Higher Biofilm Formation by Multi-Drug Resistant *K. pneumoniae* and *K. rhinoscleromatis* Strains and Effects of Lemon and Ginger Essential Oils on Biofilm Formation

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ABSTRACT

Background: *Klebsiella* strains are opportunistic pathogens forming biofilms on many surfaces. Nowadays some resistance traits' acquirements via gene transfer mechanisms in biofilm environments cause an urgency to discover new substances targeting biofilm inhibition. **Objective:** The focus of this study is to examine different *K. pneumoniae* and *K. rhinoscleromatis* strains' clinical information and antibiotic resistance results according to their biofilm formation levels and to determine lemon and ginger essential oils' effects on biofilm formation. **Methods:** Biofilm formation of different *Klebsiella* strains and effects of ginger and lemon essential oils on biofilm formation were determined by Crystal Violet Binding assay. **Results:** Strong biofilm forming *K. pneumoniae* strains were more frequently observed to be isolated from blood sample. 80% of multi-drug resistant *K. pneumoniae* strains were strong biofilm forming and all multi-drug resistant *K. rhinoscleromatis* strains were intermediate biofilm formers. 170 µl/mL of lemon essential oil caused 48.3% (±13.7) and 83.4% (±7.2) decreases in biofilm formation amounts of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains respectively. **Conclusion:** According to our results; multi-drug resistant *K. pneumoniae* and *K. rhinoscleromatis* strains form higher amount of biofilms and lemon essential oil might be used as a new anti-biofilm agent, targeting the inhibition of biofilm formation of *K. pneumoniae* and K. *rhinoscleromatis* strains.

Key words : Anti-Biofilm, Biofilm Formation, Ginger Essential Oil, *Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*, Lemon Essentil Oil.

INTRODUCTION

Klebsiella species, which exist as commensal microorganisms in human upper respiratory and human gastrointestinal tracts, are also known as opportunistic pathogens causing; bacteremia, neonatal sepsis, urinary tract and wound infections especially in immuno compromised individuals[1-3] and within them, while *Klebsiella pneumoniae* is known as one of the most frequent agents of catheter-associated urinary tract infections (CAUTIs), *Klebsiella rhinoscleromatis* strains exist as an etiological agent of rhinoscleroma disease and rarely as an agent of bacteremia.[2,4-5] By means of providing microorganisms to encase themselves and serving as a source for recurrent nosocomial infections, biofilm formation is known as one of the most important mechanisms making these commensal inhabitants dangerous both for indwelling medical device using and immuno compromised patients.[6-7] Additionally, as a result of including huge amount of extracellular DNA molecules and providing distinct microorganisms to come together and stay immobile inside them, biofilm environments also promote genetic acquirement of antimicrobial resistance genes for several microorganisms. In this respect, the possibility of some resistance traits' acquirement via gene transfer within biofilms causes an urgency to discover new substances targeting the inhibition of biofilm formation.[8-10]

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The aim of this study is to examine different *K. pneumoniae* and *K. rhinoscleromatis* strains' clinical information and antibiotic resistance results according to their biofilm formation levels and to determine lemon and ginger essential oils (EOs) effects on biofilm formation.

MATERIALS AND METHODS

Bacterial strains

In this study, *K. pneumoniae* and *K. rhinoscleromatis* strains which were obtained from different clinical materials and identified in Urkmez (2009)'s previous study were used.[11] All isolated strains were inoculated in to the Brain Heart Infusion Broth media including 10% glycerol and stored at -20°C.

Biofilm formation

The determination of biofilm formation in *K. pneumoniae* and *K. rhinoscleromatis* strains was performed by Crystal Violet Binding Assay described by O'Toole with some modifications.[12] Briefly, bacterial cells corresponding to a 2.0 McFarland optical density standard were inoculated into Brain Heart Infusion Broth medium and were incubated at 37°C at 24 h. After incubation, the culture was 1:100 diluted into a fresh BHI medium and the wells of a polysytrene plate were filled with 1 ml of the diluted inoculum. Then, the plates were incubated for 24 h at 37°C. Following this, the wells were gently washed and stained with 1% crystal violet for 45 min at room temperature. After washing the wells again, bound crystal violet in each well was solubilized by Ethanol (96.6%) solution and solubilized crystal violet for each well was read by a spectrofotometer at 540 nm. *Klebsiella* strains, having an OD value ≥ 0.4, were evaluated as biofilm producers and classified into three categories as follows:

0.4 ≤ OD < 0.8 Weak Biofilm Former (WBF)

0.8 ≤ OD < 1.2 Intermediate Biofilm Former (IBF)

OD ≥ 1.2 Strong Biofilm Former (SBF)

Effects of ginger and lemon essential oils on biofilm formation of Klebsiella pneumoniae and Klebsiella rhinoscleromatis strains

Essential oils of ginger and lemon were purchased from NU-KA Defne Essencia, TURKEY. Bacterial cells corresponding to a 2.0 McFarland optical density standard were inoculated into Brain Heart Infusion Broth medium and were incubated at 37°C for 24 h. 1:100 diluted inoculums of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains were incubated with 170 µl/mL and 1.7 µl/mL concentrations of ginger and lemon EOs for 24 h at 37°C and the biofilm formations were analyzed via "Crystal Violet Binding Assay" which has been described previously. The wells which does not include any concentration of essential oils, were evaluated as controls. Increases (%) or decreases (%) in biofilm formation were calculated by the following formula:

$$\%\ \text{of Decrease} = \frac{[(\text{OD Control} - \text{OD Treatment})}{\text{OD control}]} \cdot 100$$

$$\%\ \text{of Increase} = \frac{[(\text{OD Treatment} - \text{OD Control})}{\text{OD control}]} \cdot 100$$

Statistical Analysis

All experiments were done in triplicate and data were compared using Student's t-test, accepting $p<0.05$ as statistically significant.

RESULTS

In this study, *K. pneumoniae* and *K. rhinoscleromatis* strains which were obtained from different clinical materials and service units were classified as strong, intermediate and weak biofilm formers, according to their biofilm formation levels. When the occurrences of *K. pneumoniae* and *K. rhinoscleromatis* strains in different clinical materials are examined, all *K. pneumoniae* strains isolated from clinical materials of tracheal aspirate and blood were SBF (Figure 1) and SBF *K. pneumoniae* strains were more frequently observed to be isolated from blood sample (Figure 1). In addition; all *K. pneumoniae* strains isolated from; service units of otorhinolaryngology, internal medicine and surgical intensive care and all *K. rhinoscleromatis* strains isolated from urology unit were SBF (Figure 2).

Antibiotic resistance patterns of *K. pneumoniae* and *K. rhinoscleromatis* strains have been determined previously in Urkmez's study[11] and when the multi-drug resistant *K. pneumoniae* and *K. rhinoscleromatis* strains are examined according to their biofilm formation levels, 80% of MDR *K. pneumoniae* strains were observed as SBF and all MDR *K. rhinoscleromatis* strains were IBF (Figure 3). In the last part of this study, effects of lemon and ginger essential oils on biofilm formation of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains were determined. According to our results, 170 µl/mL and 1.7 µl/mL concentrations of ginger EO did not cause any significant decrease/increase on biofilm formations of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains (Figure 4 and 5). When the effect of 170 µl/mL concentration of lemon EO is examined; 48.3% (± 13.7) and 83.4% (± 7.2) decreases in biofilm

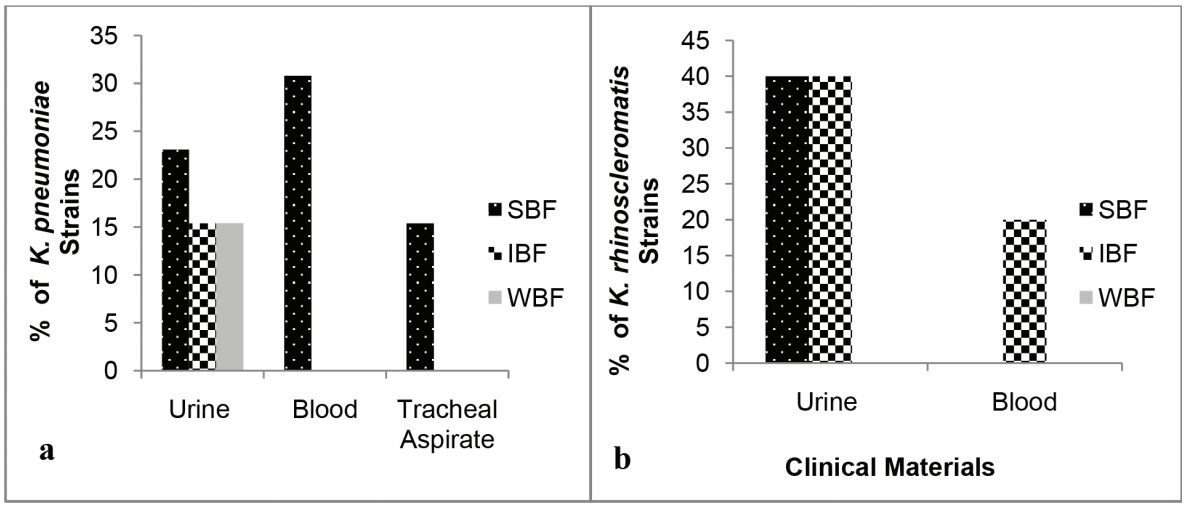


Figure 1: Percentage of (a) SBF, IBF and WBF *Klebsiella pneumoniae* strains (b) SBF, IBF and WBF *Klebsiella rhinoscleromatis* strains in different clinical materials

(*SBF: Strong Biofilm Formers, IBF: Intermediate Biofilm Formers, WBF: Weak Biofilm Formers*).

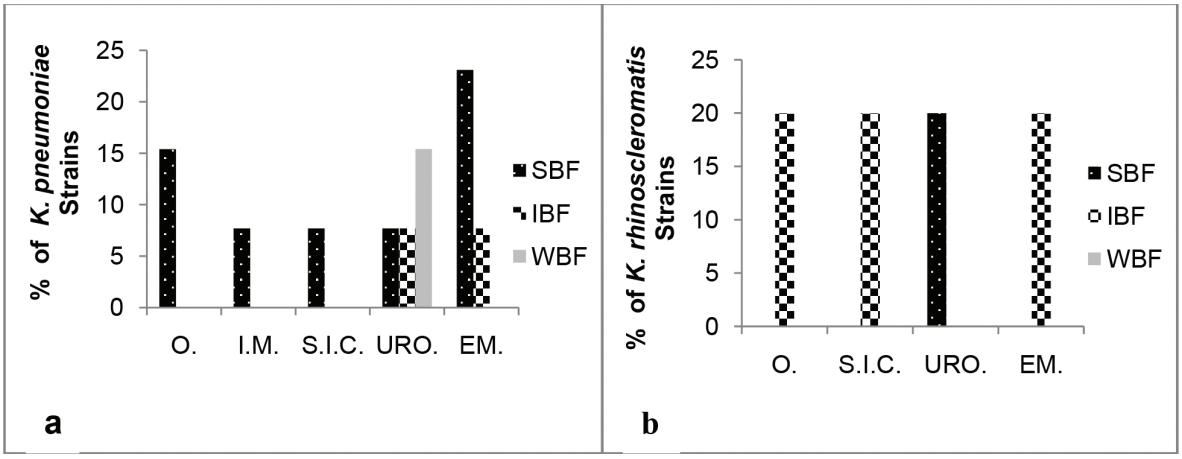


Figure 2: Percentage of (a) SBF, IBF and WBF *Klebsiella pneumoniae* strains (b) SBF, IBF and WBF *Klebsiella rhinoscleromatis* strains, in different service units

O.: Otorhinolaryngology, I.M.: Internal Medicine, S.I.C.: Surgical Intensive Care, URO.: Urology, EM.: Emergency, SBF: Strong Biofilm Formers, IBF: Intermediate Biofilm Formers, WBF: Weak Biofilm Formers).

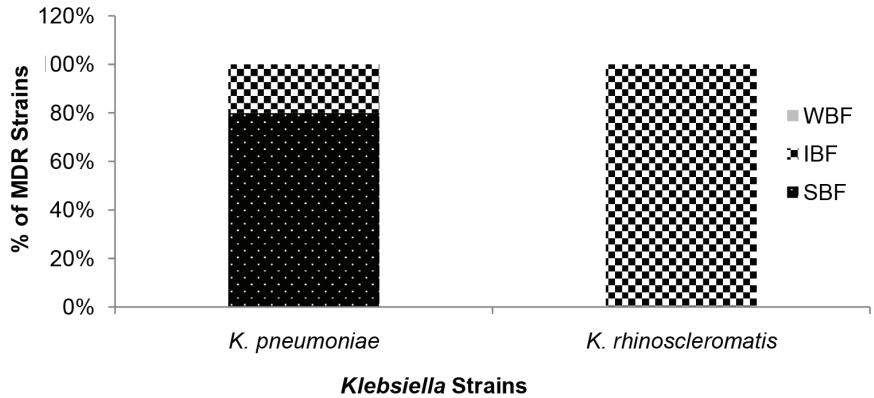


Figure 3: Percentages of Multi-Drug Resistant *K. pneumoniae* and *K. rhinoscleromatis* strains according to their biofilm formation levels

(*SBF: Strong Biofilm Formers, IBF: Intermediate Biofilm Formers, WBF: Weak Biofilm Formers*).

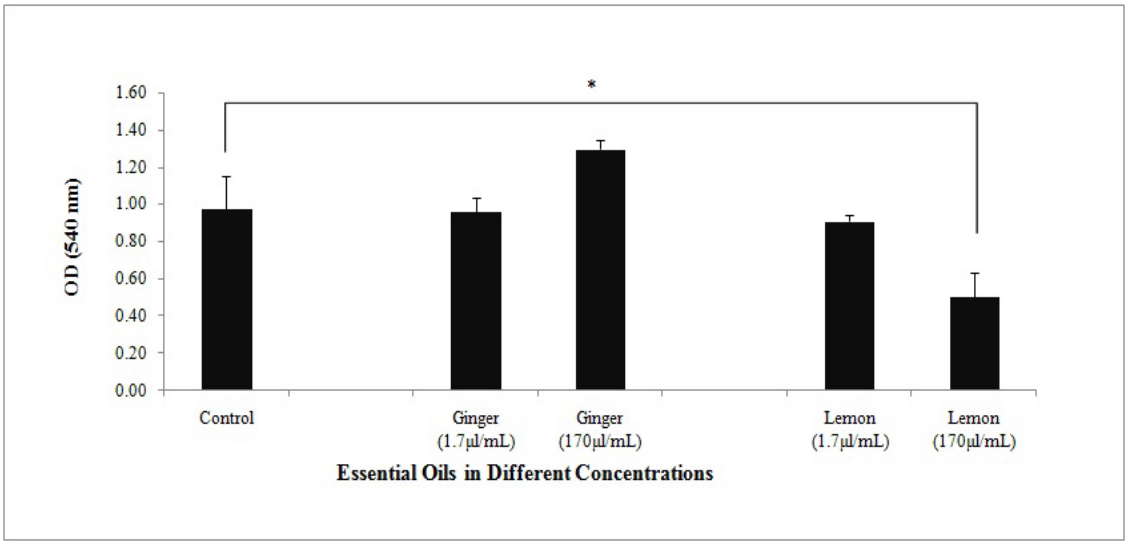


Figure 4: Effects of lemon and ginger essential oils on biofilm formation of strongest biofilm forming *K. pneumoniae* strain
* indicates a significant difference (Student's t-test; P < 0.05).

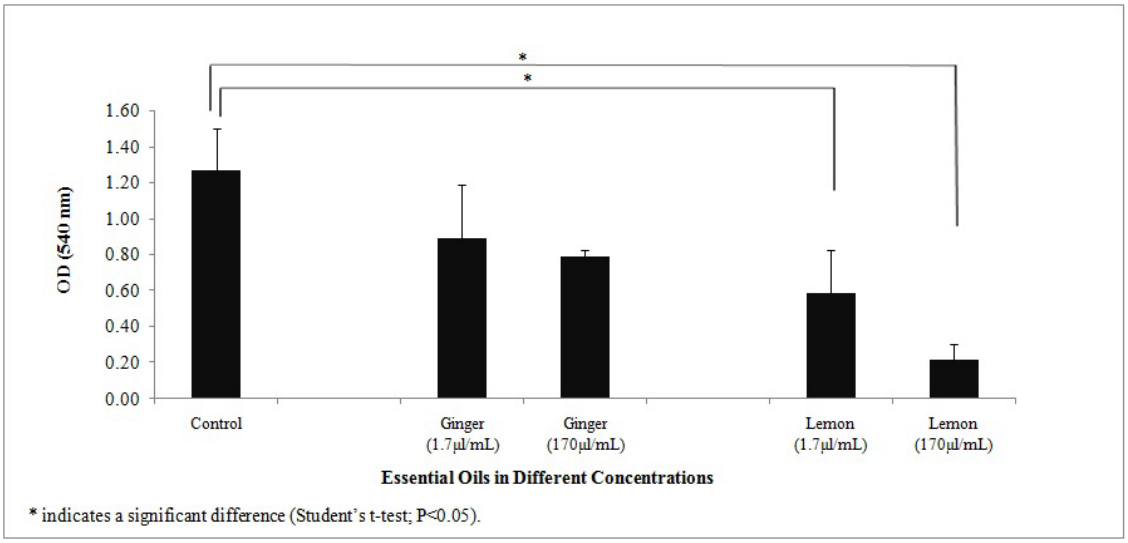


Figure 5: Effects of lemon and ginger essential oils on biofilm formation of strongest biofilm forming *K. rhinoscleromatis* strain
* indicates a significant difference (Student's t-test; P < 0.05).

formation amounts of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains were observed respectively (Figure 4, 5). Furthermore, lemon EO's 1.7 µl/mL concentration also caused a significant decrease in biofilm formation amount of strongest biofilm forming *K. rhinoscleromatis* strain (Figure 5).

DISCUSSION

Biofilms, which are known as organized matrix-enclosed communities adhering to particular living and non-living surfaces, play a major role in development of many nosocomial infections.[3] By means of providing an inert surface for the attachment of diverse microorganisms and thereby enhancing microbial colonization; indwelling biomaterials are especially known to favor biofilm formation of many pathogens such as different *Klebsiella* species.[13] Biofilm formation by these pathogenic bacteria is believed to inhibit the effectiveness of antibiotic treatment and facilitate bacterial communication leading to expression of some other virulence determinants both on particular tissues of immuno compromised hosts and on diverse indwelling biomaterials.[14] Therefore; determining biofilm formations of different *K. pneumoniae* and *K. rhinoscleromatis* strains, examining their biofilm formations according to their clinical information and

anti-microbial resistances, and investigating natural substances that can able to inhibit their biofilm formation will provide development of more comprehensive preventing strategies against *Klebsiella* related biofilm infections both in immuno compromised and indwelling biomaterial using individuals.

According to our results, similar with one of the recent studies,[15] all *K. pneumoniae* strains isolated from blood samples were SBF and blood was the most frequent clinical material for SBF *K. pneumoniae* isolation (Figure 1). When the studies applied on blood stream infections is examined; immuno suppression and extended duration of catheter placement are indicated as main risk factors for nosocomial bloodstream infections.[16-18] Therefore, by means of providing a favorable surface for bacterial attachment, Central Venous Catheters (CVCs) might be one of the reasons of biofilm related blood stream infections.[19] Apart from this, a research applied in United States indicates that; most frequent nosocomial infection sites (blood stream, urinary, and respiratory tract) in medical-surgical intensive care units (MS ICUs) were almost always found to be associated with use of an indwelling device.[20] Therefore, by means of being responsible from blood stream infections, indwelling devices also may emerge as reason of many nosocomial infections in surgical intensive care units. Hence, our finding which shows that all *K. pneumoniae* strains isolated from service unit of surgical intensive are SBF; may also be the result of enhanced indwelling biomedical device usage.

When the prevalence of *K. rhinoscleromatis* strains according to different clinical materials are examined; it was surprising to observe the isolation of these common respiratory tract infection (Rhinoscleroma) agents from sample of urine in higher frequency[5] and 50% of these *K. rhinoscleromatis* strains which were isolated from urine were SBF.

Nowadays, many investigations on correlation between biofilm production and multiple drug resistance in clinical isolates of different strains are being applied.[21-24] Because, by means of including huge amount of extra cellular DNA and providing too many distinct bacterial cells to stay in close contact and immobile, biofilm environments are known to favor genetic exchange of some anti-microbial resistance genes. Therefore, they are suggested to increase bacterial virulence and contribute to the development of multi resistant phenotypes.[9, 10, 25-26] Confirming these, in our study; 80% of MDR *K. pneumoniae* strains were observed as SBF and all MDR *K. rhinoscleromatis* strains were IBF (Figure 3).

Today, the usage of new synthetic derivatives or synthetic antibiotics is regarded as unsafe by means of their side effects[27] and biofilm mode of growth causes an urgency to discover new substances targeting biofilm inhibition. Thus, new antimicrobials like medicinal plants which have little or no toxic effect are being considered as potential sources for the inhibition of biofilm associated infections.[28] Essential oil of lemon which is extracted from *Citrus limonum* and known to pose an antioxidant activity[27] was observed to inhibit biofilm formations of *K. pneumoniae* and *K. rhinoscleromatis* in our study. Similar to our finding, one of the recent studies shows that, essential oil of *Citrus limonum* displays highest biofilm inhibition activity and greatest AI-2 (Quorum Sensing mediating molecule) reduction activity (96%) in *Campylobacter jejuni* strains.[29] Additionally, *Citrus limonum* is also indicated to reduce the levels of expression of two genes (fla A and fla B) which are crucial for bacterial pathogenesis and play an important role in biofilm formation, in highest level.[29]

CONCLUSION

In conclusion, this study shows that; MDR *K. pneumoniae* and *K. rhinoscleromatis* strains display higher biofilm formation and essential oil of lemon might be used as a new anti-biofilm agent, targeting the inhibition of biofilm formation of *K. pneumoniae* and *K. rhinoscleromatis* strains in the future. Therefore, this study not only throws light on many studies carried out on transformation of harmless commensal human natural colonizers into multidrug resistant nosocomial pathogens but also exhibits a new plant substance, that inhibits biofilm formation of *K. pneumoniae* and *K. rhinoscleromatis* strains.

CONFILCTS OF INTEREST

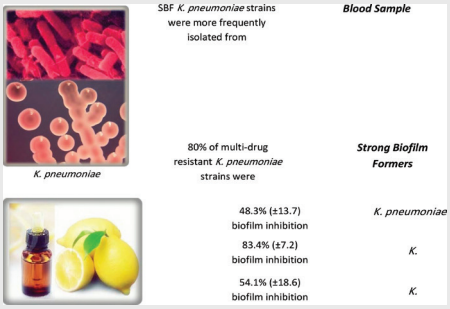
The authors declare that, there are no conflicts of interest.

REFERENCES

1. Martino PD, Cafferini N, Joly B, Darfeuille-Michaud A. *Klebsiellap neumoniae* type 3 pili facilitate adherence and biofilm formation on abiotic surfaces. Res Microbiol. 2003;154(1):9-16.
2. Stock I, Wiedemann B. Natural antibiotic susceptibility of *Klebsiella pneumoniae*, *K. oxytoca*, *K. planticola*, *K. ornithinolytica, K. terrigena* strains. J Med Microbiol. 2001;50(5):396-406.
3. Schroll C, Barken KB, Krogfelt KA, Struve C. Role of type 1 and type 3 fimbriae in *Klebsiella pneumoniae* biofilm formation. BMC Microbiol. 2010;10(1):179.
4. Brisse S, Grimont F, Grimont PAD. The Genus *Klebsiella*. The Prokaryotes 2006;159-96.
5. Pound MW, Fulton KB, Chima CO. Successful treatment of *Klebsiella rhinoscleromatis* bacteremia with levo floxacin. Pharmacotherapy. 2007;27(1):161-3.

6. Hancock V, Witsø IL, Klemm P. Biofilm formation as a function of adhesin, growth medium, substratum and strain type. Int J Med Microbiol. 2011;301(7):570-6.
7. Magesh H, Kumar A, Alam A, Priyam, Sekar U, Sumantran VN, *et al*. Identification of natural compounds which inhibit biofilm formation in clinical isolates of *Klebsiella pneumoniae*. Indian J Exp Biol. 2013;51(9):764-72.
8. Kumar CG, Anand SK. Significance of microbial biofilms in food industry: a review. Int J Food Microbiol. 1998;42(1):9-27.
9. Parsek MR, Singh PK. Bacterial Biofilms: An Emerging Link to Disease Pathogenesis. Annu Rev Microbiol. 2003;57(1):677-701.
10. Subramanian P, Shanmugam N, Sivaraman U, Kumar S, Selvaraj S. Antibiotic resistance pattern of biofilm-forming uropathogens isolated from catheterised patients in Pondicherry, India. Australas Med J. 2012;5(7):344-8.
11. Urkmez NH, Seyis Bilkay I. Investigation and typing of antibiotic resistance and plasmid profiles of *Klebsiella* strains, *Master Thesis*, University of Hacettepe, Turkey (in Turkish) 2009.
12. O'Toole GA. Microtiter dish biofilm formation assay. J Vis Exp. 2011;30(47):2437.
13. Stahlhut SG, Struve C, Krogfelt KA, Reisner A. Biofilm formation of *Klebsiella pneumoniae*on urethral catheters requires either type 1 or type 3 fimbriae. FEMS Immunol Med Microbiol. 2012;65(2):350-9.
14. Lavender HF, Jagnow JR, Clegg S. Biofilm formation *in vitro* and virulence *in vivo* of mutants of *Klebsiella pneumoniae*. Infect Immun. 2004;72(8):4888-90.
15. Nahar A, Anwar S, Saleh AA, Miah MRA. Virulence Factors and Antibiotic Susceptibility Pattern of *Acineto bacter* Species in a Tertiary Care Hospital in Bangladesh. Ibrahim Med Coll J. 2012;6(1):27-30.
16. Helou GE, Viola GM. Rapidly growing mycobacterial bloodstream infections. Lancet Infect Dis. 2013;13(2):166-74.
17. Reilly AF, McGowan KL. Atypical mycobacterial infections in children with cancer. Pediatr Blood Cancer. 2004;43(6):698-702.
18. Swanson DS. Central venous catheter-related infections due to nontuberculous mycobacterium species. Pediatr Infect Dis J. 1998;17(12):1163-4.
19. Esposito S, Purrello SM, Bonnet E, Novelli A, Tripodi F, Pascale R, *et al*. Central venous catheter-related biofilm infections: An up-to-date focus on meticillin-resistant *Staphylococcus aureus*. J Global Antimicrobial Resistance. 2013;1(2):71-8.
20. Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol. 2000;21(8):510-5.
21. Dheepa M, Rashme VL, Appalaraju B. Comparision of biofilm production and multiple drug resistance in clinical isolates of *Acineto bacter baumanii* from a tertiary care hospital in South India. Int J Pharm Biomed Sci. 2011;2(4):103-7.
22. Eyoh AB, Toukam M, Atashili J, Fokunang C, Gonsu H, Lyonga EE, *et al*. Relationship between multiple drug resistance and biofilm formation in *Staphylococcus aureus* isolated from medical and non-medical personnel in Yaounde, Cameroon. Pan Afr Med J. 2014;17:186.
23. Rao RS, Karthika RU, Singh SP, Shashikala P, Kanungo R Jayachandran S *et al*. Correlation between biofilm production and multiple drug resistance in imipenem resistant clinical isolates of *Acineto bacter baumannii*. Indian J Med Microbiol. 2008;26(4):333-7.
24. Sahal G, SeyisBilkay I. Multi drug resistance in strong biofilm forming clinical isolates of *Staphylococcus epidermidis*. Braz J Microbiol. 2014;45(2):539-44.
25. de Araujo GL, Coelho LR, de Carvalho CB, Maciel RM, Coronado AZ, Rozenbaum R *et al*. Commensal isolates of methicillin-resistant *Staphylococcus epidermidis* are also well equipped to produce biofilm on polystyrene surfaces. J Antimicrob Chemother. 2006;57(5):855-64.
26. Król JE, Nguyen HD, Rogers LM, Beyenal H, Krone SM, Top EM *et al*. Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. Appl Environ Microbiol. 2011;77(15):5079-88.
27. Shaik G, Sujatha N, Mehar SK. Medicinal plants as source of antibacterial agents to counter *Klebsiella pneumonia*. J App Pharm Sci. 2014;4(01):135-47.
28. Derakhshan S, Sattari M, Bigdeli M. Effect of cumin (*Cuminumcyminum*) seed essential oil on biofilm formation and plasmid Integrity of *Klebsiella pneumoniae*. Pharmacogn Mag. 2010;6(21):57-61.
29. Castillo S, Heredia N, Arechiga-Carvajal E, García S. Citrus Extracts as Inhibitors of Quorum Sensing, Biofilm Formation and Motility of *Campylobacter jejuni*. Food Biotechnol. 2014;28(2):106-22.

PICTORIAL ABSTRACT



ABBREVIATIONS USED

BHI: Brainheart Infusion Broth; **EO:** Essential Oil; **OD:** Optical Density; **WBF:** Weak Biofilm Former; **IBF:** Intermedaite Biofilm Former; **SBF:** Strong Biofilm Former; **MDR:** Multi-Drug Resistant; **CAUTIs:** Catheter-Associated Urinary Tract Infections; **CVCs:** Central Venous Catheters; **MS ICU:** Medical-Surgical Intensive Care Units.

SUMMARY

- Strong biofilm forming *K. pneumoniae* strains were more frequently observed to be isolated from blood sample.
- 80% of multi-drug resistant *K. pneumoniae* strains were strong biofilm forming and all multi-drug resistant *K. rhinoscleromatis* strains were intermediate biofilm formers.
- 170 µl/mL of lemon essential oil caused 48.3% (±13.7) and 83.4% (±7.2) decreases in biofilm formation amounts of strongest biofilm forming *K. pneumoniae* and *K. rhinoscleromatis* strains respectively.
- 1.7 µl/mL of lemon essential oil caused 54.1% (±18.6) decrease in biofilm formation amount of strongest biofilm forming *K. rhinoscleromatis* strain.

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