

**ANTIMICROBIAL ACTIVITY OF SELECTED BOTANICALS
AGAINST FOOD SPOILING MICROORGANISMS**

by

AMIT KUMAR

CENTRE FOR RURAL DEVELOPMENT AND TECHNOLOGY

Submitted

In fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

to the



INDIAN INSTITUTE OF TECHNOLOGY DELHI

MAY 2012

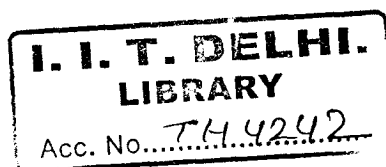
Negative ions



TH

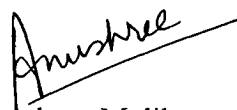
664.8

AMI-A



CERTIFICATE

This is to certify that the thesis entitled “**Antimicrobial activity of selected botanicals against food spoiling microorganisms**” being submitted by **Mr. Amit Kumar** to the Indian Institute of Technology Delhi for the award of the degree of ‘Doctor of Philosophy’ is a record of bonafide research work carried out by him. He has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis. To the best of my knowledge the results contained in this thesis have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.



Anushree Malik

Associate Professor

Centre for Rural Development and Technology

Indian Institute of Technology Delhi

ACKNOWLEDGEMENTS

It gives me an immense pleasure to express my sincere thanks to all those who have rendered their kind help during the course of this study. First and foremost, praise and thanks goes to my saviour The Almighty for the many blessings bestowed upon me.

My sincere gratitude goes to my supervisor, Dr. Anushree Malik, for her guidance, encouragement and tremendous patience during the course of this research, without which the completion of this work would not have been possible. Her critical assessment and affectionate words always kept me charged and motivated to perform best. I cede all my commendations to her, for her candid suggestions, confidence and the commitment she instilled in me. I would also like to thank Prof Maria Elisabetta Guerzoni for providing me an opportunity to work under her supervision in advanced and highly equipped Food Microbiology lab during my India4EU research exchange program at Department of Food Science, University of Bologna, Italy. I am extremely privileged and grateful for their tutelage, mentoring and friendship.

I would like to thank my SRC members, Prof. S.N.Naik, Prof. S.K.Khare and Prof. Satyawati Sharma for providing valuable suggestions and expert advice throughout the course of this study. My special thanks to Prof. Santosh Satya and Dr. P. Sudhakar for their constant encouragement, valuable suggestions and financial support through MORD project. I am also grateful to all the faculty members of Centre for Rural Development and Technology (CRDT) for extending all the necessary help in completing this work. I am grateful to Dr. Kamlesh Singh for her kind help in initiating the NAI related work. Moral support, useful suggestions and guidance rendered by Dr. H.K. Malik is highly acknowledged.

I would like to express my sincere gratitude towards Prof. S.N.Naik, Head, CRDT who provided the necessary infrastructural support for carrying out the work. Facilities at Applied Microbiology Lab (jointly CBME-CRDT) benefitted my research work extensively. Facilities availed from Department of Textile Technology/Department of Physics, IIT Delhi for SEM/AFM, Advanced instrument research facility (AIRF), JNU for GC/GC-MS, Sophisticated Analytical Instrument Facility (SAIF) – AIIMS for TEM are gratefully acknowledged.

The utmost cooperation of the office and laboratory staff especially Mr. Sabal Singh is sincerely acknowledged. I express my earnest thanks to Dr. Rosalba Lanciotti, Dr. Prasant Rout, Dr. Ashwani Kumar, Dr. Asheesh Yadav, Dr. Shweta Sharma, Dr. Prachi Kaushik, Mr. Abhishek Mishra, Ms. Kalpana Arora, Mr. Lalit Mohan Bal, Mr. Davide Gottardi, Ms. Danka Bukvicki, Mr. Manoj Kumar and Mr. Bikesh Kumar Nirala for the affection and pleasant company. All my labmates and friends at CRDT and university of Bologna, Italy deserve a special mention for their help and cooperation.

Very special thanks to my wife, Ashu Tyagi for all her concern, understanding and cooperation. I am indebted to my parents and siblings whose blessings and love brought me up to here and were a source of constant inspiration to excel. My special words of gratitude to my parents for providing the guidance, affection and support which was influential in letting me concentrate on my work.



Amit Kumar

ABSTRACT

The present study focused on the utilization of natural antimicrobial agents such as essential oils/vapours and negative air ions (NAI) against food spoiling microorganism for food preservation. To begin with, chemical composition of mentha, eucalyptus, lemon grass oils and respective vapours was analyzed by GC-MS and SPME GC-MS, respectively followed by various antimicrobial tests against 21 food spoiling microbial strains. The antimicrobial activity was found to vary depending upon the strain tested, oil composition and different methods of treatment (liquid and vapour phases). In general, Gram positive bacteria, fungus and yeast strains were more sensitive than Gram negative bacteria. Relative efficacy of various essential oils was sorted as lemon grass oil > mentha oil > eucalyptus oil. These results were in agreement with the chemical composition and relative antimicrobial efficacy of the major active components of *Cymbopogon citratus* (citral), *Mentha piperita* (menthol) and *Eucalyptus globulus* (1-8, cineole) essential oils.

Essential oil vapours had significantly higher antimicrobial activity against all the tested strains as compared to the corresponding essential oils. Chemical characterization of essential oils and their vapours showed that certain antimicrobial compounds got enriched in the vapour phase. A combination of SEM, TEM and AFM was employed for the first time to differentiate the morphological and ultrastructural changes in lemon grass oil and vapour treated *Escherichia coli* and *Candida albicans* cells. TEM observations showed that whilst the cell envelop was damaged at certain locations in lemon grass vapour treated *E. coli* cells, the overall boundary of the damaged cells was retained in lemon grass oil treated cells. This correlated well with the ghost cells or foot prints of the cells observed in the SEM of the vapour treated *E. coli* cells in contrast to oil treated cells which appeared to be aggregated and only partially deformed. This hypothesis was confirmed by AFM that showed drastic reductions in cell height and increased cell surface roughness in lemon grass vapour treated cells as compared to oil treated cells or controls. Lemon grass vapours could cause extensive cellular damage at much lower concentrations, probably due to better penetration and contact.

To estimate the kill time as well as facilitate integration with NAI, an innovative methodology (Multiple Petriplate exposure regimes) was conceived and a set-up harbouring essential oil vapour generator and NAI generator was developed. Kill time assays revealed that 100% reduction in viability occurred within 1 h (*Bacillus subtilis*) and 4 h (*C. albicans*) exposure to lemon grass oil vapours while the same required 8 h exposure to mentha or eucalyptus oil vapours. On the basis of above results it can be stated that lemon grass vapour is highly effective and its application can be further developed in food

preservation and air disinfection. Further, there is a great potential to further enhance its antimicrobial potential through integration with other hurdle technologies. To accomplish this, antimicrobial activity of NAI alone and in combination with selected essential oil vapour was observed against selected highly resistant Gram negative bacteria. The results showed that the susceptibility of bacterial strains to NAI varies depending upon the strain type, their physiological state as well as the distance/ orientation with respect to the source. Significant enhancement in viability reductions was observed by combining NAI with essential oil vapour exposure. Within 8 h exposure to the combination, 100% killing of *E. coli* was noted as opposed to 40% (NAI alone) and 78% (vapour alone). The role of both the volatile components of the essential oil and NAI was important to the effect which was proven by the morphological analysis of the treated cells at different time intervals. It is noteworthy that such a combination of NAI generated through low cost, simple NAI generators with essential oil vapours has not been studied earlier. Hence, this study makes a novel contribution towards developing a simple and more efficient technique for control of bacterial contamination that could be applied for air purification/surface disinfection or food stabilization practices

Last part of the work aimed at evaluating the in vivo antimicrobial efficacy of essential oils and vapours in real food samples against selected spoilage bacteria/yeast or the natural microflora. Representative food items from fruits (mixed fruit juice, apples), vegetables (bamboo shoots) and meat (chicken) category were tested for preservation with natural compounds. The results mirrored as well as established the in vitro observations suggesting that lemon grass oil vapours possess the highest antimicrobial efficacy against food spoilage microorganisms. To the authors' best knowledge, this happens to be the first report on the use of essential oil vapours in preservation of various food stuffs especially bamboo shoots. This shall certainly go a long way in reducing the use of synthetic compounds in food preservation.

Academically, the results constitute a pioneering report on the antimicrobial activity of selected essential oil vapours as well as its integration with NAI in a specially designed set-up. Also, it represents a novel insight on the impact of essential oils/vapours on morphology, ultrastructure and three dimensional properties such as cell height/surface roughness. In fact the study provides innovative methodologies and assay regimes for facilitating further work. On the practical grounds, the investigations testify the applicability of essential oils/vapours in actual food preservation and offer the design footprint of a simple, eco-friendly system for exposure of perishable products or surface/indoor space disinfection.

CONTENTS

CERTIFICATE	I
ACKNOWLEDGEMENTS	II
ABSTRACT	IV
LIST OF FIGURES	XI
LIST OF TABLES	XIV
Chapter 1: Introduction and literature review	1
1.1 Introduction	1
1.2 Literature review	4
1.2.1 Antimicrobial activity of essential oil against food spoiling microorganisms	4
1.2.1.1 Antimicrobial activity of mentha oil.....	5
1.2.1.2 Antimicrobial activity of eucalyptus oil	8
1.2.1.3 Antimicrobial activity of lemon grass oil	11
1.2.2 Application of essential oils (mentha, eucalyptus and lemon grass) against food spoiling microorganisms.....	14
1.2.3 Antimicrobial activity of essential oil vapours against food spoiling microorganisms	17
1.2.4 Application of the essential oil vapours against food spoiling microorganisms	23
1.2.5 Antimicrobial activity of negative air ions	28
1.2.6. Application of NAI in air disinfection and food preservation.....	32
1.2.7 Combination of Essential oil vapours and NAI against food spoiling microorganism	35
1.3 Scope of the work.....	37
Chapter 2: Material and methods	39
2.1 Chemicals	39
2.2 Chemical composition of essential oils and essential oil vapour	40
2.2.1 GC and GC-MS analysis	40

2.2.2 SPME GC-MS analysis of essential oil vapours	40
2.2.3 Identification of chemical compounds	42
2.3 Microbial strains and inoculum preparation.....	42
2.4 Antimicrobial activity of botanicals in liquid phase	43
2.4.1 Determination of MIC by agar dilution method.....	43
2.4.2 Determination of MBC and MFC using broth dilution method	43
2.4.3 Well diffusion method.....	44
2.4.4 Disc diffusion method	44
2.5 Antimicrobial activity of botanicals in vapour phase.....	45
2.5.1 Disc volatilization method.....	45
2.5.2 Determination of the kill time	46
2.6 Morphological and ultra-structural study.....	46
2.6.1 Scanning electron microscopy.....	47
2.6.2 Transmission electron microscopy	48
2.6.3 Atomic force microscopy	48
2.7 Antimicrobial activity of NAI alone and in combination with volatile compounds.....	48
2.7.1 Antimicrobial potential of NAI	48
2.7.1.1 Preparation of bacteria and NAI exposure.....	49
2.7.1.2 Effect of exposure distance and exposure time.....	49
2.7.1.3 Effect of bacterial strain.....	50
2.7.1.4 Effect of location.....	50
2.7.1.5 Effect of exposure medium	50
2.7.1.6 Effect of culture age.....	50
2.7.1.7 Effect of starvation.....	51
2.7.2 Combined effect of the essential oil vapour and NAI	51
2.7.2.1 Exposure regimes.....	51
2.8 Application of essential oils and vapours.....	53
2.8.1 Apple browning prevention by essential oil treatment.....	53
2.8.1.1 Preparation of the dipping solutions with antibrowning agents.....	53
2.8.1.2 Preparation and treatment of the apple slices	54
2.8.1.3 Measurement of variation in colour parameters	54

2.8.2 Prevention of crude juice mixture spoilage by lemon grass oil and thermal treatment	56
2.8.2.1 Preparation of crude juice mixture inoculated with <i>S. cerevisiae</i>	56
2.8.2.2 Effect of thermal treatment	56
2.8.2.3 Effect of lemongrass oil	56
2.8.2.4 Effect of lemon grass oil and thermal treatment.....	57
2.8.2.5 Sensory analysis	58
2.9 Treatment of raw chicken by essential oil vapours	58
2.10 Bamboo shoot treatment by essential oil and essential oil vapours	59
2.11 Statistical analysis	60
Results and Discussions	
Chapter 3: Antimicrobial activity of mentha oil and vapour	62
3.1 Chemical composition of mentha oil and mentha oil vapour	62
3.2 Antimicrobial activity	66
3.2.1 MIC and MBC/MFC of mentha oil	66
3.2.2 Well diffusion method	68
3.2.3. Disc volatilization method.....	69
3.3 Kill time assay.....	71
3.4 SEM observation.....	73
3.5 Conclusions	75
Chapter 4: Antimicrobial activity of eucalyptus oil and vapour	76
4.1 Chemical composition of eucalyptus oil and eucalyptus oil vapour.....	76
4.2. Antimicrobial activity	80
4.2.1 MIC and MBC/MFC of eucalyptus oil.....	80
4.2.2 Well diffusion method	82
4.2.3 Disc volatilization method.....	83
4.3 Kill time assay.....	89
4.4 Conclusions	90
Chapter 5: Antimicrobial activity of lemon grass oil and vapour.....	92
5.1 Chemical characterisation of lemon grass oil and vapour.....	92
5.1.1 Chemical characterisation of lemon grass oil.....	92

5.1.2 Chemical characterisation of lemon grass oil vapour.....	95
5.2 Antibacterial activity of lemon grass oil and vapour	96
5.2.1. MIC/MBC of lemon grass oil.....	96
5.2.2 Zone of inhibition due to lemon grass oil/vapour.....	97
5.2.3 Kill time assay	99
5.2.4 Morphological and ultra structural alteration of <i>E. coli</i>	101
5.2.4.1 SEM observation.....	101
5.2.4.2 TEM observation	103
5.2.4.3 AFM observation	107
5.3 Antifungal activity of lemon grass oil and vapour.....	114
5.3.1 MIC and MFC of lemon grass oil against fungal strains.....	114
5.3.2 Morphological deformation in fungal hyphae	115
5.4 Antiyeast activity of the lemon grass oil and vapour	117
5.4.1 Determination of MIC and MFC of lemon grass oils against various yeast strains by 96-well microplate method.....	118
5.4.2 Kill time assay (liquid phase)	121
5.4.3 Zone of inhibition due to lemon grass oil.....	121
5.4.4 Zone of inhibition due to lemon grass oil vapours	124
5.4.5 Kill time assay (vapour phase)	125
5.4.6 Morphological and ultra structural alteration of <i>C. albicans</i>	126
5.4.6.1 SEM observation.....	126
5.4.6.2 TEM observation	128
5.4.6.3 AFM observation	130
5.5 Conclusions	134
Chapter 6: Combined antimicrobial potential of NAI and essential oil vapours	136
6.1 Antimicrobial activity of NAI alone	137
6.1.1 Effect of exposure distance and time.....	137
6.1.2 Effect of NAI on different bacterial strains	137
6.1.3 Effect of plate location side	139
6.1.4 Effect of media composition.....	140
6.1.5 Effect of starvation	141

6.1.6 Effect of culture age	143
6.2 Combined effect of NAI and volatile compound	145
6.2.1. Effect of essential oil vapour and NAI combination against <i>E. coli</i>	145
6.2.2 Effect of lemon grass oil vapour and NAI combination against <i>P. fluorescens</i>	149
6.2.3. Morphological deformation in bacterial cells.....	151
Chapter 7: Application of essential oils and their vapours	155
7.1 Prevention of apple browning by essential oil treatment	155
7.1.1 Variation in color parameters	157
7.2. Prevention of crude juice mixture spoilage by lemon grass oil and thermal treatment ...	163
7.2.1. Log reduction in CFU count due to Thermal Treatment	163
7.2.2 Log reduction in CFU count due to varying concentration of lemon grass oil	165
7.2.3. Log reduction in CFU count due to combined effect of lemon grass oil and thermal treatment	167
7.2.4 Sensory analysis	170
7.3 Reduction in microbial load of chicken due to essential oils vapour exposure	170
7.4 Reduction in total microbial load of bamboo shoots due to essential oil and its vapour treatment.....	174
7.5 Conclusions	176
Chapter 8: Summary and Conclusions	178
8.1 Antimicrobial activity of mentha oil and vapour	178
8.2 Antimicrobial activity of eucalyptus oil and vapour	179
8.3 Antimicrobial activity of lemon grass oil and vapour.....	180
8.4 Combined antimicrobial potential of NAI and essential oil vapours	181
8.5 Application of essential oils and vapours.....	182
8.6 Future Prospects	184
References.....	185