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Efficacy of Ethanol Extract from Leaves Of *Malva parviflora* to Inhibit Bacterial Biofilm Formation

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Abstract

Infection due to bacterial biofilm represents a serious health threat worldwide mostly due to the appearance of antibiotic resistant strains. Treatment of bacterial biofilm infection is currently a difficult and complicated challenge for microbiologists and clinicians. Antibiotics treatment alone is often inadequate to overcome bacterial biofilm infection so the necessity of research for alternative to inhibit biofilm formation. The purpose of this study was to investigate the efficacy of leaves extract of *Malva parviflora* as antibiofilm against different pathogens .The plant extraction was done with ethanol under cold extraction. Antibiofilm effect was evaluated for *Staphylococcus aureus*, *Klebsiella pneumonia* and *Escherichia coli* using 96 wells technique. The results revealed that leaves extract of *Malva parviflora* had efficacy to eradicate bioflm formed from most of isolates.

Keywords: Biofilm, *Malva parviflora*, Anibiofilm, β-Sitosterol

1. Introduction

Biofilm is a structured consortium attached on a living or inert surface formed by microbial cells stuck to each other and is surrounded by the self-produced extracellular polymeric matrix. The formation of biofilm is considered an adaptation of microbes to hostile environments (De Fuente- Núñez et al., 2013; Hall-Stoodley et al., 2004). Biofilm-forming bacterial cells are significantly more resistant to antibiotics and host immune defense than their planktonic counterparts (Hengzhuang et al., 2011; Hengzhuang et al., 2012).

Aggressive and intensive antibiotic treatment is usually helpful to control the exacerbations of chronic biofilm infections induced by dispersed bacteria and reduce the biofilms, but cannot eradicate the biofilm infections (Høiby et al., 2010; Høiby, 2011), because the minimal concentration of antibiotic for eradication of mature biofilm is difficult to reach in vivo (Hengzhuang et al., 2011). Therefore, once a bacterial biofilm infection established, it becomes difficult to eradicate. Bacterial biofilm formation is widely found in natural environments with water and also in human diseases, especially in the patients with indwelling devices for the purpose of medical treatments (Hall-Stoodley et al., 2004; Høiby et al., 2010). The number of bacterial infections that involve biofilms varies depending on the reporting agency, with estimation being around 65% of all infections according to the Center for Disease Control (CDC), and 80% according to the National Institutes of Health (NIH).

Bacterial biofilms are characterized as highly resistant to antibiotic treatment and immune responses (Høiby et al., 2010). Although it is well known that antibiotic treatment is currently most important and effective measure for the control of microbial infections, however, antibiotic treatments are almost impossible to eradicate biofilm infections. In vitro and in vivo experiments demonstrated that the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) for biofilm bacterial cells were usually much higher (approximately10–1000 times) than the planktonic bacterial cells (Hengzhuang et al., 2011; Høiby et al., 2011).

The effective antibiotic MBC in vivo for biofilm eradication are therefore impossible to reach by conventional antibiotic administrations due to the toxicities and the side effects of antibiotics and the limitation of renal and hepatic functions. Treatment of biofilm infections becomes therefore challenging and attracts significantly scientific attention (Hong et al., 2014).

Ever since the advent of humanity on earth, plants have served as unlimited source of phytotherapeuticals for various diseases (Clardy & Walsh, 2004). In recent years, the emergence of biofilm infections have generated an urgent alarm in research and development field in seeks for novel antimicrobials from ethnomedicinal plant (Perumal & Mahmud, 2013).

Currently, there has been great interest in the search for non-lethal antibiofilm agents (Adesina et al., 2015).

Despite the various researches concerning the evaluation of multiple bioactivities of *Malva parviflora*, none has dealt with the bacterial biofilm inhibition by this plant. Therefore the aim of this study was to investigate the effect of *Malva parviflora* as an antibiofilm agent.

Malva is a widespread tropical and temperate genus of the family Malvaceae (Boulos, 2000). Plants of this family are well known for their antibacterial and antifungal activities due to the presence of alkaloids, essential oils and phenolic compunds (Abad et al., 2007). *Malva parviflora* has been widely used in many parts of the world for curing various diseases (Ododo et al., 2016).

This work aimed to screen effect of Malva parviflora extraction as antibiofilm against *Staphylococcus aureus, Escherichia coli, Klebesilla pneumonia*. To our knowledge, this is the first report regarding the use of this plant as antibiofilm.

2. Methods

2.1 Collection of Plant Materials

Fresh samples of *Malva parviflora* were collected and identified from Alrib'ania, AlQassim by Dr. Magda Gazer in January, 2015. The plant was identified and authenticated by Dr. Magda Gazer, College of Science and Art, Biology Department, Qassim University, Qassim , Saudi Arabia. (according to Chaudhary, 1999-2001 and Mighaid, 1996.) The leaves were air dried, powdered then stored in an air tight container for further analysis.

2.2 Extraction

Twenty grams of dry leaves of *Malva parviflora* was grinded into fine powder then extracted by decoction by boiling with ethanol for 5 minutes, followed by filtration and concentration of the filtrate and evaporated till dryness on water bath to afford one gram extract. For further use, the extract was stored in airtight container at 4°C in the refrigerator.

2.3 Test Organisms

The susceptibility test comprises a panel of clinically resistant Gram-negative and Gram-positive bacteria. These selected human pathogenic bacteria are capable of forming biofilms and causes severe infections. The panel of clinically resistant pathogens used in this study includes 40 isolates of *Klebsiella pneumonia*, *Escherichia coli* and *Satphylococcus aureus*. The clinical isolates were obtained from Clinical Laboratory Department of Al Fayha Clinic in Buraydah, Qassim, Saudi Arabia. The isolates were propagated on nutrient agar plates and maintained on the plate at 4°C. The isolates were sub-cultured in nutrient agar at 37°C for 24 h. prior to further studies.

2.4 Inocula Preparation

All bacterial strains were recovered on a fresh tryptic soy agar (Difco, USA) plate 24 h prior to antimicrobial test. To prepare the inoculum, colonies from fresh tryptic soy agar were transferred into sterile Mueller Hinton (MH) liquid growth medium and incubated at 37°C overnight. Aliquots (500 µl) were transferred to 10 ml of fresh MH broth and incubated at 37°C. Cells were harvested by centrifugation(3,000 g, 5 min at 4°C), washed in 10 mmol/L phosphate-buffered saline (PBS) (pH 7.4), and re-suspended in MH broth to an approximate cell density of 1.0 $\times 10^5$ CFU/ml. The final cells concentration was confirmed by viable counts.

2.4.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The minimum inhibitory concentrations (MIC) of leaves extract of *Malva. parviflora* were determined by using assay of serial broth microdilution method by 96 well as described by (Eloff,1998). This assay was performed using flat-bottomed polystyrene96-well clear microtiter plates with standard plate layout as proposed by Cos et al. (2006). Briefly, ethanol extract of leaves of *Malva parviflora* was dissolved in DMSO and an identical two fold serial dilution using MH broth were made to form 31.25-1000 mg/ml .One hundred microliters of bacterial

inoculum was added and mixed thoroughly in all the wells (15.625 – 500 mg/ml) as the highest in-test concentration.

The microtiter plates were sealed with parafilm tape and incubated overnight at 37°C. An appropriate mixture of solvent DMSO, medium and inoculum were included as drug-free control and the final concentration of DMSO in the well was ensured to be less than 1% (v/v). An additional non-infected medium was included for sterility check. The MIC of leaves extract of *Malva parviflora* was determined by using recovery broth method according to Yue et al. (2009) and the least concentration inhibited the growth was determined.

For the determination of minimum bactericidal concentration (MBC), 20µl of culture medium from the microtiter plate wells that showed no growth of bacteria was re-inoculated on MH agar plates. After 24 h of incubation at 37, MBCs were determined as the lowest concentration that yielded no bacterial growth on MH agar plates.

2.5 Biofilm Formation

The bacterial cells were grown in tryptic soy broth (TSB) at 37°C overnight. The cultures was then harvested by centrifugation (14,000 rpm, 15 min at 4°C) and rinsed with phosphate-buffered saline (PBS, pH 7.4). The washed bacterial cells were then resuspended in tryptic soy broth to approximately 1×10^7cfu/ml (determined by plate count assay)200 µl/ well of bacterial suspension was transferred to sterile 96 well microtiter plates for 24 h. at 37°C. After 24 h of biofilm formation the supernatant of each well was removed and washed twice with 200 µl of sterilized water, biofilms formed in the inner surfaces of each well of the microtiter plates subsequently treated (Stepanovic et al., 2000).

2.5.1 Biofilm Detection

Biofilm mass of different isolates was detected by crystal violet (CV) staining method, adapted from (Stepanovic et al., 2000). For that, the microtiter plates containing the biofilms were left to air dry for 30 min, and 200 µl of 98 % methanol were transferred to each well in order to fix the remaining attached cells, for 15 min. Afterwards, the plates were emptied and left to air dry. The fixed cells were stained with 200 µL of CV (Gram's staining; Merck) per well, for 5 min. After this staining step , the plates were washed with running tap water , air dried and filled with 200 µl of 33%(v/v) of glacial acetic acid in order to resolubilize the CV bound to the adherent biofilm . After that, the quantitative analysis of biofilm mass was performed through the measure of optical density at 750nm of each well using a microtiter plate reader.

2.5.2 Inhibition of Biofilm Formation

The effect of leaves extract of *Malva parviflora* to inhibit biofilm formation was measured using microplate based assay, modified from (Stepanovic et al., 20007). Briefly, the bacterial cells were grown in tryptic soy broth (TSB) at 37°C overnight. The cultures was then harvested by centrifugation (14,000 rpm, 15 min at 4°C) and rinsed with phosphate-buffered saline (PBS, pH 7.4). The washed bacterial cells were then resuspended in tryptic soy broth to approximately 1×10^7cfu/ml (determined by plate count assay).

An amount of 100µL of the leaves extract of *Malva parviflora* solution was then pre-mixed with the bacterial inocula (100µL) to attain final concentration ranging from 125 – 1000 mg/ml. One hundred microliters of this mixture for each concentration was added to three separate wells in the 96-wellmicroplates for replicate testing. Wells containing mixture of dilute DMSO and inoculum were included as control with DMSO final concentration of 1% (v/v). After 24 h of incubation at 37°C, the supernatant containing TSB and planktonic cells were gently removed from the microplate wells and the wells were washed twice with 150 µL of PBS.

After rinsing, 100 µl of TSB were added and the suspension was incubated for 24 h at 37°C. The minimum biofilm inhibitory concentration (MBIC) is defined as the lowest concentration of an antimicrobial agent required to inhibit the formation of biofilms was determined by observing the growth of bacteria in the microplate wells.

2.5.3 Eradication of Biofilms

Biofilm bacterial culture was set up by a modification of an established method of (Deighton et al., 2001). Briefly, a bacterial suspension of McFarland 0.5 (~ 108 CFU/ml) was diluted1:100 into Trypticate Soya Broth (TSB). A hundred microliters of the diluted bacterial suspensions were pipetted into each well in a 96-well flat-bottom microplate and incubated for 18-24 h at 35°C without shaking. After overnight incubation, the bacterial suspensions were aspirated and the wells were rinsed twice with 100 µL of phosphate buffer saline (PBS) per well to remove non adherent bacteria.

The biofilms established for 24 hours in the each well were subsequently treated with two-fold serial dilutions of leaves extract of *Malva Parviflora* extract (2000 mg/ml as the highest in-test concentration). The microtiter plates were sealed with parafilm tape and incubated overnight at 37°C. After overnight incubation, the suspensions were

discarded and washed twice times with 100 µL of PBS and new broth were added for another 24 h. After 24 h. incubation 150 µl of the contents in each well of the microplate was serially diluted and plated onto solid media for viable count, this method is defined as the broth recovery method according to (Yue et al., 2009).

2.6 Statistical Methods

Data obtained were statistically analyzed using Analysis of Variance (ANOVA) Microsoft EXCEL 2010.

Table 1. Biological activity of ethanol extract of leaves of *Malva parviflora* (mg/ml) against resistant clinical isolates strains

Resistant clinical isolates	*Malva parviflora*ethanol extract			
	MIC (mg/ml)	MBC (mg/ml)	MBIC (mg/ml)	MBEC (mg/ml)
Kl.pneumonia isolate 1	125	250	500	1000
Kl.pneumonia isolate 2	125	250	500	1000
Kl.pneumonia isolate 3	250	250	250	2000
Kl.pneumonia isolate 4	125	250	500	2000
Kl.pneumonia isolate 5	250	250	500	1000
Kl.pneumonia isolate 6	250	250	500	2000
Kl.pneumonia isolate 7	250	250	500	2000
Kl.pneumonia isolate 8	125	250	250	1000
Kl.pneumonia isolate 9	250	250	500	2000
Kl.pneumonia isolate10	125	500	250	2000
Kl.pneumonia isolate11	250	250	500	1000
Kl.pneumonia isolate12	125	500	500	10000
S. aureus isolate 1	62.5	125	250	500
S. aureus isolate 2	31.25	125	250	1000
S. aureus isolate 3	31.25	125	250	500
S. aureus isolate 4	31.25	125	250	1000
S. aureus isolate 5	62.5	125	250	1000
S. aureus isolate 6	31.25	125	250	1000
S. aureus isolate 7	31.25	125	250	1000
S. aureus isolate 8	31.25	125	250	1000
S. aureus isolate 9	62.5	125	250	1000
S. aureus isolate 10	31.25	125	250	500
S. aureus isolate 11	31.25	125	250	1000
S. aureus isolate 12	31.25	125	250	1000
S. aureus isolate 13	31.25	125	500	1000
S. aureus isolate 14	31.25	125	500	1000
S. aureus isolate 15	31.25	125	250	500
S. aureus isolate 16	31.25	62.5	500	1000
S. aureus isolate 17	31.25	62.5	500	1000
S. aureus isolate 18	31.25	62.5	250	1000
E. coli isolate 1	31.25	125	250	500
E. coli isolate 2	31.25	125	500	1000
E. coli isolate 3	31.25	125	250	500
E. coli isolate 4	31.25	250	250	1000
E. coli isolate 5	31.25	125	500	1000
E. coli isolate 6	31.25	125	500	1000
E. coli isolate 7	62.5	250	500	1000
E. coli isolate 8	62.5	250	500	1000
E. coli isolate 9	62.5	250	500	1000
E. coli isolate 10	62.5	250	500	1000

3. Results

3.1 Isolated Bacteria

The 40 selected isolates were distributed as following: 12 *Klebseilla pneumonia*, 18 *Staphylococcus aureus*, and 10 *Escherichia coli*.

3.2 Biofilm Mass

All test strains formed biofilms were detected by the crystal violet assay. The most substantial biofilms were produced by *Klebseilla pneumonia* isolate no 1, 4, 5, 6, 7 as shown in figure 1 (C4, C9, D8, F1, F8) respectively.

3.3 Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC)

In this experiment the ethanol extract of leaves of *Malva parviflora* was tested for the inhibitory effect against growth of planktonic bacteria of clinical pathogens.

The potency was quantitatively assessed by the MIC, MBC, MBIC and MBEC as presented in Table 1 and qualitatively assessed MBIC and MBEC by crystal violet as shown figure 2.

The results revealed that MIC and MBC of leaves extract of *Malva parviflora* ranged from (125-250 mg), (250-500 mg) respectively for *Klebseilla pneumonia* while in case of *Staphylococcus aureus* it was (31.25-62.5 mg), (62.5-125 mg) respectively and with *E. coli* it was (31.25-62.5 mg), (125-250 mg) respectively.

Also, the MBIC and MBEC ranged from (250-500mg), (1000-2000 mg) respectively for *Klebseilla pneumonia* while in case of *Staphylococcus aureus* and *E. coli* it was (250-500 mg), (500-1000 mg) and (250-500 mg), (500-1000 mg) respectively.

4. Discussion

Biofilms are robust communities of microbes that are held by extracellular matrix .They can form on almost any surface where they can potentially cause disease or contaminate medical devices. Due to the numerous challenges of biofilms to health care, there is a continuous need to search for new antimicrobial compounds that can handle incidences and re-infection that could arise from biofilm contaminated medical devices (Adesina et al., 2015).

Herbal drugs have received greater attention in recent times because of their diversity of curing diseases, safety and being well tolerated remedies when compared to the conventional medicines. Biofilm infections represent a serious health threats worldwide today mostly due to the appearance of antibiotic resistant strains. Contemporary testing on MIC which measures only planktonic susceptibility may be the possible explanation for treatment failures and resistant development among bacterial biofilms (Perumal & Mahmud, 2013).

In the present study, the results of the MIC, MBC, MBIC and MBEC have highlighted the interesting activity of ethanol extract of leaves of *Malva parviflora* leaf extracts against *Klebseilla pneumonia*, *Staphylococcus aureus* and *E. coli*.

Medicinal plants are rich of secondary metabolites which some of them are directly involved in plant defense mechanisms against microorganisms (Cowan, 1999).

To begin our investigation on antibacterial and antibiofilm activities, the growth inhibition effect of ethanol extract of leaves of *Malva parviflora* was determined first on planktonic cultures of resistant clinical isolates. Ethanol extract of leaves of *Malva parivflora* exhibited broad antibacterial spectrum against gram positive and gram negative tested. The difference in susceptibility between gram negative and gram positive may be due to different cell wall composition according to (Fennel et al., 2004) Gram positive bacteria are often found to be more susceptible to plant extracts than the Gram negative bacteria. It is well known that the outer membrane present only in the Gram negative bacteria play an important role as an effective barrier.

Ethanol extract of leaves of *Malva parviflora* displayed distinct bactericidal mode of action against most of the bacteria tested. The bactericidal activity is confirmed by the obtained MBC values which are usually two to four higher than the corresponding MICs.

Ethanol extract of leaves of Malva parviflora showed antimicrobial activity against gram negative and gram positive bacteria. This finding agree with (Islam et al., 2010; Miri et al., 2013; Kalayou et al., 2012; Ododo et al., 2016).

Ethanol extract of leaves of *Malva parviflora* exhibited antibiofilm activity against all resistant isolates. The MBEC was 4 times that of planktonic bacteria This is consistent with previous reports indicating that bacteria in biofilms are more resistant to antibiotics and chemical agents than planktonic cells in suspension (Stewart, 2001).

The increase in MBIC and MBEC due to bacteria living as biofilm are often difficult to eradicate compared to the planktonic mode of growth. Planktonic cells forms biofilms by adhering to each other strongly via formation of pili (Permaul & Mahud, 2013).

Antibiofilm effect may be due to presence of 2S albumin proteins (Salas et al., 2015), these proteins have the characteristic molecular weight, cationic residues, and disulfide bonds of antimicrobial peptides (Nawrot et al., 2014). Antimicrobial peptides kill pathogens by interaction with phospholipids and membrane permeabilization (Nawrot et al., 2014). Also , antibiofilm effect may be due to the presence of β-sitosterol (Ododo et al., 2016; Abdel-Ghani et al., 2013) which is a natural product found in the cells and membranes of all oil-producing plants, fruit, vegetables, grains, seeds and trees (Sen et al.,2012). Leaves of *Malva parviflora* were reported to contain β-sitosterol which has been proven to be a safe, nontoxic, effective nutritional supplement and has amazing potential health benefits in many diverse applications including antibacterial activity (Sen et al., 2012).

Also, leaves of *Malva parviflora* contains gallic acid (Ereifej et al., 2015) and it was reported that gallic acid has antibiofilm activity against *Staphylococcus aureus* (Liu et al., 2017).

In conclusion, based on the obtained result ethanol extract of leaves of *Malva parviflora* may provide a new gate for treatment of biofilm related infections. The antibiofilm activities of the studied plant need more investigation as this is preliminary work.

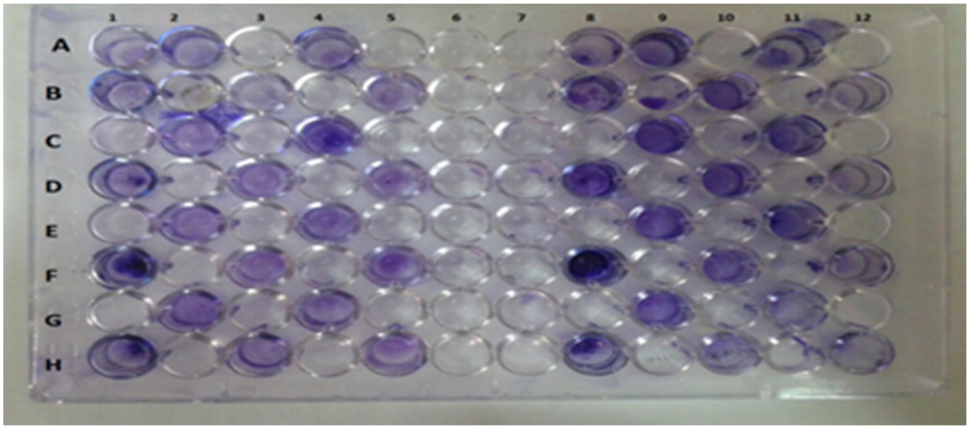

Figure 1. Crystal violet stained biofilm of *Klebseilla pneumonia* **.**The most substantial biofilms were produced by *Klebseilla pneumonia* (C4,C9 , D8,F1, F8) respectively

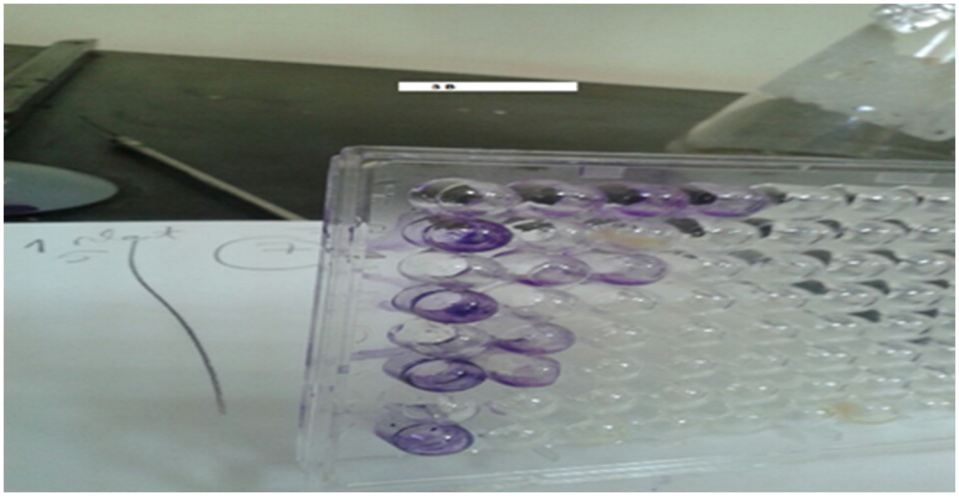

Figure 2. Qualitive determination MBIC and MBEC by crystal violet of biofilm of *Klebseilla pneumonia* after addition of ethanol extract of leaves of *Malva parviflora*

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