal, repellent, oral and dental, dermatological, nasal and many other effects (2).

Biofilms are complex sessile microbial colonies that form on both living and nonliving surfaces (3). Due to limited penetration into the biofilm matrix, the extracellular polymeric material, and increasing resistance to antibacterial drugs, biofilms formed on surfaces are up to a thousand times more resistant to antibiotic therapy than planktonic counterparts (4). As a result of the emergence of multidrug resistant pathogens, a search for novel therapeutic alternatives among plants and essential oils with significant antibacterial capabilities became necessary (5) and the discovery of new and natural plant products with low cytotoxicity and potent antibacterial action for the prevention and management of biofilm-related bacterial

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infection (6), so this study aimed to use of *Eucalyptus* liquid extract as antibacterial and antibiofilm formation.

Material and methods:

Plant materials :

Eucaylptus leaves were obtained from the gardens of the veterinary medicine college in Baghdad. These leaves were washed with tap water, then distilled water, and then dried for five days in the shade by air at room temperature. Using an electric grinder, the dry sections of the plants were ground into powder.

Crude extractions of plants

Eucalyptus extract was made by combining 50 grams of leaves powder with 250 ml of 99% ethanol in a flask, leaving it at room temperature for three days, then filtering through gauze, Whatman filter paper No. 1, and air drying it.

Preparation of microorganism:

The antibacterial effect of *Eucalyptus* extract was tested on gram positive and negative bacteria such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Proteus, Citrobacter, Listeria*, and *Enterococcus,* which were obtained from zoonotic research unit /veterinary medicine college / university of Baghdad. These bacteria were inoculated on brain heart infusion agar for refreshing overnight, and then a 108 cfu ml was generated for each bacterium by diluting cultures in saline and adjusting to 0.5 McFarland turbidity standards.

Assessment the bactericidal activity of Eucalyptus extract

The bactericidal activity was determined using the agar diffusion method, which was described by (30). After inoculating each of the tested bacterial cultures on Mueller Hinton agar plates with sterilized swabs, two-fold serial dilutions of the *Eucalyptus* extract (20, 10, 5, 2.5, 1.25, 0.6,0.3 mg/ml) with normal saline were performed, and 100 l of each concentration was put in each well. For 24 hours, the culture plates were incubated at 37°C. The diameter of the zones of inhibition was measured to determine antibacterial activity (7).

Determination of Minimum Inhibitory Concentration (MIC):

The MIC was calculated for each bacteria using test tubes containing two-fold serial dilutions of *Eucalyptus* extract (20, 10, 5, 2.5, 1.25, 0.6, and 0.3 mg/ml). Each tube received 1ml of 0.5 McFarland turbidity standard containing 108 cfu/ml of each bacteria and was incubated aerobically for 18-24 hours at 37°C. After that, each tube was filled with 0.5 ml (0.04 mg/ml) piodonitrotetrazolium violet (trazolium salt) and incubated for 6 hours at room temperature. Color change was used to determine the MIC, and the MIC was indicated by the first clear tube that did not turn red when compared to the other red tubes (8).

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Minimum Bactericidal Concentration (MBC)

The MBC was calculated by subculturing a loop of MIC tubes that showed no apparent growth and no color change onto extract free agar plates and incubating for another 24 hours at 37°C, then determining the lowest concentration of MIC that caused no growth on solid medium (8).

Antibiofilm activity:

The antibiofilm activity of *Eucalyptus* extract was estimated in glass sterile test tubes, a 1 ml of 20 mg/ml concentration of *Eucalyptus* extract was added for the first time to1ml of the 0.5 McFarland turbidity standard of each tested bacteria inoculated on muller hinton and sucrose broth and incubated for 24 hours at 37°C (zero time before biofilm formation) and the second time was added after 24 hr. from incubation (after biofilm formation). Then negative control tube was depended (containing bacteria and broth only). The tubes were then emptied and washed three times with sterile Phosphate Buffered Saline (PBS), air-dried, oven-dried at 60 °C for 45 minutes, and stained with 1ml of 1% crystal violet and incubated at room temperature for 15 minutes. After that, the tubes were washed three times with sterile distilled water to remove unabsorbed stain, and for biofilm formation determination, 1ml of ethanol was added to each (9).

Inhibition of biofilm formation was calculated by the following equation (7):

$$\text{ratio of biofilm inhibition} = \frac{\text{O.D. of control - O.D. of treatment}}{\text{O.D. of control}} \times 100$$

Results and Discussion:

Antimicrobial activity of *Eucalyptus* by zone of inhibition:

Disc diffusion method was used to assess antimicrobial activity of *Eucalyptus* extract, zone of growth inhibition of selected bacteria by *Eucalyptus* extract was ranged from 20-35 mm at concentration 20 mg/ml. This result considered highly effective according to the standard zones of inhibition alleged by (10) shown in (Table 1)

Table 1: Standard inhibition zones claimed by (Guevara, 2005)

Zone of inhibition	Inferences
< 10	May be expressed as inactive
10-13	Partially active
14-19	Active
>19	Very active

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Our results agree with (11) who study antimicrobial activity of *Eucalyptus* which highly potential against gram-positive and negative bacteria, and also agreed with (9) who mentioned that *Eucalyptus* was highly active against 8 out of ten bacterial isolates.

This activity is due to *Eucalyptus* compounds (1,8 –cineole) which have significant potential antibacterial, therapeutic agents, as well as water-soluble and able to diffuse further through the agar (12).

Antimicrobial Activities of the *Eucalyptus* Extract by MIC and MBC:

Our results proved the antibacterial activity of the *Eucalyptus* leaf extract**,** table (2) as well as figure (1,2) showed that minimum inhibitory concentration (MIC) ranged from (0.6 -20) mg/ml, the extract of *Eucalyptus* was highly effective against *Pseudomonase* at 0.6 mg/ml and at 20 mg/ml against *Citrobacter*.

Table (2): Antimicrobial activity of *Eucalyptus* with different concentration mg / ml (MIC / MBC)

Bacteria	Antimicrobial activity of *Eucalyptus* with different concentration mg / ml	
	MIC	MBC
Pseudomonase	0.6	0.3
Protease	5	10
Citrobacter	20	No effect
Staphylococcus	2.5	10
E.coli	5	No effect
listeria	10	2.5
Enterococcus	5	0.3

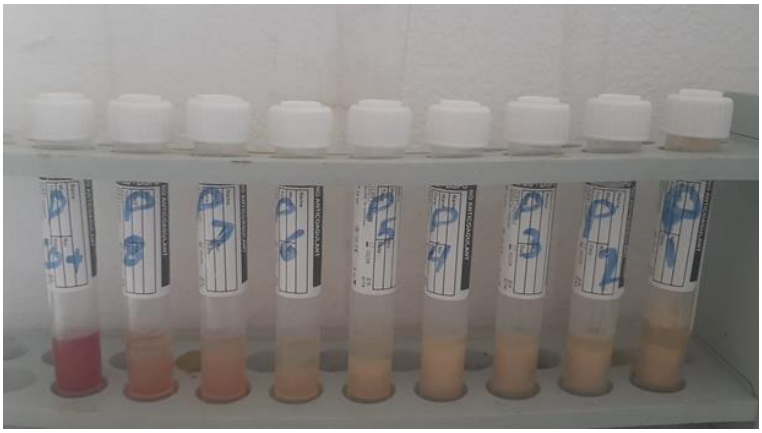

Figure (1): MIC of *Eucalyptus* against *Pseudomonas* using tetrazolium as indicator for viable cell at different concentrations starting from 20 mg/ml at left (no bacterial growth) and ending with control at right (shows darken redness color which indicate live bacterial cells) tube 7 considered the MIC.

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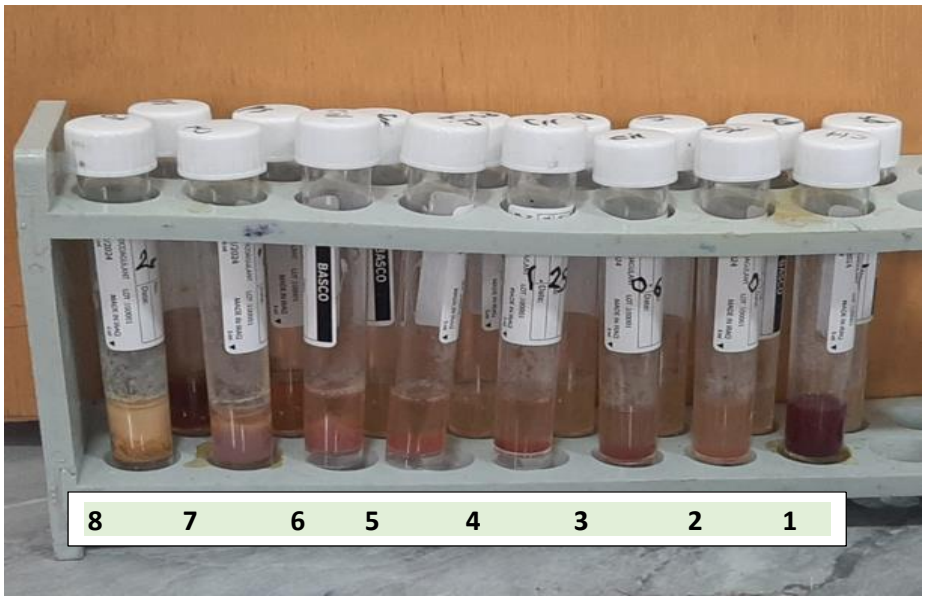

Figure (2): MIC of *Eucalyptus* against *Citrobacter* using tetrazolium as indicator for viable cell at different concentrations starting from 20 mg/ml at left (no bacterial growth) and ending with control at right (shows darken redness color which indicate live bacterial cells) tube 8 considered the MIC.

These results were confirmed with (13) who mentioned that ethanolic and aqueous extracts of *Eucalyptus camaldulensis* leaves have the greatest effect on *Staphylococcus aureus*, and also agreed with (14) who said that *Eucalyptus polybractea* oil was the only oil that inhibited the growth of all organisms.

The antibacterial effect of *Eucalyptus* plant attributed to its contents of essential oils, phenols, flavonoids and tannins that were considered as natural antibiotics for several infectious microorganisms (14).

1, 8-cineoled is the main component in Eucalyptus essential oil. Cineole is a monoterpenoid cyclic ether that can influence bacteria's cytoplasmic membrane (15).

Table (2) also shows minimum bactericidal concentration (MBC) of *Eucalyptus* which was the lowest at 0.3 mg/ml against *Pseudomonas* and *Enterococcus* and the highest at 10 mg/ml against *Staphylococcus* and *Proteus* and no effect against *E. coli* and *Citrobacter*. Figure (3,4).

This result was similar to the results of (13) who mentioned that ethanolic and aqueous extracts of *Eucalyptus* leaves have the highest effect on gram-positive bacteria such as *Staphylococcus aureus*, while *Escherichia coli* is resistant to most aqueous and ethanolic *Eucalyptus* extracts.

Seyyednejad SM *et al*., (16) recorded their results that leaf extracts of native *Eucalyptus microtheca* in Khuzestan Both ethanolic and methanolic extracts had the most effect on *E.*

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coli, methanolic extract had the greatest effect on *P. aeruginosa*, while *S. typhi* and *K. pneumonia* had the least effect and *S. aureus* had no effect.

A hydrophilic outer membrane, composed of lipids and LPS, with the property of selective permeability, is an important factor in gram-negative bacteria's resistance to antimicrobial compounds, according to Burt *et al.* (17), and some bacteria have polysaccharide capsules that can act as a barrier to the entrance of active antibacterial compounds (18).

Figure (3): MBC of *Eucalyptus* extract against *Pseudomonas* showing no growth at all concentration.

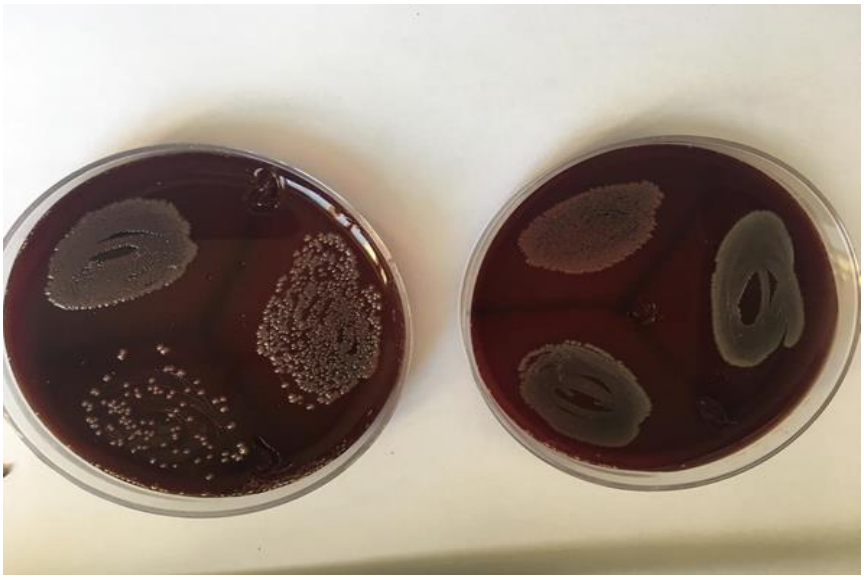

Figure (4): MBC of *Eucalyptus* extract against *Citrobacter* showing growth at all concentration.

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Activity of *Eucalyptus* Extract on biofilm formation and removal:

The ability of *Eucalyptus* to inhibit the biofilm formation was tested on *Pseudomonas* and *Staphylococcus aureus* only (figure 5,6).

The results showed that the inhibition was (28.3, 25 %) on *Pseudomonas* and *Staphylococcus aureus* at zero time and (26.6, 25 %) after 24 hr. from incubation respectively (table 3, figure 7).

This result agreed with (19) who confirmed that the antibiofilm activity was in ½ MICs, with inhibition percentages of 50 to 70% of *S. aureus* biofilms. Many studies support the ability of *Eucalyptus* extract to inhibit biofilm formation by bacteria, this was due to essential oils' significance in preventing biofilm formation. (20). Sandeep Kaur (21) conducted that the aqueous *Eucalyptus* extract have shown strong antibacterial activity against *Stasph.aureus* and *Pseudomonas aeruginosa* biofilm cells. The antibiofilm impact of Eucalyptus against *P. aeruginosa* was investigated, and it was discovered that biofilm cells were 65.43 % sensitive to eucalyptus oil Sambhyal *et al.*, (22). The decrease in bacterial populations could be owing to successful microbial respiration inhibition and enhanced plasma membrane permeability, resulting in bacterial cell death due to large ion leakage, or it could be due to the hydrophilic nature of the bacterial cell wall. (23).

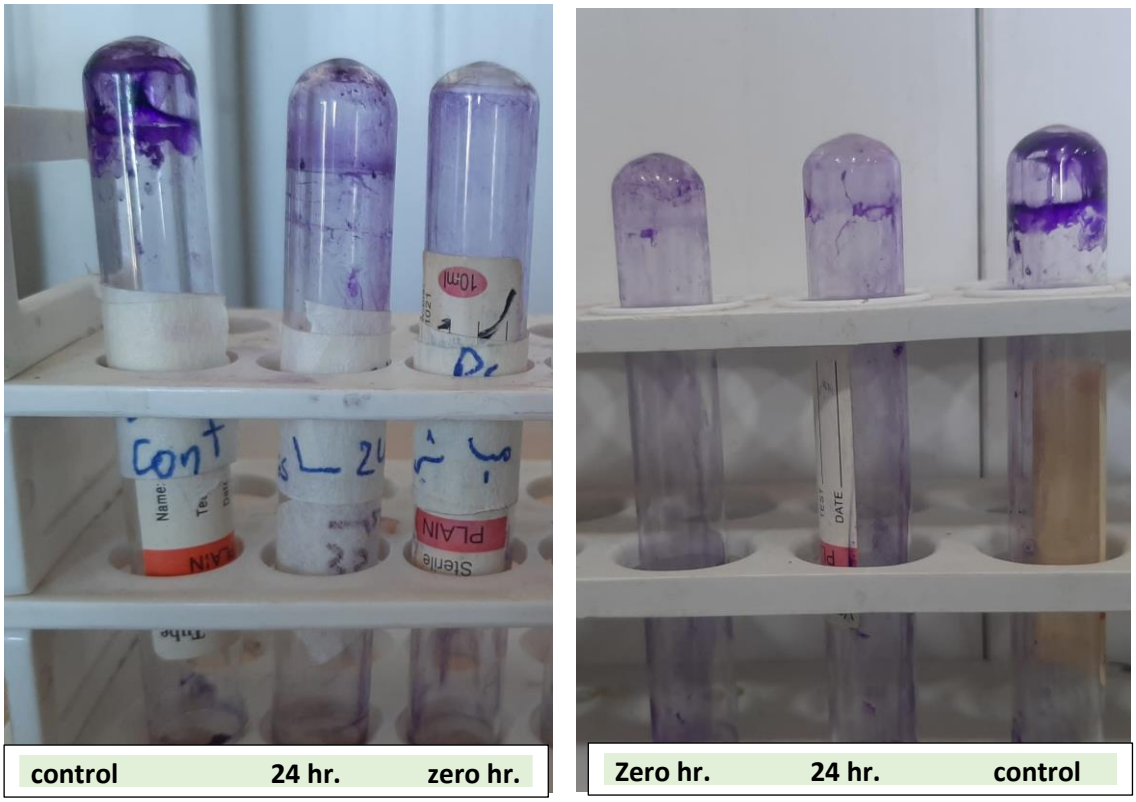

Figure (5,6): Activity of *Eucalyptus* Extract on biofilm formation against *Pseudomonas* (left) and *Staphylococcus aureus* (right)

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Table 3: antibiofilm effect of *Eucalyptus* Extract against *Pseudomonas* and *Staphylococcus aureus*

OD(570 nm)	***Pseudomonas***	***Staphylococcus aureus***
Direct (zero time)	0.043 (28.3%)	0.042 (25%)
24hr	0.044 (26.6%)	0.042 (25%)
control	0.06	0.056

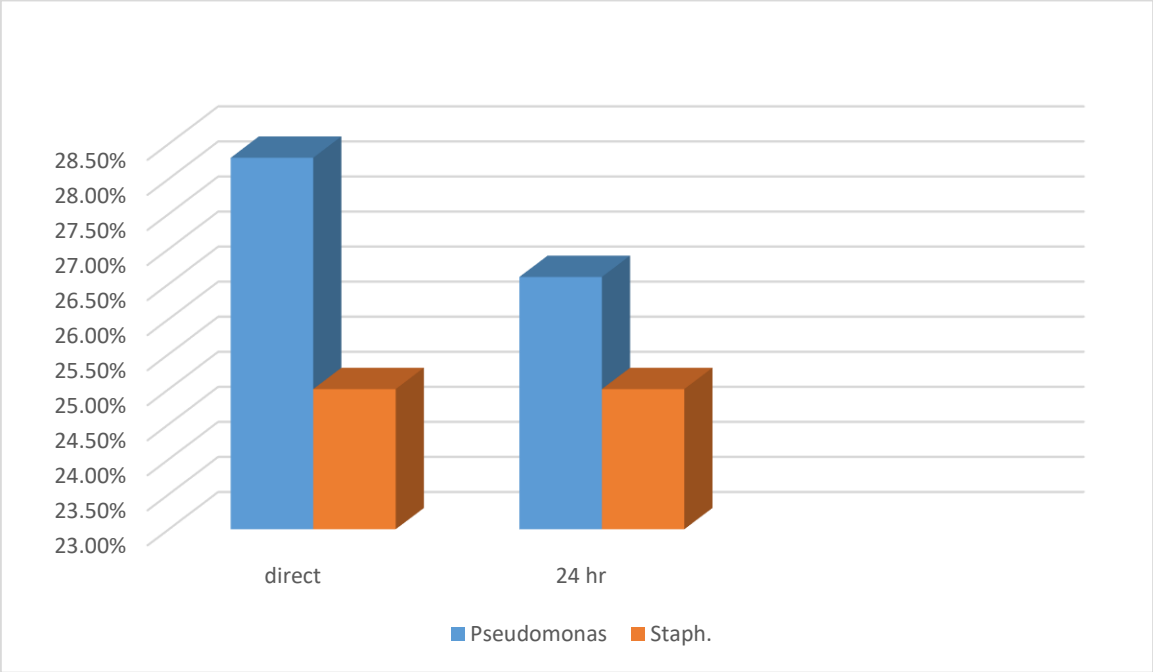

Fig (7): Activity of *Eucalyptus* Extract on biofilm formation against *Pseudomonas* and *Staphylococcus aureus.*

Conclusion:

It was concluded from result above that the antibacterial effect of *Eucalyptus* extract depending on its concentration and it was active in high concentration.

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