EVALUATION OF *PHYLLANTHUS EMBLICA* EXTRACT AS ANTIBACTERIAL AND ANTIBIOFILM AGAINST BIOFILM FORMATION BACTERIA

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ABSTRACT

The objective of this study was to evaluate the antibacterial effect of *Phyllanthus emblica* extract by (ethanol: methanol 1:1) against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* at different concentration started with 20, 10, 5, 2.5, 1.25 and 0.625 mg/ml. The antibacterial activity was determined by the agar well diffusion method to investigate the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The alcoholic extract of *Phyllanthus emblica* had the highest antibacterial activity at 20 mg/mL and 5 mg/mL except in *Pseudomonas aeruginosa* where the value of inhibition was between 20 mg/mL and 10 mg/mL whereas The MIC concentrations were mostly very high and ranged from 5 to 1.25mg/ml while MBC range from 10 to 2.5 mg/ml against tested bacteria. In this study, we evaluated the effect of *Phyllanthus emblica* against *Pseudomonas aeruginosa* biofilm formation was evaluated and the biofilm inhibitory concentrations of the *Phyllanthus emblica* extract was 40-6.25mg/ml.This implies that they may contain valuable substances for application directed against pathogenic biofilms. The use of herbal extract such as Phyllanthus emblica represent a new date for antimicrobial therapy after increasing the antibiotic resistance to microbes.

Key word: *Phyllanthus emblica*, antibacterial, antibiofilm, *Pseudomonas aeruginosa* biofilm

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تقييم مستخلص الاملج كمضاد بكتيري ومضاد لتكوين الغشاء الحيوي لبعض انواع البكتريا المكونة للغشاء الحيوي

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المستخلص:

هدفت الدراسة الحالية الى دراسة تأثير المستخلص الكحولي المثيلي للاثيلي بنسبة 1:1 لنبات الاملج كمضاد بكتيري ضد الزوائف الزنجارية والمكورات العنقودية فضلا عن الايشيريشيا القولونية بتراكيز مختلفة بدءا ب 20 ،10،5،2.5،1.25 و0.625 ملغم/مل، درس المضاد البكتيري للنبات بطريقة الانتشار بالوسط الزرعي،اقل تركيز مثبط MIC واقل تركيز قاتل MBC والتي اظهرت فاعلية مضادية بتراكيز تراوحت من20 ملغم/ مل الى 5 ملغم/ مل لجميع الجراثيم عدا الزوائف الزنجارية حيث اظهر المستخلص فاعليته بتركيزي 20 و10 ملغم/مل، اما اقل تركيز تثبيطي فقد سجل اعلى النتائج تراوح بين 5 الى 1.25 ملغم/مل وكذلك اقل تركيز قاتل تراوح من 10 الى 2.5 ملغم/مل كمضاد بكتيري. درس تأثير المستخلص الكحولي لنبات الاملج على تكوين الغشاء الحيوي بدءا من تركيز 40 الى 6.25 ملغم /مل، ان النتائج المستحصلة من المستخلص النباتي ضد الجراثيم قد تعود الى التركيب النباتي، ان استخدام الاملج كمضاد بكتيري في هذه الدراسة يوعز بدراسات لاحقة لاستعمالة كمضاد جرثومي لاسيما بعد ازدياد نسبة المقاومة البكتيرية للمضادات الحياتية.

الكلمات المفتاحية: الاملج، المضاد البكتيري، مضاد الغشاء الحيوي، الغشاء الحيوي للزوائف الزنجارية

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INTRODUCTION

Recently the resistance of microorganisms to any antibiotic has increased (21). Inadequate usage of antibiotics the most important factor of antibiotic resistance (51).Drug resistant bacteria, particularly , *Staphylococcus aureus*, *Klebsiella pneumonia*, *Escherichia coli* and *Pseudomonas aeruginosa*, are most important in healthcare (49) because no new antimicrobial agents are currently available for treatment of infected patients (8,19,51) else bacterial virulence factor play a role in diseases mechanism which is targets in drug evolution,moreover the ability of pathogens to form biofilms award a selected advantage for bacteria to militate under harsh environmental conditions lead to resistance to antimicrobial agents (52) *Pseudomonas aeruginosa*, and, *Escherichia coli* are examples of bacteria that form biofilms (39,35).Therefore alternative therapeutic agents from plants is One strategy to avoid antibiotic resistant bacteria, safe and have low cost(2,33,36,46). Consequently this study aimed to assess the in vitro antibacterial activities of *Phyllanthus emblica* extracts against bacterial clinical isolates *Staphylococcusaureus* , Gram-negative bacilli: *Escherichia coli*, *Klebsiella pneumonia* and antibiofilm effect against *Pseudomonas aeruginosa* and *Escherichia coli*.

MATERIALS AND METHODS

Preparation of plant extract: The dried plant was purchased from markets in Baghdad. The powdered plant material (250 g) was extracted in a 1000 ml conical flask with 500ml ; solvents(ehanol:methanol,1:,1,v:v) for 14 days in freeze after that filterated using Whattman No 4 filter paper. The filtrate obtained was concentrated byevaporated to dryness to obtain the crude extract. and kept it at 4°C until further uses.

Preparation of microorganism and inoculums

Microorganisms were medical isolates collected from the culture collections of the zoonotic diseases unit /veterinary medicine college at the university of Baghdad. Organisms were as follows: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, Organisms were maintained on brain heart infusion agar overnight, Inocula were prepared by diluting overnight cultures in saline and adjusted to 0.5 McFarland turbidity standards to approximately 10^8 cfu ml for each bacterium.

Assay for antibacterial activity

The screening of antibacterial activity was carried out by using the agar diffusion method as described by Lino and Deogracios (30) with slight modifications. Each of the bacterial cultures tested inoculated (0.2 ml each) using the sterilized swabs onto Mueller Hinton agar (MHA, Oxoid) plates (diameter: 15 cm),Then A sterilized stainless steel borer was used to formed four wells (6 mm diameter), The *Phyllanthus emblica* extract was separately redissolved in sterile distilled water at concentrations of (20, 10, 5 and 2.5 mg/ml),then 100 µl of each concentration of the plant extract were filled each well .The culture plates were allowed to stand then incubated at 37°C for 24 h. antibacterial activity was determined by measurement of diameter zones of inhibition (mm) (against the test organisms) around the extracts (30).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC and the MBC of the *Phyllanthus emblica* were determined by using test tubes where two- fold serial dilutions with muller hinton broth were made to the various concentrations (20, 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml) of the *Phyllanthus emblica* extract for each bacteria. Specifically 1ml of 0.5 McFarland turbidity standard 10^8 cfu/ml was added to each tube and incubated aerobically at 37 C^o for 18-24hrs. The MIC assay was determined by visualize the bacterial growth. 0.5 ml(0.04mg/ml) of *p*-iodonitrotetrazolium violet(trazolium salt)((INT) was added to each tube and incubate all tubes were incubated at room temperature for 6 hrs. The tubes were examined for color change and the MIC was indicated by the first clear tube that not changed to red color when compared with the control tubes or none inhibited concentrations.

Minimum Bactericidal Concentration (MBC)

The MBC was determined by subculturing a loop ful of the MIC tubes that showing no visible growth and no colour change onto extract free agar plates that incubated for a further 24 hours at 37 C^o then the lowest

concentration of MIC at which no growth on solid medium was regarded as MBC.

The effect of *Phyllanthus emblica* extract on the bacterial biofilm formation

Biofilm formation was assessed in plastic sterile test tubes, where seven appropriate concentrations (20,10,5,2.5,1.25,0.625 and 0.312 mg/ml) of extract were prepared from a serial two-fold dilutions method in muller hinton broth and eight tubes were inoculated with 1ml of the 0.5 McFarland turbidity standard and incubated for 4-5 hours to allow cell attachment and then add 1ml of each concentrations was added to each tube. All the tubes were further incubated for 24 hours at 37°C, the eight tube was containing bacteria and muller hinton broth only (negative control).

The ability of adherence bacteria

The adhered cell biomass was determined using 1% crystal violet staining. At first, plastic tube was emptied and washed three times with sterile Phosphate Buffered Saline (PBS). The tubes were air-dried and then oven-dried at 60 ^{o}C for 45 min. then the tubes were stained with 1ml of 1% crystal violet and incubated at room temperature for 15 min after which the tubes were washed 5 times with sterile distilled water to remove unabsorbed stain after that 1ml of ethanol was added to each tube and the absorbance was determined at 540nm using a spectrophotometer

RESULTS AND DISCUSSION

Antimicrobial Activities of the Extract by agar diffusion method: Table 1 shows the diameters for the zones of inhibition (mm) of *Phyllanthus emblica* extract at different concentrations(mg/ml). At 20mg/ml, *E. coli* had a higher zone of inhibition of 27mm(fig.1) while *S aureus* and *P. aeruginosa* had 25mm,15mm respectively. At 10 mg/ml of the extract concentration showed the highst zone of inhibition in *s.aureus* 21mm (fig.2) and *E.coli* 20mm while in *P. aeruginosa* was the least 10mm At 5 mg/ml, *E. coli* had the highest zone of inhibition of 14mm and the least zone of inhibition of *S. aureus* was 10mm while *P. aeruginosa* was not sensitive to the extract of *Phyllanthus emblica*. In contrast,all bacteria were not sensitive to the extract of *Phyllanthus emblica* at 2.5mg/ml (fig.3). The attention of researchers at the present time search for medicinal plants instead of antibiotics as a result of antibiotic resistant bacteria(4, 24,37, ,40) As well as the ease of access to medicinal plants and the availability and affordability(7,22,41). Actually, there is a rising interest to research the effect of natural compounds of plants extracts, on the habitation of the microorganisms. It has been reported that Zingiber officinale (3) Citrullus colocynthis (6) and *Berberis lycium* (19) *B. ciliata* (roots), *J. officinale* (leaves), and *S. album* (wood) (25) and Nigella sativa (13) exhibited potentially useful antibacterial properties towards testing microorganisms. The present study has shown that the extract of *Phyllanthus emblica* exhibited antibacterial activities.

Antimicrobial Activities of the *Phyllanthus emblica* Extract by MIC and MBC

The extracts of ***Phyllanthus emblica*** had strong bactericidal activity with MICvalues against *Staphylococcus aureus* , *P.aeuroginosa* and *Escherichia coli* are presented in Table 2. and fig4 ranging from 1.25 to 5 mg/ml fig.5,6 and 7 depending on the species of bacteria, thereby demonstrating the potential of this extract as antibacterial agents, likewise alcoholic extract of ***Phyllanthus emblica*** also showed bactericidal activity against all test strains at MBC values ranging from 2.5 to 10mg/ml fig.8,9.The statistical analysis using Anova singal factor shown in table (3). The resemblance of the MBC and MIC values of the herbal extract could be due to the sensitivity of the tube dilution method in detecting a minimum amount of turbidity which was the indicator of the growth of the test organisms than visual inspection on the other hand the MIC and MBC increased value against *E. coli* and *P.aeruginosa* rather than *S.aureus* that could be related with the differences in cell wall composition of testing microorganisms. The composition of cell membrane of Gram-negative bacteria involving many layer that prevent the permeation of antimicrobial agent therefore the bacteria become more resistance (16) as well *P.aeruginosa* have multiple technique of resistance to antibiotics and disinfectants like quartenary ammonium compounds (QACs), dyes and soaps (11,14, 43,48,50) such resistance including, target structure

alteration, enzymatic degradation, multidrug efflux pumps ,down regulation of outer membrane porins, β- lactamases (1,5,27,28,29,32,38) which may play a role in the low-level drug resistance else the decrease antibacterial effectiveness of this plant against *P.aeruginosa* shown in the present study should consequently to the resistance of bacteria strains tested that related to pump efflux system (18,42)

Anti-biofilm activity on plastic tube quantification with *P.aeruginosa*

The results of *in vitro* anti-biofilm activity of alcoholic extract of *Phyllanthus emblica* against *P.aeruginosa* found to inhibit the biofilm formation on the plastic surface and showed decreases in the turbidity when the optical density (OD) was taken at 590 nm (nanometer) are presented in fig.10. The bacteria used in this part of the investigation have been selected from the bacteria used for antibacterial activity depending on their biofilm formation potential. Inhibition of biofilm formation on plastic tube surfaces for *P.aeruginosa* by *Phyllanthus emblica* were additionally visualized by ccrystal violet assay which is illustrated in Fig. 11

Table 1. Antibacterial activity of ethanolic extract from *Phyllanthus emblica*

Bacteria	Zone of inhibition of *Phyllanthus emblica*					
	20 mg/ml	10 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml
E. coli	27mm	20mm	14mm	-	-	-
S aureus	25mm	21mm	10mm	-	-	-
P. aeruginosa	15mm	10mm	-	-	-	-

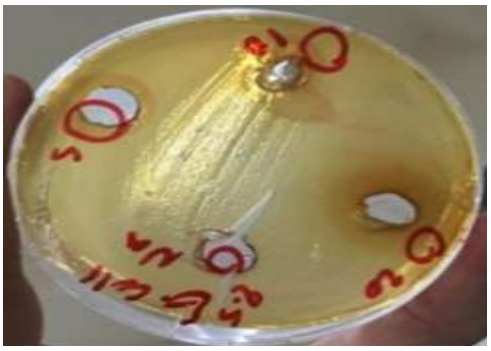

Fig.1. Antibacterial activity of Phyllanthus emblica extract against E.coli at different concentration

Fig.2. Antibacterial activity of Phyllanthus emblica extract against S.aureus at different concentations

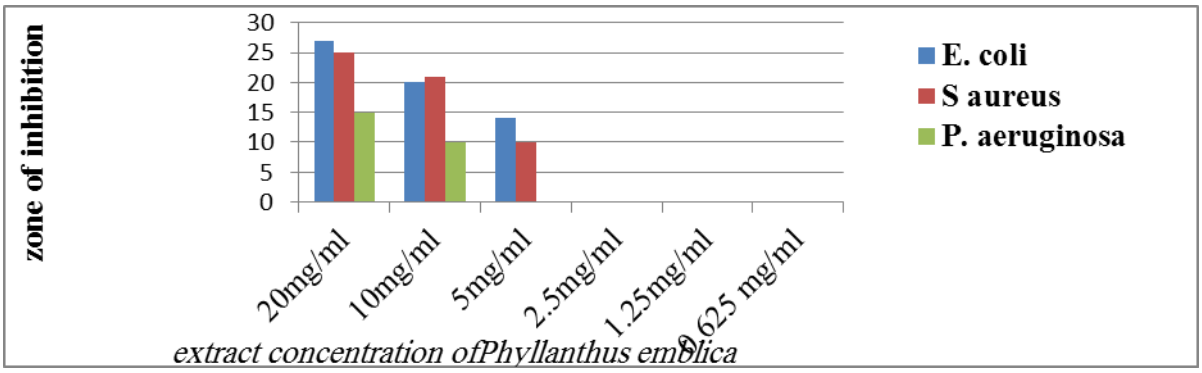

Fig.3 Antibacterial activity of *yllanthus emblica* extract on *E. coli,S.aureus* and *P. aeruginosa*

Table 2. MIC and MBC values for crude extract of *Phyllanthus emblica*against three microorganisms

Microorganisms	Antimicronial activity of *Phyllanthus emblica*	
	MIC mg/ml	MBC mg/ml
E. coli	5	10
S aureus	1.25	2.5
P. aeruginosa	2.5	5

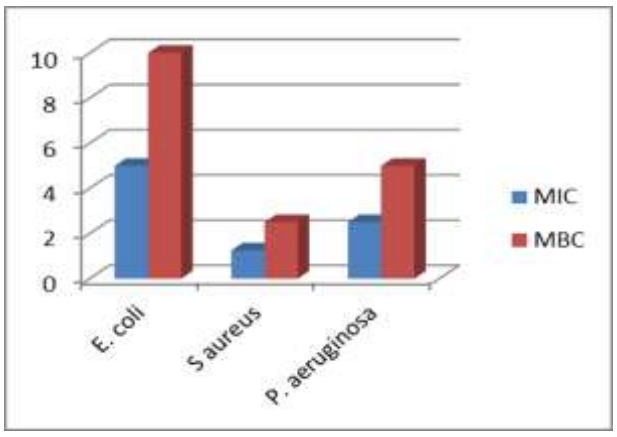

Fig.4. MIC and MBC of *Phyllanthus emblica*

Fig.5. MIC of *Phyllanthus emblica* against *E. coli* (left),*S. aureus* (middle) against *E.coli* and *P. aeruginosa* (right)

Alcoholic extract of *Phyllanthus emblica* exhibit antimicrobial activity against Gram positive and negative bacteria, the diameters of the inhibition zones in the presence of 5 µl of extract were smaller than in the presence of 10 µl, indicating a concentration dependent effect, Such an activity could bestrictly related to the chemical composition of extract, All three bacterial strains tested(*E. coli*,*S.*aureus, P.*aeruginosa*) were sensitive to *Phyllanthus emblica* extract which is

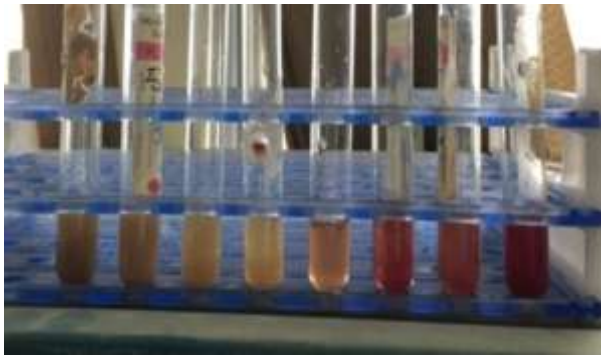

Fig.6. MIC of *Phyllanthus emblica* against *S. aureus* using tetrazolium as indicator for vailable cell at different concentration start from left 20 mg/ml ended with the right control that show darkn redness color which indicate vigorous vailable cell

Fig.7. MIC of *Phyllanthus emblica* against *P. aeruginosa* using tetrazolium as indicator for vailable cell at different concentration start from left 20 mg/ml ended with right control that show dark redness color which indicate vigorous vailable cell

Table 3: Anova:Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column1	**3**	**8.75**	**2.916667**	**3.64583**
Column 2	**3**	**17.5**	**5.833333**	

ANOVA

Source of variance	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between groups	**12.76042**	**1**	**12.76042**	**1.4**	**0.302242**	**7.708647**
Within groups	**36.45833**	**4**	**9.114583**			
Total	**49.21875**	**5**				

potential source of antibacterial agent as well as our result agree with previous study(17).This result shows that the plant might have important compounds which may active against test organisms. moreover *P.aeruginosa* formation of biofilms that resist the entrance of chemical agents (45,47) likewise *P. aeruginosa* biofilms are found on

many surfaces in hospital tools like dialysis membranes and catheters (20,23,34), on the other hand the continuance of the biofilm of this bacteria on the tools makes it very difficult to eliminate and finally caused subsequent in infected patient like cystic fibrosis, otitis media (15,44,9) however the uses of conventional antibiotic for eradicate the biofilm forming is very hard because of substantial and acquired mechanisms of resistance (10,31).

Fig.8.MBC of *Phyllanthus emblica* against *E. coli* showing no growth.at 20 and 10 mg/ml no growth

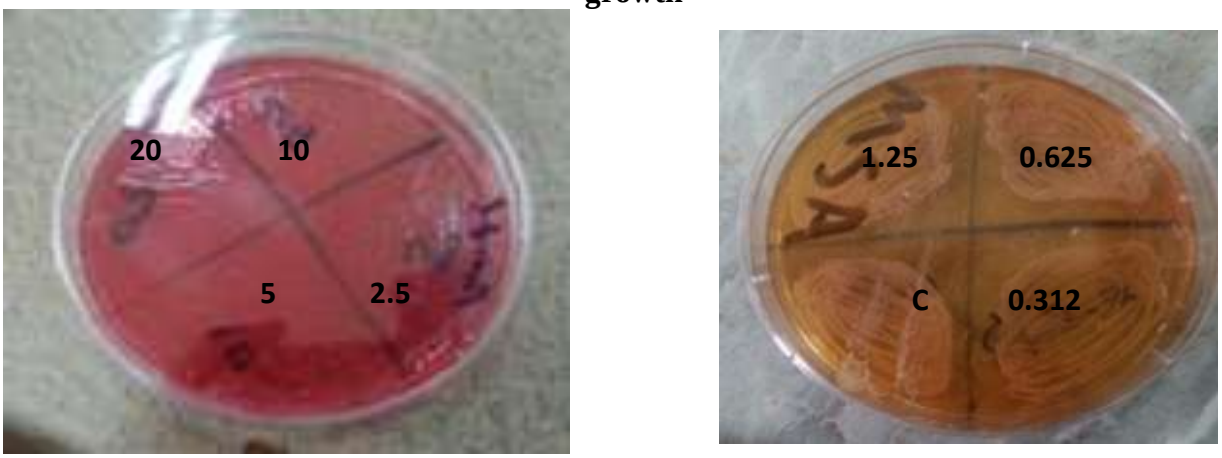

Fig.9.MBC of *Phyllanthus emblica* against*S.aureus.* showing no growth at 20,10,5 and 2.5 mg/ml no growth

Fig.11. biofilm formation on plastic tube of *P.aeruginosa*

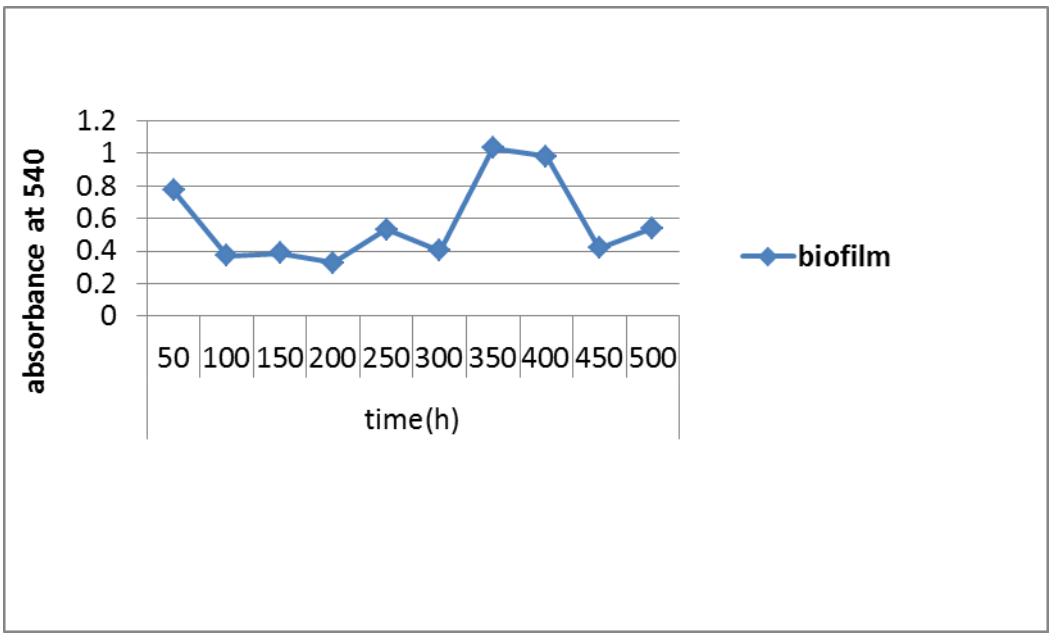

Fig10: Antibiofilm of *Phyllanthus emblica* against *p.aeruginosa*

In comparison with the cell membrane of Gram-positive bacteria which is single layered and hence show small or no resistance to the entrance of materials like antimicrobial agents. The plant showed a positive anti biofilm effect on the *P.aeruginosa* adherence formation on the plastic surface, These active herbal extract was found to inhibit the biofilm formation a dose dependent manner on the plastic surface and showed decreases in the turbidity when the OD was taken at 590 nm. The success of *Phyllanthus emblica* extract in inhibiting biofilm formation of *P.aeruginosa* in this study is a promising tool for reducing microbial colonisation on surfaces and epithelial mucosa which subsequently leads to infections .The facility of *Phyllanthus emblica* plant extract can inhibited the cell adherence is confirmation with prior studies that was found where it was found that inhibition of cell attachment to a substrate is easier to attain than inhibiting the growth of an already established biofilm (12). In this study, suppression of cell adherence was effective with most extracts showing suppression more than 50%. There are multiple way success for inhibiting cell adherence. Finally we found that *Phyllanthus emblica* can effect on the planktonic state which refers to the condition where the bacteria were allowed to grow as a suspension in the test tubes. *Phyllanthus emblica* can significantly damage the adhesion of the early colonizers. This subsequently will interfere with the initial stage of biofilm development.

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