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ANTIBIOFILM ACTIVITY AND THE SYNERGISTIC EFFECT OF LEMON GRASS ESSENTIAL OIL WITH ANTIBIOTICS AGAINST A.BAUMANNII COMPLEX – A PRELIMINARY REPORT

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ABSTRACT

Introduction- The epidemiology and antibiotic resistance of A.baumannii have been extensively reviewed but the pathogenesis and virulence remain unclear. Biofilm formation is a well-known pathogenic mechanism in device related infections. High incidence of resistance to pharmaceutical antibiotics among microbes in hospital environment prompts the search for alternative source of antimicrobial chemicals with anti-biofilm activity. **Materials and Methods-** Biofilm formation and antibiofilm activity of Lemon grass essential oil [LGO] in 251 Acinetobacter clinical isolates was detected by polystyrene tissue culture plate method and modified crystal violet assay respectively. The broad-spectrum antimicrobial activity of LGO in combination with antibiotics against multidrug resistant & extremely drug resistant A.baumannii complex was also investigated. **Results and Discussion-** Among 251 A.baumannii isolates, 70% isolates showed biofilm formation which indicated that they were established nosocomial pathogens. About 65-79% of anti-biofilm activity was seen at the dilutions 0.625 µl/ml to 0.156 µl/ml, indicating that LGO had a significant role in inhibition of biofilm formation by A.baumannii. There was almost two fold enhancement of the antibacterial activity of the antibiotics on A.baumannii when used in combination with LGO. **Conclusion-** The antibiofilm activity of Lemon grass essential oil with its potentiating effect on antimicrobials by twofold might open a new avenue for determining better treatment option for A.baumannii infections as well as preventing the dissemination of nosocomial MDR & XDR A.baumannii.

KEYWORDS: Cymbopogon flexuosus [Lemon grass], Essential oil [EO], Biofilm, Anti-biofilm activity, Synergy, MDR/XDR AB.

INTRODUCTION

The epidemiology and antibiotic resistance of *A.baumannii* have been extensively reviewed but the pathogenesis and virulence remain unclear. Even though there have been studies defining important bacterial components expressed by *A.baumannii*, very few reports are available defining the mechanisms of virulence and persistence.[1-2] Along the course of time, *A.baumannii* has gained virulence determinants which have made it an all-rounder in the nosocomial field. It is essential to understand the molecular mechanisms contributing to virulence to identify the novel drug targets.[3-4] The characteristics or factors that had made *A.baumannii* the most effective nosocomial pathogen includes AbaR resistance islands, biofilm formation, β-lactamase production, efflux pumps and outer membrane proteins that helps in adhesion.

About 60-70% of nosocomial or hospital acquired infections are associated with the implantation of a biomedical device. Biofilm formation is a well-known pathogenic mechanism in device related infections. Biofilms are assemblages of surface microbial cells that are enclosed in an extracellular polymeric matrix [EPS]. Biofilm formation increases resistance to detergents and antibiotics, as the dense extracellular matrix and the outer layer of cells protect the interior of the community. Only few studies were available reporting about the virulence or persistence strategies of *A.baumannii*.[1,5] Biofilm formation is an important feature of most clinical isolates of *Acinetobacter* spp. The ability of *Acinetobacter* species to adhere to the surfaces, form biofilms, display antibiotic resistance and gene transfer means that there is an urgent need to study the factors responsible for their spread. There are few studies demonstrating anti-biofilm activities of various agents such as antibiotics, chemicals and even herbal extracts.[6-10]

High incidence of resistance to pharmaceutical antibiotics among microbes in hospital environment prompts the search for alternative source of antimicrobial chemicals. According to recent CDC report, superbugs needed natural medicine since they resist nearly all antibiotics within the conventional drug armamentarium.[11] The optimal treatment for *A.baumannii*, especially nosocomial infections resulting from MDR/XDR strains, remains to be established. New experimental approaches are warranted to develop and evaluate novel therapeutic strategies for dealing with *A.baumannii* infections.

A largely unexploited resource in this regard is the herbal extracts used in traditional medicine. Historically plants have provided a source of inspiration for novel drug compounds. They serve as sources of valuable compounds with therapeutic potential. Essential oils are volatile, natural, fragrant liquid that are synthesized by plants through complex metabolic pathways and protect the plants from diverse pathogenic microbes. Due to their remarkable bioactivities, they could be used for the treatment of wide range of microbial infections.[12] According to El-Hosseiny, *et al*, plant essential oils might be used to enhance the activity of antibiotics against non-fermenters.[13]

In view of the finding that the volatile oils/essential oils from the plants inhibited the growth of various bacteria including *A.baumannii*, the aim of the present study was to investigate the frequency of biofilm formation and the effect of *Cymbopogon flexuosus*/Lemon grass Essential oil [LGO] on the biofilm formation in *Acinetobacter* clinical isolates. An attempt was also done to demonstrate the broad-spectrum antimicrobial activity of LGO alone and in combination with antibiotics against MDR & XDR *A.baumannii* complex so that it could be used as an economic and effective antiseptic topical agent against this resistant pathogenic microorganism.

MATERIALS AND METHODS

A total of 251 isolates of *Acinetobacter* were collected from various clinical samples from patients of ICU and chronically ill patients suffering from various forms of malignancies. The isolates were identified, confirmed and antimicrobial susceptibility testing was performed to detect MDR and XDR isolates.

a. Detection of Biofilm formation

According to the standard procedure[14] biofilm formation in these 251 *Acinetobacter* clinical isolates was detected by polystyrene tissue culture plate method**.** The optical density (OD) was measured at 570nm. The isolates were divided into 3 categories – non-adherent (biofilm –ve), weakly adherent (biofilm moderately +ve) and strongly adherent (highly +ve) based on the OD values.[14]

Mean value (OD in nm)	Adherence	Biofilm formation
< 0.120	Non-adherent	Negative
0.120 – 0.240	Weakly adherent	Moderately positive
>0.240	Strongly adherent	Highly positive

The number 0.120 was chosen for a guideline because it was 3 standard deviations (0.023) above the mean OD (0.050) of a clean tissue culture plate stained by the above method.[14]

b. Determination of Anti-biofilm activity of Lemon grass Essential oil: A modified crystal violet assay using tissue culture plate was employed to test the effect of lemon grass EO on biofilm formation in *A.baumannii*.[15] Ninety two biofilm producing isolates were chosen for the assay [34 highly positive and 58 moderately positive]. The optical density (OD) was measured at 570 nm using an ELISA microplate reader. All tests were performed in triplicates [Fig-1A,B]. The biofilm inhibition concentration [BIC_{50}] was defined as the lowest concentration of EO that showed 50% inhibition on the biofilm formation.

The percentage of biofilm inhibition [BIC_{50}] was calculated using the formula

$$\frac{[\text{OD of growth control} - \text{OD of sample}] \times 100}{\text{OD of growth control}}$$

Where growth control is bacteria+broth and sample is bacteria+broth+LGO.

c. Evaluation of Synergistic effect of Lemon grass essential oil with antibiotics

Antibacterial activity of Lemon grass oil with various dilutions was first checked against 251 MDR *Acinetobacter* clinical isolates by disc diffusion method.[16] Then assay to check the synergistic activity of LGO with antibiotics was carried out on these clinical isolates. The antibiotics chosen were those to which the isolates were previously resistant, such as Imipenem, Cefipime, Cotrimoxazole, Amikacin, Ciprofloxacin, Piperacillin/Tazobactum, Cefoperazone and Gentamicin.

The assay was done by Kirby-Bauer disc diffusion method.[13] The test isolates [standardized to 0.5 McFarlands turbidity] were lawn cultured on the dry surface of 2 Mueller Hinton Agar plates, one normal and another that contained 0.5% Tween 20 to enhance the diffusion of essential oil. In the first plate, which was normal, the above mentioned antibiotic discs [HiMedia] were placed on the lawn culture. In the second plate containing 0.5% Tween 20, same antibiotic discs impregnated with 2µl of the lemon grass essential oil were placed. The plate that had antibiotic plus LGO was

maintained at 4°C for two hours to facilitate the diffusion of LGO and then incubated at 37°C for 18-24 hours. The diameters of the resulted zones of inhibition were measured in millimetres. The test was repeated in triplicates with appropriate controls.

RESULTS

Among 251 *A.baumannii* isolates, about 70% isolates showed biofilm formation which indicated that they were established nosocomial pathogen [Table-1].

Table. 1: Biofilm formation in 251 *A.baumannii* isolates.

Biofilm positive 175 (69.7)	Highly positive n (%)	34 (13.5)
	Moderately positive n (%)	141 (56.2)
Biofilm negative	76 (30.3)	

$\chi^2 = 39.04$, p = 0.000 < 0.01, HS

Significantly higher number of Imipenem resistant *A.baumannii* isolates [75%] produced biofilm, indicating that biofilm formation not only contributed to the virulence, but helped in persistence and survival of the resistance strains in the hospital environment. [Table-2].

Table. 2: Biofilm production in correlation with Imipenem susceptibility.

Imipenem susceptibility	Total number of isolates n (%)	Biofilm production	
		Positive n (%)	Negative n (%)
Resistant	202 (80.5)	152 (75.2)	50 (24.8)
Sensitive	49 (19.5)	23 (46.9)	26 (53.1)

$\chi^2 = 14.9$, p = 0.000 < 0.01, HS

About 65-79% of anti-biofilm activity was seen at the dilutions 0.625 µl/ml to 0.156 µl/ml, indicating that lemon grass essential had a significant role in inhibition of biofilm formation by *A.baumannii* [Table-3].

Table. 3: Anti-biofilm activity of lemon grass EO on 92 biofilm positive *A.baumannii* isolates.

Number of isolates (%)	Percentage of biofilm inhibition	BIC_{50}
28 (30)	67	0.625 µl/ml
34 (37)	79	0.313 µl/ml
30 (33)	73	0.156 µl/ml

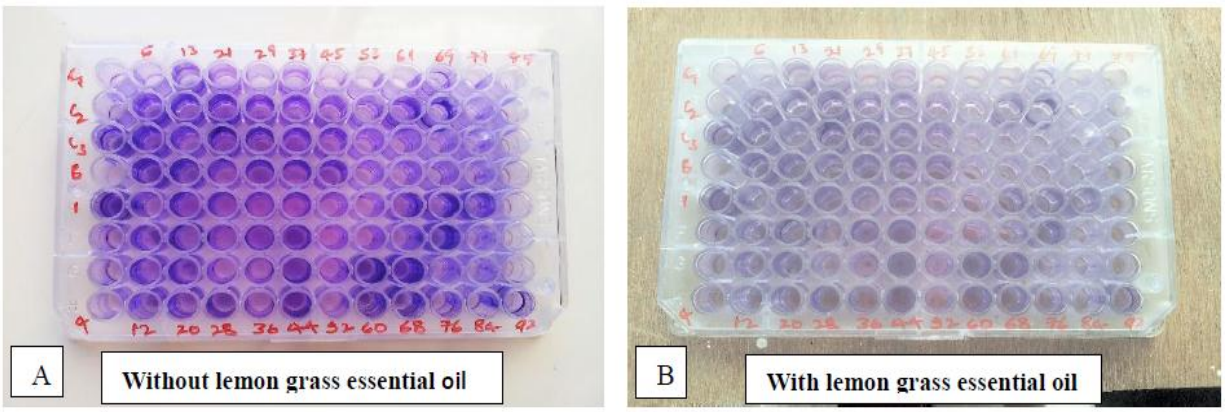

Fig. 1: A- Biofilm production & B- Anti-biofilm activity at 0.313 µl/ml dilution of LGO.

All the clinical the clinical isolates showed varying degree of susceptibility to various dilutions of lemon grass oil, i.e., 10-28 mm. Two fold enhancement of the antibacterial activity of the antibiotics on *A.baumannii* was observed when used in combination with lemon grass EO [Table-4 & Fig-2 A,B,C].

Table. 4: Synergistic action of lemon grass essential oil with antibiotics.

Antibiotics tested	Disc concentration	Zone of inhibition when tested alone (in mm)	Sensitivity pattern	Zone of inhibition with antibiotic & lemon grass EO (in mm)	Sensitivity pattern
Imipenem	10 mcg	6-20	Resistant	28-31	Sensitive
Cefipime	30 mcg	6- 14	Resistant	21	Sensitive
Co-trimoxazole	1.25/23.75 mcg	6-10	Resistant	21	Sensitive
Amikacin	30 mcg	10-16	Resistant	20-28	Sensitive
Ciprofloxacin	5 mcg	6-15	Resistant	22	Sensitive
Piperacillin/ Tazobactum	100/10 mcg	12-17	Resistant	25-26	Sensitive
Cefoperazone	75 mcg	6-15	Resistant	20	Sensitive
Gentamicin	10 mcg	6-12	Resistant	18-24	Sensitive
Lemon grass EO	5-10 microliters	10-28	Sensitive	21-44	Sensitive

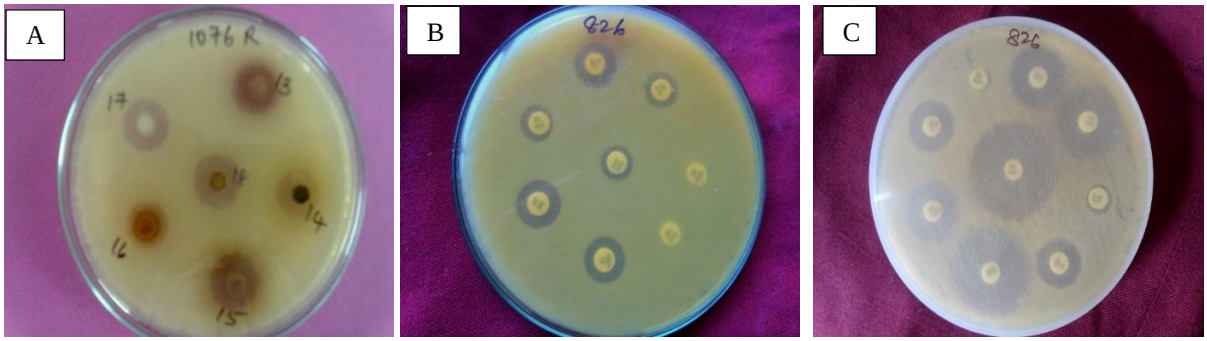

Fig. 2: A- Lemon grass EO alone; B-Antibiotics alone; C-Antibiotics in combination with LGO showing enhancement of zone of inhibition.

DISCUSSION

Plant derived medicines have made large contributions to human health and well-being. The antibacterial properties of plant essential oils have been known for many centuries and were accepted to be effective against infectious diseases. It is reported that plant derived antimicrobials have higher minimum inhibitory concentrations than antibiotics of bacterial and fungal origin.[17-20] They may enhance the effects of conventional antimicrobials, which will probably decrease the costs and improve the treatment quality.

EOs are usually considered as safe antimicrobials due to their natural origin and have been reported to show a little or no toxicity. This provides the rationale for research into the potential outcome of employing plant derived essential oils as treatment option. Essential oils and their multiple components are candidate agents that were proposed to act on several targets, meanwhile reducing the probability of development of resistance.

Biofilm, a polymeric matrix that encloses the bacteria, acts as a protective mechanism to survive in harsh environment and also against host defence during infection. Lateral gene transfer is greatly facilitated in biofilms and leads to a more stable biofilm structure. Bacteria living in a biofilm are reported to become more resistant to antimicrobial agents and disinfectants. In some cases antibiotic resistance can be increased a thousand fold. Hence biofilm represents an important virulence factor.

It is clear from the epidemiological evidence that biofilm producing *Acinetobacter* play a role in infectious diseases such as cystic fibrosis, periodontitis, bloodstream infections and UTI because of their ability to indwell medical devices.[21] The studies have also shown that formation of biofilm at the air-liquid interface was four times higher in *A.baumannii* and *A.nosocomialis* than *A.pittii*.[21] According to few workers, iron limitation plays an important regulatory role in the formation of strong or weak biofilm, thereby helping *A.baumannii* to survive in less available micronutrient environment and making it a more successful nosocomial pathogen.[22]

Out of the 251 *Acinetobacter* strains tested for biofilm production in the present study, 70% of strains produced biofilm. The results of similar studies conducted by various investigators employing less number of isolates showed around 63% of biofilm production.[23,7] The number of isolates subjected to biofilm production and the positivity in the present study were higher compared to earlier studies indicating that the prevalence has increased and the isolates were established nosocomial pathogens.

The observations made by Pour NK, et al[1], showed that the *A. baumannii* strains were able to attach and form biofilm on different surfaces such as glass, polycarbonate and polypropylene which are used widely in the fabrication of medical environments. In the present study, among the 202 imipenem resistant isolates, 75%

showed biofilm formation on polystyrene surface suggesting that these isolates can establish nosocomial infections related to medical devices.

The 251 isolates chosen for biofilm formation in this study were all multidrug resistant, i.e., resistant to more than 3 classes of antibiotics. Among them 80.5% isolates were imipenem resistant, the result was almost similar to the result of the study done by Pour NK, et al, whose data showed 83.3% of imipenem resistance. The results of the present study in accordance with the investigations conducted by other workers[1] indicated that the antibiotic resistance is higher among biofilm producing isolates than in the non-biofilm producers. On the contrary, the results obtained in the study conducted by Rodriguez, et al, showed higher biofilm production in antibiotic sensitive isolates than in resistant isolates.[23]

The antibacterial activity of various EOs have been extensively studied against various pathogenic microorganisms and their biofilms.[7-8,17-19] They have also exhibited remarkable activity against viruses like bacteriophages and H1N1.[12] In the present study, antibiofilm activity of lemon grass essential oil was tested against biofilm producing *Acinetobacter* isolates and the results indicated that lemon grass essential oil had a significant role in inhibition of biofilm formation by *A.baumannii*.

It has been reported that plants either contain antimicrobials that can operate in synergy with antibiotics or possess compounds that have no intrinsic antibacterial activity but are able to sensitize the pathogen to an ineffective antibiotic.[13] EOs alone are good antimicrobial agents while their effect seems to be enhanced when studied in synergy with commercial antibiotics. In this context, the lemon grass EO in the current study not only displayed intrinsic antibacterial activity[16] but also significantly potentiated the activity of the eight co-tested antibiotics which were ineffective when tested alone. Hence mechanism of their antimicrobial action needs to be addressed to understand the complexity of synergistic potential between natural essential oils and antibiotics against bacteria.

It has been reported that most of the antibacterial activity is found in oxygenated terpene components are mainly by membrane disruption. They act on cell membrane integrity by changing the membrane permeability. But it has also been shown that the antimicrobial mechanism of action varies with the type of EO or the strain of microorganism. It is reported that gram positive bacteria are more susceptible to EOs than gram negative bacteria.[12] It has also been demonstrated that whole EOs, alone and when used in combination with organic acids, antibiotics and nanomaterials, usually have higher activity than the mixture of their major components.[12,24-25]

In this study it was observed that the lemon grass EO with its high content of oxygenated terpenes like Citral, Geraniol, etc., exhibited the highest antibacterial activity when used singly or jointly with the tested conventional antibiotics. The activity of these antibiotics was significantly enhanced especially those targeting the cell wall including imipenem, piperacillin/tazobactum, cefipime, etc.

The antibiofilm activity of lemon grass essential oil with its synergistic effect on antimicrobials demonstrated in this study might open a new avenue for determining better treatment option for *A.baumannii* infections as well as preventing the dissemination of nosocomial MDR & XDR *A.baumannii*.

An attempt should be made to identify, purify and evaluate the active components in the *Cymbopogon flexuosus* extracts and essential oil which should be followed by carefully carried out, controlled clinical trials. For this, the pharmaceutical chemists, microbiologists and practitioners of traditional and allopathic medicine have to come together and facilitate the discovery of the vital drugs for the treatment of infection with a holistic approach in the coming decades.

CONCLUSION

Cymbopogon flexuosus [Lemon grass] essential oil proved to be more potent showing highest *in-vitro* antibacterial and anti-biofilm activity. It potentiated the antimicrobial activity of previously resistant antibiotics as well, indicating that there is a scope for developing it into an alternative drug that could be used in conjunction with antibiotics to treat MDR & XDR-AB infections. However, further pharmacokinetic and pharmacodynamic tests are needed for routine clinical use. Since LGO reduced the biofilm formation in *Acinetobacter* isolates, use of LGO [either topical agent or vapours] as disinfectant in hospital environment and ICU might help in reducing the colonization of this nosocomial pathogen. *Cymbopogon flexuosus* [Lemon grass] essential oil is occupying increasingly and vastly varied significance in pharmaceuticals and medicine owing to their potential bioactivities. The easy availability, pleasant olfactory properties and insignificant toxicity of *Cymbopogon flexuosus* essential oil can make it the most promising candidate for the treatment of infections and chronic diseases.

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