

**ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL OF *CYMBOPOGON FLEXUOSUS*  
(LEMON GRASS) AGAINST CLINICAL ISOLATES OF MULTIDRUG-RESISTANT  
*ACINETOBACTER BAUMANNII*: A PRELIMINARY *IN-VITRO* STUDY**

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**ABSTRACT**

**Aim** – In recent years, the incidence of nosocomial infections due to *A.baumannii* has increased to a point of concern. Rapid spread of multidrug and extremely drug resistant *A.baumannii* in clinical settings has made treatment options difficult for clinician. It has also increased the morbidity and mortality in immunocompromised patients. The aim of this study was to investigate the efficacy of essential oil of a traditional medicinal plant, *Cymbopogon flexuosus* (lemon grass) against the problematic multidrug resistant *A.baumannii*. **Methodology** – Essential oil of lemon grass was distilled by Neo-Clavenger's method and the antibacterial activity was tested against 102 multidrug resistant *A.baumannii* clinical isolates by punch-well and disc diffusion methods. The minimum inhibitory concentration of lemon grass oil was determined by Macrobroth dilution method. **Results** – Majority of *A.baumannii* isolates were inhibited by lemon grass oil, inhibition zone ranging from 13mm to 33mm, mean inhibition zone being 23mm and the minimum inhibitory concentration of lemon grass oil was 6.25µl/ml. **Conclusion** – essential oil lemon grass showed good antibacterial activity against *A.baumannii* and might be considered as an alternative treatment option against multi-drug resistant *A.baumannii* infections. However, further pharmacokinetic and pharmacodynamic studies are needed for routine clinical use.

**Key words:** MDR-AB, Lemon grass, Essential oil, MIC

**INTRODUCTION**

In recent years, the most serious issue in medicine is increasing drug resistance among bacteria due to inappropriate use of antibiotics. This is a cause for emergence of untreatable superbugs and high mortality rates throughout the world particularly among immunosuppressed patients. Gram negative pathogens are particularly worrisome because they have acquired resistance to nearly all drugs that would be used for treatment. This is a significant issue in developed as well as developing countries where antibiotic policy is still insufficient and tends to use broader spectrum antibiotics. *A.baumannii* (AB) was isolated from the environment in the early 20<sup>th</sup> century, worldwide. Until 1970s, it was considered to be a rare cause of nosocomial infections. However, recently the incidence of AB infections has increased to a point of concern. It is characterized by its rapid development of drug resistance to most of the classes of antibiotics including high adaptability to the selective pressure from the extensive use of antibiotics. The rapid spread of multidrug resistant *A.baumannii* (MDR-AB) in clinical settings has made the treatment options difficult for clinicians and has also increased morbidity and mortality in immunocompromised patients. *Acinetobacter* is also noted for its apparent ability to survive on artificial/inanimate surfaces for an extended period of time due to biofilm formation. At present, fully active antibiotic options available to treat nosocomial infections due to MDR-AB are extremely limited. Hence it is considered as one of the notorious nosocomial pathogen which desperately requires novel treatment strategies. Prevention of dissemination of multidrug resistance in *A.baumannii* is not an easy task. While multiple drug resistance is increasing in this pathogen and carbapenem resistance is rapidly spreading cross-continently, there is a sharp decline in the development of new antimicrobial agents that can control MDR-AB.

The lack of therapeutic options for treating MDR-AB calls for systematic pharmacokinetic and pharmacodynamic studies of rational combination therapies until new, powerful anti-*Acinetobacter* drug appear in clinical practice. New experimental approaches are warranted to develop and evaluate novel therapeutic strategies for dealing with *A.baumannii* infections. Since, there has been a continued decline in the number of newly approached drugs, returning to the pre-antibiotic era has become a reality and greater need for alternative treatments arises.

Historically plants have provided a source of inspiration for novel drug compounds. Higher plant products with an evidence based action against fungi and pathogenic bacteria are currently playing a growing role. It is reported that plant derived antimicrobials have higher minimum inhibitory concentrations than bacterial and fungal produced antibiotics (Prabuseenivasan S, et al., 2006, Vyas P, 2012, Prakasam G, et al.,2014). Plant derived essential oils have long been used as flavouring agents and food preservatives. In view of the dearth of new antibiotics against MDR and extremely drug resistant (XDR) *A.baumannii*, plants provide a potential source for therapeutically useful compounds.

The lemon grass oil, extracted from leaves, is reported to have strong activity against bacteria and dermatophytes (Hamza I.S, et al., 2009, Jafari B, et al., 2012). In this context, the current study investigated the *in vitro* antibacterial activity of lemon grass essential oil against MDR-*A.baumannii* clinical strains that were isolated in a tertiary care hospital.

## MATERIALS AND METHODS

The study was conducted in a tertiary care hospital in Mangaluru, Coastal Karnataka, India. 102 *Acinetobacter* isolates were collected from different clinical samples, identified and confirmed as *A.baumannii* [gram negative coccobacilli with non-fermenting colonies on MacConkey agar, oxidase negative, non-motile, alkaline slant and no reaction in the butt in triple sugar iron (TSI) agar, citrate utilization positive, urease and indole negative, attacked sugars oxidatively and grown at both 37° C and 44° C]. Antimicrobial susceptibility testing was performed according to CLSI guidelines to detect MDR isolates. These isolates were then subjected to various phenotypic tests such as Combined Disc Test (CDT) and Modified Hodge Test (MHT) to detect the MBL production. They were also checked for AmpC production and ESBL production by Phenotypic Confirmatory Disc Diffusion Test (PCDDT) using Ceftazidim and Ceftazidim/Clavulanic acid antibiotic discs (HiMedia, Pvt. Ltd. India). The isolates were then tested for Imipenem MIC using Imipenem E-test strips (bioMerieux SA, France).

### Preparation of Herbal Extracts

The leaves of *Cymbopogon flexuosus* (Nees ex Steud) Watson, belonging to Poaceae family were selected for extraction of essential oil. The extract was prepared in the department of Pharmacognosy, Al-Ameen college of Pharmacy, Bengaluru, Karnataka, India. The leaves of *Cymbopogon flexuosus* were obtained from Horticulture department, Gandhi Krishi Vignana Kendra (GKVK), University of Agricultural Sciences, Bengaluru and submitted for authentication to National Ayurvedic Dietetics Research Institute [Central Council for research in Ayurveda & Siddha, Dept. of AYUSH, Ministry of Health & F.W, Govt. of India], Bengaluru. The authenticated and certified leaves of *Cymbopogon flexuosus* (Nees ex Steud.) Watson, were then subjected for extraction procedure.

The essential oil (EO) was extracted by Neo-Clavenger's method using Clavenger's apparatus. The shade dried leaves were reduced to coarse powder. The powder was taken in a round bottom flask with glycerine and distilled water. The flask was then fitted to the Clavenger's apparatus and fixed on the mantle for extraction. The mantle temperature was set to 50°C and the mixture was boiled for about 8 hours. The oil was collected in the graduated tube on top of the water level in the form of droplets. After 8 hours of boiling (exhaustion time), when all the oil was extracted, the boiling was stopped and the oil was separated and collected in a vial. The total yield was noted. The oil was tested for antimicrobial action against *A.baumannii* isolates, both by Punch-Well and Disc diffusion methods. The preliminary, screening susceptibility test was done by using 100 µl/ml dilution of oil. The dilution was prepared by using DMSO (Di-methyl Sulfoxide) as the solvent. Antimicrobial susceptibility testing by punch-well method was done on Mueller-Hinton agar (MHA) plate. Two wells of 6mm diameter were bored on the surface of MHA plates with the aid of sterile metallic template and cultures of test isolates along with standard control strains (opacity adjusted to 0.5 McFarland opacity Standard) were lawn cultured on them. To one well 200 µl of DMSO was added and was considered as control. 200µl of 100µl/ml of lemon grass essential oil was added to the second (test) well and refrigerated for 2 hours to allow the diffusion of oil into the medium. Then the plates were incubated at 37°C overnight. The zone size was measured in millimetres.

### Disc diffusion method

20µl of essential oil (100µl/ml) were placed onto individual 6 mm sterile, blank Whatmann no.2 filter paper discs. Standard inoculum of *Acinetobacter* isolates adjusted to 0.5 McFarland opacity was lawn cultured on dry MHA plates to produce confluent growth.

The lemon grass essential oil impregnated discs were then placed onto the inoculated surface of MHA plates and incubated at 37°C overnight. The antimicrobial effect of EO was assessed by measuring the diameter of zone of inhibition in millimetres (Doran A.L, et al., 2009, Miyasaki Y, et al., 2010). The test was carried out in triplicates, along with controls. Essential oil of Lemon grass was further diluted into various dilutions- 50µl/ml, 10µl/ml and 5µl/ml and tested on all the isolates by disc diffusion method. Then the Minimum Inhibitory Concentration (MIC) of Lemon grass oil was determined.

#### Determination of MIC of Lemon grass (*Cymbopogon flexuosus*) essential oil

The MIC of essential oil of Lemon grass against clinical strains of *A.baumannii* was determined by Macro Broth Dilution method (Ahanjan.M, et al., 2014). The MIC procedure was standardized using visual method (according to CLSI M07 guidelines) as it was convenient than colorimetry and turbidimetry. Control organism used was *A.baumannii* MTCC 1425. McFarland's standard opacity tube 0.5 [150 million bacteria/ ml] was used to compare the growth turbidity (according to CLSI M07-A6 document). The medium used was Mueller Hinton Broth (MHB) with 0.5% Tween-20, for uniform mixing of lemon grass oil in the broth.

An eight-fold serial dilution of the essential oil in DMSO, including 100µl/ml, 50µl/ml, 25µl/ml, 12.5µl/ml, 6.25µl/ml, 3.125µl/ml, 1.56µl/ml, 0.78µl/ml and 0.39µl/ml was prepared in sterile test tubes. These dilutions were added to 1 ml Mueller Hinton broth with 0.5% tween-20 containing 150 million bacteria/ ml (0.5 McFarland's). 3 tubes were used as controls – positive, negative and blank. The test tubes were incubated at 37°C for 24 hours. The dilution that showed complete inhibition of growth was observed and recorded as MIC. The procedure was done in triplicates and was repeated for all the 102 clinical isolates of *A.baumannii* along with the control strain and the results were recorded.

## RESULTS

A total 102 *A.baumannii* clinical isolates were investigated for their antibacterial susceptibility against lemon grass essential oil. All strains were multi drug-resistant. Among 102 MDR isolates, 82 (80.4%) were resistant to Carbapenems and 20 (19.6%) were sensitive. Antibacterial activity of Lemon grass oil with various dilutions was checked against all these isolates by punch-well and disc diffusion method. Disc diffusion method was found to be of same efficiency as punch well method, easy to perform, less cumbersome, consumed less quantity of solvents and extracts and could be compared with disc diffusion of antibiotics. All the 102 isolates showed varying degree of susceptibility to lemon grass oil by disc diffusion method (Fig-1). Table-1 shows the result of disc diffusion assay. The minimum inhibitory concentration of lemon grass oil against majority of isolates was 6.25µl/ml.

**Table-1:-Susceptibility pattern of 102 *A.baumannii* isolates to various dilutions of Lemon grass oil:**

Susceptibility pattern	100µl/ml		50µl/ml		10µl/ml		5µl/ml	
	n	%	n	%	n	%	n	%
+	12	(11.8)	16	(15.7)	75	(73.5)	74	(72.5)
++	27	(26.5)	25	(24.5)	05	(5.0)	08	(7.8)
+++	29	(28.4)	28	(27.4)	08	(7.8)	09	(8.9)
++++	34	(33.3)	33	(32.4)	14	(13.7)	11	(10.8)

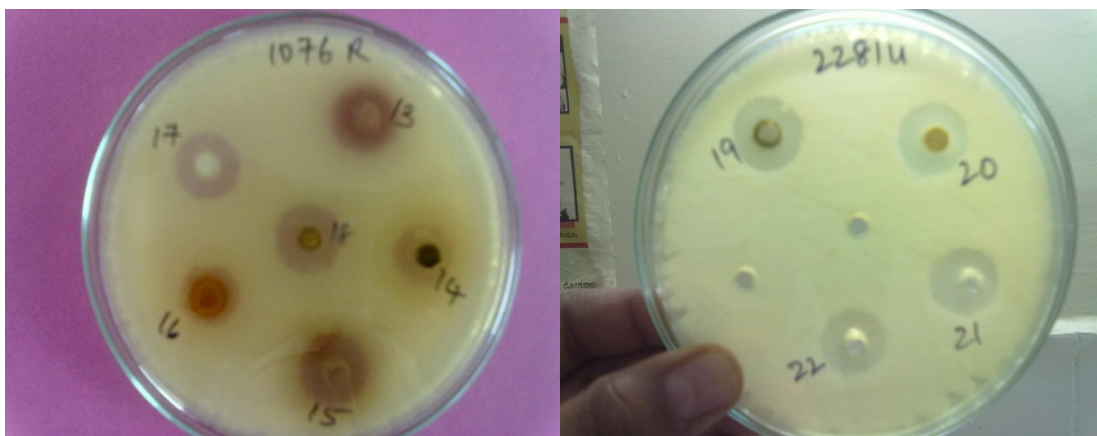
Key: + = 10-13mm; ++ = 14-17mm; +++ = 18-21mm; ++++ = >21mm

**Table-2:- MIC results of Lemon grass essential oil against 102 *A.baumannii* isolates:**

<i>A.baumannii</i> Isolates n (%)	Total number n (%)	Dilutions of lemon grass essential oil in µl/ml & number of isolates showing no growth							
		50	25	12.5	6.25	3.125	1.56	0.78	0.39
Carbapenem Resistant	82 (80.4)	-	-	-	-	+	+	+	+
		-	-	-	-	-	+	+	+
Carbapenem Sensitive	20 (19.6)	-	-	-	-	+	+	+	+
		-	-	-	+	+	+	+	+
Control <i>A.baumannii</i>	MTCC 1425	-	-	-	-	+	+	+	+

-no growth;+growth present

Table-2 shows the MIC values of both carbapenem resistant and carbapenem sensitive clinical isolates of *A.baumannii*. In the present study, while determining the MIC of lemon grass oil against carbapenem sensitive *Acinetobacter* isolates, an unusual finding was observed. Except two isolates, the growth of all the 20 isolates were inhibited at a concentration of 6.25µl/ml lemon grass oil. The two carbapenem sensitive isolates were inhibited at 12.5µl/ml of lemon grass oil, the result contradicting the disc diffusion assay.



**Fig-1:- Antimicrobial susceptibility testing of lemon grass essential oil against *A.baumannii* by Disc diffusion & Punch well method respectively.**

## DISCUSSION

Antibiotic resistance is increasing resulting in a decreasing number of fully active antimicrobial agents available for the treatment of MDR bacteria. Plant derived medicines have made large contributions to human health and wellbeing. Phytomedicines have shown great promise in treatment of various infections. The medicinal properties of plants have been investigated, in the light of recent scientific developments, throughout the world due to their potent pharmacological activities and economic viability (Dahiya P, et al., 2012, Miyasaki Y, et al., 2013).

The antibacterial properties of plant essential oils have been known for many centuries and were accepted to be effective against infectious diseases. They are usually considered as safe due to their natural origin. It has been reported that plants either contain antimicrobials that can act on pathogens or can operate in synergy with antibiotics or possess compounds that has no intrinsic antibacterial activity but are able to sensitize the pathogen to an ineffective antibiotic (Maizura M, et al., 2007, Kon K.V, et al., 2012). This provides the rationale for research into the potential outcome of employing plant derived essential oils as a treatment option.

Lemon grass belongs to the family Poaceae. Out of 500 described species, two species – *Cymbopogon citratus* and *Cymbopogon flexuosus* – are generally called as lemon grass. Medicinal use of *Cymbopogon flexuosus* is known to mankind since antiquity. The essential oil, extracted from leaves, is used to cure various ailments like cough, cold, haemoptysis, rheumatism, lumbago, digestive problems, bladder problems, leprosy, cholera, colic, obstinate vomiting, etc. it was also used as mouth wash for tooth ache and swollen gums. It has stimulating, diuretic, anti-purgative, analgesic, antipyretic, anti-tumour, antifungal and bactericidal properties which can be comparable to penicillin in its effectiveness.

Many reports are available on the antibacterial activity of lemon grass oil against various gram positive, gram negative bacteria and fungi like *S.aureus*, *P.aeruginosa*, *E.coli*, *S.typhi*, *Aeromonas*, *Bacillus*, *Aspergillus*, *Candida*, etc (Inouye S, et al., 2001, Revathi K, et al., 2012, Chamdit S, et al., 2012, Arputha B.M, et al., 2012, Choi J.Y, et al., 2013, Thompson E, et al., 2013, Korenblum E, et al., 2013, Sharma P, et al., 2013, Tarek N, et al., 2014). Reports on antibacterial activity of lemon grass oil exclusively against *A.baumannii* are lacking. Very few authors have reported the activity of lemon grass oil on *A.baumannii* along with other bacteria (Miyasaki Y, et al., 2010, Kon K.V, et al., 2012, Miyasaki Y, et al., 2013). Rapid dissemination of resistance genes against most of the antibiotics including carbapenems, which was considered as the last option for the treatment of MDR-AB, has decreased the number of active antibiotics that can be used against them. This study has shown that the lemon grass oil has significant effect against this multi-drug resistant *A.baumannii*.

The MDR-AB isolates were obtained from various clinical samples of hospitalized patients and hence, could be extremely problematic. This adds an additional value to this work. In our study, two carbapenem sensitive isolates showed a contradictory type of MIC.

They were inhibited at concentration of 12.5µl/ml of lemon grass oil, while rest showed MIC of 6.25µl/ml. This contradicting observation should be evaluated further. It could be due to various reasons such as, the antibiotics and other drugs used for the treatment of patient, might have altered the *Acinetobacter* strain leading to the change in the MIC value.

We already know that this medicinal herb has been used for the treatment of various diseases in traditional medicine. Therefore, use of lemon grass oil to obtain antibacterial effect in humans will be much easier than many synthetic materials. But being natural does not exclude toxic or allergic properties of plant derived products. Although most essential oils are regarded as safe, some of them may cause irritation, sensitization, photo toxicity or allergic reactions including anaphylaxis. Hence, the studies on toxic and irritant properties of essential oil are imperative, especially when considering for human use. Further *in vitro* and *in vivo* studies have to be conducted on lemon grass essential oil before clinical use.

## CONCLUSION

This study shows that the essential oil of *Cymbopogon flexuosus* (lemon grass) has bactericidal effect on multidrug resistant *Acinetobacter baumannii* and it is a promising finding. The scope for developing it into an alternative drug in conjunction with other antibiotics for treatment of MDR-AB infections may be considered. However, further pharmacokinetic and pharmacodynamic tests are needed for routine clinical use.

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