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Research Article
Antibacterial and Anti-Biofilm Activity of Selected Plant Extracts Against Some Human Pathogenic Microorganisms

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Abstract

Background and Objective: Biofilm is a complex microbial community highly resistant to antimicrobials agents. The formation of biofilms in biotic and abiotic surfaces is associated with high rates of morbidity and mortality and considered as a major factor of bacterial pathogenicity. In the present study the antimicrobial and antibiofilm activities of 5 plant extracts (*Glycyrrhiza glabra* roots, *Laurus nobili*, *Malus domestica* peels, *Melissa officinalis* and *Lagenaria siceraria* peels) were evaluated against clinical isolated microorganisms (*E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumonia*). **Materials and Methods:** The extracts of medicinal plants were prepared using microwave assisted method by ethanol and water. **Results:** The results of this study revealed that, the aquatic extract of *M. domestica* peels showed powerful antimicrobial activity against *E. coli* with MIC value 1.56 mg mL^{-1}, the ethanolic extract of *G. glabra* roots showed good antimicrobial activity against *P. aeruginosa* with minimum inhibitory concentration (MIC) of 12.5 mg mL^{-1}. Most plant extracts in this study gave the minimum bactericidal concentration (MBC) ≥200 mg mL^{-1} against tested bacteria. However, the aquatic extract of *L. nobili* at concentration of 12.5 mg mL^{-1} showed the highest antibiofilm activity (86.7%) against *S. aureus* biofilm formation. The ethanolic extract of *M. domestica* peels at concentration of 25 mg mL^{-1} showed the highest inhibition rate (90%) against *P. aeruginosa* biofilm formation. **Conclusion:** These results revealed the importance of the tested extracts in the control of common human pathogenic micro-organisms. Plant extracts used in this study may contain potential antimicrobial and antibiofilm components that may be of great use for the development of new therapies against the most common infectious bacterial isolates.

Key words: Anti-biofilm, plant extracts, antibacterial effect, minimum inhibitory concentration, minimum bactericidal concentration

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Excessive use of antibiotics may enhance the resistance of bacterial species that cause human diseases[1]. The development of bacterial resistance to available antibiotics has stimulated researchers to find alternative antimicrobial agents, despite the role of antibiotics to prevent the growth of pathogens[2].

Despite the fact that bacteria are unicellular organisms, they often show group behavior; the attachment of the bacterial biofilm to the food product is a serious public health risk[3]. Microbial biofilms are communities of bacteria, embedded in a self-producing matrix, forming on living and nonliving solid surfaces[4]. Biofilm-associated cells have the ability to adhere irreversibly on a wide variety of surfaces, including living tissues and indwelling medical devices as catheters, valves, prosthesis and so forth[5]. Bacteria protected within biofilm exopolysaccharides up to 1,000 times more resistant to antibiotics than planktonic cells (free-floating)[6] which generates serious consequences for therapy and severely complicated treatment options[7]. Approximately, 75% of bacterial infections involved in biofilms formation are protected by an extracellular matrix[8].

Medicinal plants are important natural resources that can be constantly renewed and have an effective role in protecting people from disease; it is also an important source for containing huge amounts of antimicrobial agents[9,4]. Plant extracts and other biologically active compounds isolated from plants have gained wide spread interest for the treatment of disease since ancient times.

Considering the above and based on previous results obtained in our laboratory[10-12], the present study was proposed to evaluate the antibacterial and antibiofilm effect of 5 extract plants against 4 clinical isolated pathogens.

MATERIALS AND METHODS

Plant material: Fresh and healthy plants of *G. glabra* (roots), *L. nobilis* (leaves), *M. officinalis* (leaves), *M. domestica* (peel) and *L. siceraria* (peel), growing wild around the Gaza Strip community, were obtained from the local markets.

Bacterial strains and culture conditions: Pathogenic isolates of *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae* were obtained from microbiology department of Al-Shifa hospital and maintained on brain heart infusion agar (BHA) medium slant at 4°C and microbial diagnosis were confirmed using biochemical tests.

Methods

Preparation of plant extracts: Extracts were prepared following the methodology proposed by Jameela *et al.*[13], with minor modifications. Briefly, 10 g of air-dried plant parts powder was soaked with 150 mL of 80% ethanol and150 mL distilled water and placed in the microwave for one minute and left for a minute to cool. Extraction was repeated12 times and then the extract was filtered through Whatman filter paper number 1 and evaporated in oven at 45°C. Stock solutions (200 mg mL^{-1}) were prepared in Dimethyl sulfoxide (DMSO) and stored at -4°C in the dark for further experiments[13].

Evaluation of antimicrobial activity: Antimicrobial activity of plant extracts was performed using the agar-well diffusion bioassay. Briefly, 100 µL of fresh culture (approximately 10° CFU mL^{-1}) was uniformly spread onto Mueller-Hinton agar (MHA) plates by sterile Driglasky loop. Then, inoculated plates were allowed to dry at room temperature for 20 min. After that, wells of 6 mm in diameter were made in the agar using a sterilized cup-borer and 100 µL of each extract was poured in the wells. Methanol was used as control. Plates were incubated at 37°C for 18 h. Antibacterial activity was evidenced by the presence of clear inhibition zone around each well. The diameter of this zone was measured and recorded.

Disc diffusion method: Agar disc-diffusion method was followed to determine the antibacterial activity. A suspension of bacterial inoculum was adjusted to McFarland standard 0.5 and introduced to MHA (cooled to 45-50°C) swirl gently to mix well. After solidification, sterile filter paper discs approximately 6 mm in diameter were impregnated with stock extracts of 200 mg mL^{-1} concentration and placed on the surface of agar plate. After Incubation for 24 h at 37°C, the antibacterial activity was evaluated by measuring the diameter of zones of inhibition for microbial growth surrounding the plant extracts[14].

Well diffusion method assay: MHA plates were inoculated with bacterial isolates under aseptic conditions and wells (diameter = 6 mm) were filled with 50 µL from plant crude extract (200 mg mL^{-1}). The plates were allowed to stand at room temperature for 1h for proper diffusion and thereafter incubated at 37°C for 24 h. The resulting diameter of inhibition zones were measured in millimeters (mm)[15].

Determination of MIC and MBC of plant extracts: A serial dilution of plant extracts were performed using a sterile diluent of Mueller-Hinton broth (MHB) to reach a concentration range from 100-0.1953 mg mL^{-1} and 50 µL of the inoculum was added to each well. The inoculated plates with bacterial suspension (adjusted to McFarland standard 0.5) were incubated at 37°C for 18 h. Then 2,3,5-Triphenyl Tetrazolium Chloride (TTC) was added to the wells and the plate was incubated for another one hour. Tetrazolium salt is known to be colorless and turns red when biologically active bacteria are grown, the inhibition of growth can be detected when the solution in the well remains clear after incubation with TTC[15].

For MBC assay, from the lowest extract concentration that visibly inhibited the bacterial growth; 5 µL were taken and subcultures onto agar plates. The plates were incubated at 37°C for 24 h. MBC was determined according to the lowest concentration that did not exhibit any bacterial growth on the freshly inoculated agar plates[16].

Biofilm formation assay

Tube method: Biofilms can be formed in test tubes; for this reason, 0.1 mL of bacterial isolates (obtained by adjusting turbidity to 0.5 McFarland standards) were transferred to glass test tubes containing 10 mL nutrient broth (NB) and incubated at 37°C for 72 h. Then, the medium was removed and the tubes were washed with distilled water (DW), air-dried and biofilm formation was assayed by crystal violet[17].

Tissue culture plate method: Three wells of sterile 96-microtiter U-bottomed plate were filled with 200 µL of bacterial suspension. After incubation for 24 h at 37°C, wells were washed three times with 250 µL of DW. After 15 min, plates were stained for 10 min with 200 µL of 0.1% crystal violet per well; excess stain was removed and rinsed off by placing the plates under running tap water and the plates were air-dried. The adherent cells were re-solubilized with 160 µL of 95% (V/V) methanol per well and the optical density (OD) of each well was measured at 570 nm using plate Elisa reader[18].

Biofilm inhibition assay: *S. aureus* and *P. aeruginosa* from fresh agar were inoculated in BHI broth with 1% glucose and incubated overnight at 37°C in stationary condition. The concentration at which the extract depleted the plankton growth of bacterial by at least 50% was labelled as the sub-PMIC50 and used for anti-biofilm assay. 96 well U bottom tissue culture plates were filled with sub-PMIC50 concentration of plant extract and 100 µL suspension bacteria plates were incubated overnight at 37°C; then the well contents were removed by tapping the plate, washed with sterile DW to remove planktonic bacteria[19].

Adherent organisms in plates were stained with crystal violet (0.1% W/V) for 10 min, excess stain was rinsed off by DW and plates were dried; re-suspended in 200 µL 95% (v/v) methanol and transferred to 96 well-flat bottom plates. Optical density of stained adherent bacteria was determined with micro-ELISA reader at wavelength of 570 nm. The percentage of inhibition was then compared with the control[20].

$$\text{Inhibition (\%)} = \frac{\text{1 - (A570of the test)}}{\text{1 - (A570 of non - Treated control)}} \times 100$$

RESULTS

Antibacterial activity and MIC of plant extracts: Plant crude extracts showed varying degrees of antibacterial activities against the tested pathogenic bacteria, with a range of inhibition zone (7-25 mm in diameters) as shown in Table 1. Our results showed that most used plant extracts

Table 1: Antimicrobial activity of plant extracts on bacteria that used in this study by well and disc diffusion method

	G. glabra roots		*L. nobilis*		*M. domestica* peels		*M. officinalis*		*L. siceraria* peels	
Plant extracts	E	W	E	W	E	W	E	W	E	W
Well diffusion method										
E. coli	25.3±0.577	15.3±0.577	22.3±0.577	20.0±0.000	18.3±0.577	20.0±0.000	0.0	0.0	0.0	5.3±0.577
S. aureus	24.0±0.000	23.3±0.577	20.0±0.000	18.3±0.577	20.0±0.000	25.3±0.577	12.0±0.0	14.0±0.0	0.0	0.0
P. aeruginosa	0.0	13.3±0.577	0.0	0.0	0.0	0.0	0.0	0.0	6.3±0.577	9.0±0.000
K. pneumonia	23.3±0.577	9.0±0.000	17.3±0.577	15.3±0.577	10.3±0.577	13.3±0.577	0.0	0.0	9.0±0.000	11.0±0.000
Disc diffusion method										
E. coli	7.3±0.577	7.3±0.577	7.3±0.577	7.3±0.577	7.3±0.577	7.3±0.577	7.3±0.577	8.0±0.000	7.3±0.577	7.3±0.577
S. aureus	7.3±0.577	7.3±0.577	11.0±0.000	11.0±0.000	7.3±0.577	7.3±0.577	7.3±0.577	7.3±0.577	6.3±0.577	6.3±0.577
P. aeruginosa	7.3±0.577	8.0±0.000	6.3±0.577	6.3±0.577	6.3±0.577	6.3±0.577	7.3±0.577	8.0±0.000	7.3±0.577	7.3±0.577
K. pneumonia	9.0±0.000	9.0±0.000	10.3±0.577	10.3±0.577	10.3±0.577	10.3±0.577	9.0±0.000	8.0±0.000	9.0±0.000	7.3±0.577

*E: Ethanol, W: Water, (N = 3) Values are mean±SD of three separate experiments

have antibacterial activity against *E. coli*, except *M. officinalis* extracts which showed low inhibition zone by disc diffusion method with no effect with well diffusion method (Table 1).

The ethanolic and aquatic extracts of *G. glabra* roots showed strong antibacterial activities against *E. coli* with inhibition zone 25.3 and 15.3 mm, respectively using well diffusion method. The aqueous extract of *M. domestica* peels showed strong antibacterial activity against *S. aureus* with zone of inhibition (25.3 mm) as shown in Table 1.

The ethanolic and aquatic extracts of *G. glabra* roots showed strong antibacterial activities against *S. aureus* by well diffusion method with inhibition zones of 24 and 23.3 mm, respectively. Also, the aquatic extract of *G. glabra* roots showed antibacterial activity against *P. aeruginosa* using well diffusion method with inhibition zone of 13.3 mm (Table 1). All plant extracts showed good results of antibacterial activity against *K. pneumonia* with inhibition zones of 9-23.3 mm using well diffusion assay except *M. officinalis*; a range of inhibition zone (7.3-10.3 mm) were found by all plant extracts against all pathogens used by disc diffusion method as shown in Table 1.

All plant extracts were evaluated for their MIC against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumonia*. The MIC value of aquatic extract of *M. domestica* peels showed the best value against *E. coli* followed by ethanolic extract of *L. nobilis* which gave MIC values of 1.56 and 3.125 mg mL^{-1}, respectively (Table 2). The MIC value of ethanolic extract of *G. glabra* roots against *P. aeruginosa* was 12.5 mg mL^{-1}.

The ethanolic and aquatic extracts of *G. glabra* roots, *L. siceraria* peels and *M. officinalis* against *S. aureus* gave intermediate activity with MIC value of 25 mg mL^{-1}. The aquatic extract of *G. glabra roots* and *L. nobilis* against *K. pneumonia* gave intermediate activity with MIC value of 25 mg mL^{-1}. Ethanolic extract of *M. officinalis* and *M. domestica* peels against *K. pneumonia* gave intermediate MIC values. Also, the ethanolic and aquatic extracts of *L. siceraria* peels gave intermediate MIC values against *K. pneumonia* (Table 2).

Minimum bactericidal concentration (MBC): The ethanolic and aquatic extracts of *G. glabra* roots showed antibacterial activities against *E. coli* and *S. aureus* with MBC value of 200 mg mL^{-1}. The MBC of aquatic extract of *M. officinalis* against *P. aeruginosa* and *K. pneumonia* was 200 mg mL^{-1}. The MBC of ethanolic extract of *L. siceraria* peels against *K. pneumonia* was 200 mg mL^{-1} but the aquatic extract of *L. siceraria* peels showed MBC at 100 mg mL^{-1} against *K. pneumonia.* Other plant extracts gave MBC more than 200 mg mL^{-1} against tested bacteria (Table 3).

Plant extracts activity on biofilm formation of *P. aeruginosa*: The five plant extracts used in our study reduced *P. aeruginosa* biofilm formation by ≥ 83%. Our results showed that ethanolic extract of *M. domestica* peels at concentration of 25 mg mL^{-1} showed 90% inhibition on *P. aeruginosa* biofilm formation (Table 4).

Table 2: Minimal inhibitory concentrations (MIC) of the plant extracts on bacteria that used in this study

	MIC (mg mL^{-1})									
	G. glabra roots		*L. nobilis*		*M. domestica* peels		*M. officinalis*		*L. siceraria* peels	
Plant extracts	E	W	E	W	E	W	E	W	E	W
E. coli	12.5±0.0	12.5±0.0	3.125±0.0	6.25±0.0	6.25±0.0	1.56±0.0	12.5±0.0	6.25±0.0	6.25±0.0	6.25±0.0
S. aureus	25.0±0.0	25.0±0.0	25.000±0.0	50.00±0.0	50.00±0.0	50.00±0.0	25.0±0.0	25.00±0.0	25.00±0.0	25.00±0.0
P. aeruginosa	1.25±0.0	12.5±0.0	25.000±0.0	50.00±0.0	25.00±0.0	25.00±0.0	25.0±0.0	25.00±0.0	25.00±0.0	25.00±0.0
K. pneumonia	50.00±0.0	25.0±0.0	50.000±0.0	20.00±0.0	25.00±0.0	50.00±0.0	25.0±0.0	50.00±0.0	25.00±0.0	25.00±0.0

(N = 2) Values are mean±SD of two separate experiments

Table 3: Bactericidal concentrations (MBC) of the plant extracts against tested bacteria

	MBC (mg m^{-1})				
Plant/solvent	*G. glabra* roots	*L. nobilis*	*M. domestica* peels	*M. officinalis*	*L. siceraria p*eels
E (A)	200	>200	>200	>200	>200
W (A)	200	>200	>200	>200	>200
E (B)	200	>200	>200	>200	>200
W (B)	>200	>200	>200	>200	>200
E (C)	>200	>200	>200	>200	>200
W (C)	>200	>200	>200	200	>200
E (D)	>200	>200	>200	200	200
W (D)	>200	>200	>200	200	100

Table 4: Effects of ethanolic and aquatic extracts on biofilm formation and biofilm inhibition of *P. aeruginosa*

	[a]Sub-PMIC50 (mg mL^{-1})	MBIC50 (mg mL^{-1})	Reduction (%)
Plants ethanolic extracts			
G. glabra roots	3.12	1.56	89.6
L. nobili	12.50	6.25	86.8
M. domestica peels	50.00	25.00	90.0
M. officinalis	3.12	1.56	84.3
Reduction (%)			
G. glabra roots	6.25	3.12	87.5
L. nobili	25.00	12.50	88.5
M. domestica peels	50.00	25.00	86.7
M. officinalis	25.00	12.50	83.2
L. siceraria peels	25.00	12.50	89.0

[a]Concentration below the MIC that not affecting on the microbial growth

Table 5: Effects of ethanolic and aquatic extracts on biofilm formation and biofilm inhibition of *S. aureus*

	[a]Sub-PMIC50 in mg mL^{-1}	(MBIC50) in mg mL^{-1}	Reduction (%)
Plant ethanolic extracts			
G. glabra roots	12.5	6.25	77.1
L. nobili	50.0	25.0	81.4
M. domestica peels	50.0	25.0	77.5
M. officinalis	25.0	12.5	77.1
L. siceraria peels	25.0	12.5	82.0
Aquatic extracts			
G. glabra roots	25.0	12.5	82.9
L. nobili leaves	25.0	12.5	86.7
M. domestica peels	50.0	25.0	80.6
M. officinalis	50.0	25.0	78.3
L. siceraria peels	25.0	12.5	83.1

[a]Concentration below the MIC that not affecting on the microbial growth

Ethanolic extract of *G. glabra* roots at concentration of 1.56 mg mL^{-1} showed 89.6% of inhibition on biofilm formation followed by aquatic extract of *L. siceraria* peels and *L. nobili* with 89 and 88.5% inhibition on *P. aeruginosa* biofilm formation, respectively (Table 4).

Aquatic extract of *G. glabra* roots at concentration of 3.12 mg mL^{-1} showed 87.5% inhibition of *P. aeruginosa* biofilm formation while ethanolic extracts of *L. nobili* at concentration of 6.25 mg mL^{-1} showed 86.8% of inhibition followed by *M. domestica* peels aquatic extract that showed 86.7% inhibition at 25 mg mL^{-1} (Table 4).

Ethanolic extract of *M. officinalis* at concentration of 1.56 mg mL^{-1} showed 84.3% inhibition of *P. aeruginosa* biofilm formation while aquatic extracts of *M. officinalis* showed 83.2% inhibition of *P. aeruginosa* biofilm formation at concentration of 12.5 mg mL^{-1}. Also, ethanolic extract of *L. siceraria* peels at concentration of 3.12 mg mL^{-1} showed 81.5% inhibition on biofilm formation of *P. aeruginosa*.

Plant extracts activity on biofilm formation of *S. aureus*: The five plant extracts used in our study reduced *S. aureus* biofilm formation by ≥86%. Our results showed that the ethanolic extract of *M. domestica* peels at concentration of 25 mg mL^{-1} showed 90% inhibition of *S. aureus* biofilm formation while the aquatic extracts of *L. nobili* at concentration of 12.5 mg mL showed 86.7% inhibition followed by aquatic extracts of *L. siceraria* peels that showed 83.1% inhibition of *S. aureus* biofilm formation (Table 5).

Aquatic extracts of *G. glabra* roots at concentration of 12.5 mg mL^{-1} showed 82.9% inhibition of *S. aureus* biofilm formation while ethanolic extracts of *L. siceraria* peels at concentration of 12.5 mg mL^{-1} showed 82% inhibition (Table 5).

L. nobili ethanolic extracts concentration of 25 mg mL^{-1} showed inhibition 81.4% followed by aquatic extract of *M. domestica* peels that showed inhibition of 80.6% of *S. aureus* biofilm formation at 25 mg mL^{-1} (Table 5).

Aquatic extract of *M. officinalis* at concentration of 25 mg mL^{-1} showed 78.3% inhibition of *S. aureus* biofilm formation followed by ethanolic extract of *M. domestica* peels that showed 77.5% inhibition at 25 mg mL^{-1} (Table 5).

Ethanolic extract of *G. glabra* and *M. officinalis* showed 77.1% inhibition of *S. aureus* biofilm formation at different concentrations (Table 5).

DISCUSSION

Bacterial infectious diseases still represent an important cause of morbidity and mortality among humans worldwide[21]. For a long period of time, it has been found that many

compounds found in herbs have antimicrobial activities and are an important source of treatment for pathogenic microbes[8,22,23]. Therefore, particular attention is oriented nowadays to use these compounds in control of some human pathogenic microorganisms especially multidrug resistance strains i.e. *P. aeruginosa* and *S. aureus*.

Although, antibiotics are one of the most successful forms in combating bacterial infections, frequent use and overprescribing them has resulted in the development of resistant bacteria and made them less effective or useless at all[24]. Therefore, increased attention has been paid to the development of antimicrobial agents to treat bacterial infections.

The results of the current study showed that well diffusion method has higher activities than disc diffusion method which could be because the paper disk which was saturated with plant extracts may retain the active component and was not allowed to diffuse into the MHA. MIC was selected to test the antimicrobial activities of plant extracts by micro broth dilution method because it provides quantitative results and is considered as the most appropriate and reliable method[25].

All extracts showed low MIC value which is in agreement with Adwan and Mhanna[26] and the five plant extracts that used in the presentstudy reduced *P. aeruginosa* biofilm formation by ≤90% and reduced *S. aureus* biofilm formation by ≥86%. Biofilm formed by *P. aeruginosa* was more sensitive against plant extracts than biofilm formed by *S. aureus*, which could be due to the difference in the structure of the bacterial cell wall[14,27]. The results of this study showed the effectiveness of plant extracts in inhibiting the formation of *P. aeruginosa* and *S. aureus* biofilm.

Our results agree with the findings of Chakotiya *et al.*[28], who reported that the *G. glabra* roots extract has a significant inhibitory effect on biofilm formation against *P. aeruginosa* and correlated with the effectiveness of the extract to its active compounds such as glycyrrhizic acid.

The crystal violet method was used to test the activity of the plant extracts and biofilm formation[17]. In biofilm assay, it was noticed that the presence of glucose in broth media stimulated the formation of biofilms[29]. Also, the flat-bottomed 96-well plate for the bacterial adhesion was an essential step for biofilm formation[30]. Extract concentrations of Sub-PMIC50 were used for anti-biofilm assay to ensure that the used extract was not affecting the microbial growth. These results revealed the importance of the tested extracts in the control of common human pathogenic micro-organisms.

CONCLUSION

This study suggested that five plant extracts (*Glycyrrhiza glabra* roots, *Laurus nobili*, *Malus domestica* peels, *Melissa officinalis* and *Lagenaria siceraria* peels) may contain potential source of: natural antimicrobial components that may be of great use for the development of new therapies against *E. coli*, *S. aureus*, *P. aeruginosa* and *K. pneumonia* and the antibiofilm activities of these plant extracts against the biofilm formed by *S. aureus* and *P. aeruginosa.* All plant extracts used in this study contain potential antimicrobial and antibiofilm components that may be of great use for the development of therapies against common infectious bacterial isolates.

SIGNIFICANCE STATEMENT

This study discovered the importance of the tested extracts in the control of common human pathogenic micro-organisms. Plant extracts used in this study may contain potential antimicrobial and antibiofilm components that may be of great use for the development of new therapies against the most common infectious bacterial isolates.

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