

**EFFECT OF SELECTED BACTERIA ON THE  
ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF  
*CENTELLA ASIATICA***

**ARASH RAFAT**

**THESIS SUBMITTED IN FULFILMENT OF THE  
REQUIREMENT FOR THE DEGREE OF  
DOCTOR OF PHILOSOPHY**

**INSTITUTE OF BIOLOGICAL SCIENCES  
FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2012**

**In the name of God, the most compassionate the merciful**

Specially dedicated to:

My kind parents  
**Nader and Masoumeh**

**UNIVERSITI MALAYA**

**ORIGINAL LITERARY WORK DECLARATION**

Name of Candidate: Arash Rafat

(I.C/Passport No: R12477817)

Registration/Matric No: SHC080055

Name of Degree: Doctor of Philosophy (PhD)

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"): Effect of selected bacteria on the antibacterial and antioxidant activities of *Centella asiatica*

Field of Study: Microbial Biotechnology

I do solemnly and sincerely declare that:

- (1) I am the sole author/writer of this Work;
- (2) This Work is original;
- (3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
- (4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
- (5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
- (6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature

Date

Subscribed and solemnly declared before,

Witness's Signature

Date

Name:

Designation:

## ABSTRACT

The role of selected naturally distributed bacteria on the antibacterial and antioxidant activities and production of phenolic compounds in one of the most important Asian medicinal plants namely *Centella asiatica* was investigated based on the effects of beneficial and pathogenic bacteria on the metabolic pathways of plants as reported in literature. Firstly, antibacterial and antioxidant properties and production of phenolic compounds of two subspecies of *C. asiatica* which are commonly used in Malaysia (University of Malaya Herbarium Voucher Specimens: KLU047364 and KLU047552) were evaluated and the most potent of them was chosen for the rest of the study. Different antioxidant assays were used to investigate the antioxidant activities of different samples while a disc diffusion method was applied to measure the antibacterial activity of these samples. Total phenolic contents of different parts of *C. asiatica* were also evaluated using Folin-Ciocalteu method. The selected *C. asiatica* subspecies was then treated with selected pathogenic and beneficial bacteria. Antibacterial and antioxidant activities and production of phenolic compounds in different parts of the plant inoculated with the pathogenic bacteria (*Enterobacter sp.*) were studied at early and late stages of infection. The antibacterial and antioxidant capacities as well as production of phenolic compounds were also investigated in different parts of the selected subspecies of *C. asiatica* after treatment with the beneficial bacteria (*Pseudomonas sp.*). Production of phenolic compounds in the treated plants was also analyzed using high performance liquid chromatography (HPLC) and then compared to the non-treated plants (controls). Based on the importance of endophytic bacteria and their interactions with the host plant, some endophytic bacteria

associated with *C. asiatica* were also isolated and identified using 16S rDNA sequencing method. Antibacterial and antioxidant potential of the cell-free supernatant of these endophytic bacterial cultures were also evaluated. A multiple callus-subculturing method was used to produce endophytic bacteria-free calli after optimization of an *in vitro* callus induction protocol for *C. asiatica*. The results confirmed the promising antibacterial and antioxidant activities of both examined subspecies of *C. asiatica*. However, the subspecies KLU047364 showed better antibacterial and antioxidant activities compared to KLU047552 and thus selected for the rest of the study. *C. asiatica* in early response to *Enterobacter sp.* infection increased the production of phenolic compounds. Both antibacterial and antioxidant activities of the plant were also enhanced in early stage of infection while the production of phenolic compounds and antioxidant activity of plant was reduced in late stage of infection. Antibacterial and antioxidant activities as well as total phenolics production of *C. asiatica* were increased after treatment with the beneficial *Pseudomonas sp.* bacteria. HPLC results showed irregular changes in amount of phenolic compounds produced in *C. asiatica* after treatment with the bacteria. All isolated endophytic bacteria could inhibit the growth of *Pseudomonas aeruginosa* except *Bacillus gibsonii*. The isolated endophytic bacteria also showed fair antioxidant potentials. The best result for callus production from leaf explant of *C. asiatica* was obtained from the combination of 6-benzylaminopurine at concentration of 3mg/ml and 1-naphthaleneacetic acid at a concentration of 3mg/ml. The multiple callus-subculturing method resulted in 75% endophytes-free callus.

## ABSTRAK

Kajian terhadap peranan sebilangan bacteria semulajadi keatas aktiviti anti-bakteria dan anti-oksidan dan penghasilan sebatian fenolik dalam satu tumbuhan perubatan yang terkemuka, *Centella asiatica*, telah dijalankan. Kajian dijalankan keatas dua sub-species tumbuhan ini yang sering mendapat kegunaan tempatan (University of Malaya Herbarium Voucher Specimens: KLU047364 and KLU047552). Sub-species yang menunjukkan potensi yang lebih dipilih untuk kajian berlanjutan. Asai anti-oksidan yang berlainan digunakan untuk mengkaji kesan anti-oksidan manakala kaedah disk diffusion digunakan untuk mengkaji kesan anti-bakteria. Kandungan sebatian fenolik bahagian bahagian *C. asiatica* telah ditentukan oleh kaedah Folin-Ciocalteu. Sub-species *C. asiatica* yang dipilih dirawat oleh bacteria yang pathogenic dan yang berguna. Aktiviti anti-oksidan dan anti-bakteria dan penghasilan sebatian fenolic oleh bahagian bahagian sub-species ini dikaji pada peringkat awal dan peringkat lanjutan. Penghasilan sebatian fenolik oleh tumbuhan yang dirawat juga dikaji dengan menggunakan kaedah HPLC dan dibandingkan dengan tumbuhan yang tidak di rawat. Bacteria endofitik yang terlibat dengan *C. asiatica* diasingkan dan dikenali dengan menggunakan urutan 16S rDNA. Kesan anti-bakteria dan anti-oksidan supernatant bebas sel daripada kultur bacteria ini juga di kaji. Kaedah multikultur digunakan untuk mengasihkan tisu callus bebas bacteria selepas satu protocol in vitro untuk pengasihan calli dioptimumkan. Keputusan kajian menunjukkan bahawa kedua sub-species ada potensi anti-bakteria dan anti-oksidan tetapi KLU047364, yang di pilih untuk kajian lanjutan, lebih berkesan berbanding dengan KLU047552. Dalam respons awal kepada jangkitan *Enterobacter* sp., *C. asiatica* meninggikan menghasilkan bahan fenolik. Kedua dua aktiviti anti-bakteria dan anti-oksidan dirangsangkan pada peringkat awal

jangkitan dengan bacteria , manakala penghasilan bahan fenolik dan aktiviti anti-bakteria dan anti-oksidan dikurangkan pada peringkat lanjutan. Keputusan analisa HPLC menunjukkan penghasilan bahan fenolic dalam quantity yang tidak menentu. Semua bacteria kecuali *Bacillus gibsonii* endofitik yang diasingkan berupaya merencat ketumbuhan *Pseudomonas aeruginosa*. Bacteria endofitik juga menunjukkan sifat anti-oksidan yang sederhana. Keputusan yang terbaik untuk penghasilan callus adalah dengan kombinasi 6-benzylaminoourin pada kepekatan 3mg/ml dan asid 1-naphthaleneacetik pada kepekatan 3mg/ml. Kaedah multikultur menghasilkancallus 75% bebas daripada bacteria endofitik

## ACKNOWLEDGEMENTS

All praises to the Almighty God for affording me the strength and determination to complete this study.

It is my pleasure to express my sincere gratitude and appreciation to my supervisor Associate Professor Dr. Koshy Philip from the Department of Microbiology, Faculty of Science, not only for his guidance and valuable advice but for his constant encouragement and the freedom that he provided to work and think throughout my research.

I am thankful to Prof. Dr. Sekaran Muniandy as my co-supervisor from the Department of Molecular Medicine, Faculty of Medicine for his valuable suggestions and useful discussions during my research.

I am also grateful to Assoc. Prof. Dr. Kamaruzaman Bin Sijam from the Department of Plant Pathology and Mohammad Bagher Javadi from the Department of Agriculture Technology, University Putra Malaysia for kindly providing the *Enterobacter sp.* and *Pseudomonas sp.* bacteria respectively.

I would like to thank University of Malaya for the use of laboratory facilities and for providing research grant PS151/2009A.

It's my pleasure to offer my thanks to all my laboratory mates, colleagues and friends especially Wuen Yew Teoh, Jeffery Saravana Kumar, Abdul-Muhsin M. Shami, Arash



Khorasani, Chee Poi Leng, Reza Ebrahimi, Behrooz Banisalam, Rajib Imdadul Haq, and Fazilia Mohd Hatta for maintaining a pleasant research atmosphere and making my stay in Malaysia an enjoyable one.

Last but not least, it is difficult to word my gratitude towards my family members for their encouragement and support during this period. I am grateful to my parents and my brother for supporting me in every possible ways.

## TABLE OF CONTENTS

<b>ORIGINAL LITERARY WORK DECLARATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ABSTRAK</b>	v
<b>ACKNOWLEDGEMENTS</b>	vii
<b>LIST OF FIGURES</b>	xiii
<b>LIST OF TABLES</b>	xv
<b>LIST OF SYMBOLS AND ABBREVIATIONS</b>	xvii
<b>LIST OF APPENDICES</b>	xix
<b>CHAPTER</b>	
<b>1 INTRODUCTION</b>	1
<b>2 LITERATURE REVIEW</b>	5
2.1 Bioactive Compounds Generated by Bioactivities in Plants	5
2.1.1 Antibacterial Activity	6
2.1.2 Antioxidant Activity	7
2.1.3 Phenolic Compounds	11
2.2 Biotechnological Techniques to Enhance the Bioactive Compounds Production in Plants	15
2.2.1 Cell and Tissue Culture	16
2.2.2 Genetic Transformation	17
2.2.3 Physical Treatment	19
2.2.4 Chemical Treatment	19
2.2.5 Microbial Treatment	21
2.3 Plant-Bacteria Interactions	22
2.3.1 Plant Beneficial Bacteria	23
2.3.2 Plant Pathogenic Bacteria	27
2.3.3 Endophytic Bacteria Associated with Plants	32
2.4 <i>Centella asiatica</i>	42
2.4.1 Botanical Information	42
2.4.2 <i>C. asiatica</i> in Malaysia	43
2.4.3 Nutrient and Chemical Constituents	43
2.4.4 Bioactive Compounds and Medicinal Value	44
2.4.5 Phenolic Contents of <i>C. asiatica</i>	46
<b>3 METHODOLOGY</b>	47
3.1 Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of Two Different Subspecies of <i>Centella</i> <i>asiatica</i>	47
3.1.1 Plant Material	47
3.1.2 Sample Preparation and Extraction	48
3.1.3 Antibacterial Activity Assay	49

3.1.4	Antioxidant Activity Evaluation Assays	49
3.1.5	Total Phenolic Content Determination	52
3.2	Evaluation of Antibacterial and Antioxidant Activities and Total Phenolic Contents of <i>Centella asiatica</i> after Inoculation with Pathogenic Bacteria	53
3.2.1	Preparation of Bacterial Suspension	53
3.2.2	Inoculation Method and Growth Condition	53
3.2.3	Sample Preparation and Extraction	54
3.2.4	Evaluation of Antibacterial Activity of Plants Inoculated with Pathogenic Bacteria	55
3.2.5	Evaluation of Antioxidant Activity of Plants Inoculated with Pathogenic Bacteria	55
3.2.6	Determination of Phenolic Compounds	55
3.3	Evaluation of Antibacterial and Antioxidant Activities and Total Phenolic Contents of <i>Centella asiatica</i> after Inoculation with Beneficial Bacteria	57
3.3.1	Bacterial Suspension Preparation	57
3.3.2	Inoculation Method and Growth Condition	57
3.3.3	Sample Preparation and Extraction	58
3.3.4	Evaluation of Antibacterial Activity of Plants Inoculated with Beneficial Bacteria	58
3.3.5	Evaluation of Antioxidant Activity of Plants Inoculated with Beneficial Bacteria	58
3.3.6	Determination of Phenolic Compounds	58
3.4	<i>C. asiatica</i> Associated Endophytic Bacteria	59
3.4.1	Isolation of Bacteria	59
3.4.2	Identification of Isolated Bacteria	60
3.4.3	Evaluation of Antibacterial and Antioxidant Activities of Isolated Endophytic Bacteria	61
3.5	Production of Bacteria-Free <i>C. asiatica</i> Callus	62
3.5.1	Optimization of <i>C. asiatica</i> Callus Induction Protocol Using BAP and NAA Growth Regulators	62
3.5.2	Callus Multi-Subculture Method to Obtain Bacteria-Free <i>C. asiatica</i> Callus	64
3.6	Statistical Analysis	64
<b>4</b>	<b>RESULTS</b>	65
4.1	Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of Two Different Subspecies of <i>Centella asiatica</i>	65
4.1.1	Antibacterial Activity	65
4.1.2	Antioxidant Activity	66
4.1.3	Determination of Total Phenolic Content	69
4.2	Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents After Inoculation with Pathogenic Bacteria	70

4.2.1	Antibacterial Activity	71
4.2.2	Antioxidant Activity	72
4.2.3	Determination of Phenolic Compounds	74
4.3	Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of <i>Centella asiatica</i> after Inoculation with Beneficial Bacteria	78
4.3.1	Antibacterial Activity	78
4.3.2	Antioxidant Activity	79
4.3.3	Determination of Phenolic Compounds	81
4.4	<i>Centella asiatica</i> Associated Endophytic Bacteria	85
4.4.1	Isolation and Identification of <i>Centella asiatica</i> Associated Endophytic Bacteria	85
4.4.2	Antibacterial Activity Evaluation of the Endophytic Bacteria Isolated from <i>C. asiatica</i>	86
4.4.3	Antioxidant Activity Evaluation of the Endophytic Bacteria Isolated from <i>C. asiatica</i>	87
4.5	Production of Bacteria-Free <i>Centella asiatica</i> Callus via Tissue Culture	89
4.5.1	Callus Induction from Leaf Explant	89
4.5.2	Bacteria-Free <i>C. asiatica</i> Callus Tissue Production	90
<b>5</b>	<b>DISCUSSION</b>	91
5.1	Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of Two Different Subspecies of <i>Centella asiatica</i>	91
5.1.1	Plant Subspecies	91
5.1.2	Plant Morphological Parts	91
5.1.3	Plant Extraction	92
5.1.4	Antibacterial Activity of <i>C. asiatica</i> Subspecies	93
5.1.5	Antioxidant Activity of <i>C. asiatica</i> Subspecies	93
5.1.6	Estimation of Total Phenolic Content	97
5.2	Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents of <i>Centella asiatica</i> after Inoculation with Pathogenic Bacteria	98
5.2.1	Antibacterial Activity of Inoculated Plants	99
5.2.2	Antioxidant Activity of Inoculated Plants	101
5.2.3	Total Phenolic Content of Inoculated Plants	102
5.2.4	Phenolic Compounds Determination Using High Performance Liquid Chromatography	104
5.3	Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents of <i>Centella asiatica</i> after Inoculation with Beneficial Bacteria	106
5.3.1	Antibacterial Activity of Treated Plants	107
5.3.2	Antioxidant Activity of Treated Plants	108
5.3.3	Total Phenolic Contents of Treated Plants	109

5.3.4	Phenolic Compounds Determination Using High Performance Liquid Chromatography	110
5.4	Endophytic Bacteria Associated with <i>C. asiatica</i>	111
5.4.1	Isolation of Endophytic Bacteria	111
5.4.2	Identification of Isolated Endophytica Bacteria	112
5.4.3	Antibacterial Activity of Isolated Endophytic Bacteria	113
5.4.4	Antioxidant Activity of Isolated Endophytic Bacteria	114
5.5	Production of Endophytes-Free Callus	115
5.5.1	Callus Induction	117
5.5.2	Callus Multi-Subculture Method to Obtain Bacteri-Free <i>C. asiatica</i> Callus and Screening	119
<b>6</b>	<b>CONCLUSION</b>	121
	<b>APPENDICES</b>	125
	Appendix A – Material and Method	125
	Appendix B – HPLC Chromatograms	126
	Appendix C – 16S rRNA Gene Sequences	134
	Appendix D – ANOVA Tables	140
	<b>BILBIOGRAPHY</b>	150

## LIST OF FIGURES

Figure		Page
3.1	<i>Centella asiatica</i> (pegaga). a- Subspecies A (University of Malaya Herbarium Voucher Specimen: KLU047364). b- Subspecies B (University of Malaya Herbarium Voucher Specimen: KLU047552).	48
3.2	Inoculation procedure was carried out inside a class II laminar air flow cabinet and the treated plants were kept under quarantine condition using the wet plastic bags.	54
4.1	Hemolysis of rabbit erythrocytes is expressed as percentage values. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$ .	67
4.2	DPPH assay of samples. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$ .	68
4.3	SOD activities of the examined samples are presented as inhibition rates. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$ .	69
4.4	<i>Centella asiatica</i> inoculation with <i>Enterobacter sp.</i> as the pathogenic bacteria. a- Non-inoculated <i>C. asiatica</i> as the control plant; b- <i>C. asiatica</i> three days after inoculation with the pathogenic bacteria; c- <i>C. asiatica</i> seven days after inoculation with the pathogenic bacteria.	71
4.5	DPPH assay of <i>C. asiatica</i> inoculated with the pathogenic bacteria. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$ .	73
4.6	SOD activities of <i>C. asiatica</i> inoculated with the pathogenic bacteria are presented as inhibition rate. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$ .	74

- 4.7. DPPH assay of *C. asiatica* treated with the beneficial bacteria. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at  $p = 0.05$ . 80
- 4.8. SOD activities of *C. asiatica* treated with the beneficial bacteria are presented as inhibition rate. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at  $p = 0.05$ . 81
- 4.9. Hemolysis of rabbit erythrocytes is expressed as percentage values for *C. asiatica* associated endophytic bacteria samples. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at  $p = 0.05$ . 87
- 4.10. DPPH assay of endophytic bacteria associated with *C. asiatica*. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at  $p = 0.05$ . 88
- 4.11. SOD activities of the endophytic bacteria associated with *C. asiatica* are presented as inhibition rate. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at  $p = 0.05$ . 89

## LIST OF TABLES

Table		Page
2.1	Nutrient table of 100 g edible portion of <i>Centella asiatica</i>	44
3.1	Gradient elution condition	57
3.2	The combination of different concentrations of BAP and NAA for induction of callus from leaf explants of <i>C. asiatica</i>	63
4.1	Antibacterial activity of extracts and controls against bacterial species tested by disc diffusion assay.	66
4.2	Total phenolic content of samples presented as gallic acid equivalent.	70
4.3	Antibacterial activity of extracts of <i>C. asiatica</i> inoculated with pathogenic bacteria and controls against bacterial species tested by disc diffusion assay.	72
4.4	Total phenolic content of <i>C. asiatica</i> inoculated with pathogenic bacteria and the control plants presented as gallic acid equivalent.	75
4.5	Retention times for standard phenolic compounds.	76
4.6	Number of detected phenolic compounds and the amount of the detected phenolic compounds based on standard phenolic compounds in different parts of <i>C. asiatica</i> inoculated with the pathogenic bacteria and non-inoculated <i>C. asiatica</i> (Control).	77
4.7	Antibacterial activity of <i>C. asiatica</i> treated with beneficial bacteria and controls against bacterial species tested by disc diffusion assay.	79
4.8	Total phenolic content of <i>C. asiatica</i> treated with the beneficial bacteria and the control plants presented as gallic acid equivalent.	82



4.9	Number of detected phenolic compounds and the amount of the detected phenolic compounds based on standard phenolic compounds in different parts of <i>C. asiatica</i> treated with the beneficial bacteria and non-treated <i>C. asiatica</i> (Control).	84
4.10	Isolated <i>Centella asiatica</i> endophytic bacteria.	85
4.11	Antibacterial activity of a suspension of endophytic bacteria against the bacterial species tested by disc diffusion assay.	86
4.12	Callus formation and average of callus fresh matter after 6 weeks of culture on MS medium with different NAA and BAP concentrations.	90

## LIST OF SYMBOLS AND ABBREVIATIONS

ANOVA	analysis of variance
BAP	6-benzylaminopurine
BHT	Tert-butylated hydroxytoluene
bp	base pairs
cfu	colony forming unit
CRD	completely randomized design
cv.	cultivar
ddH <sub>2</sub> O	distilled deionized water
DNA	deoxy ribonucleic acid
DNMRT	Duncan New Multiple Range Test
DPPH	2,2 –diphenyl-1-picrylhydrazil
EDTA	ethylene diamine tetra acetic acid
<i>et al.</i>	et alia
IAA	indole-3-acetic acid
mg	milligram
$\mu\text{mol m}^{-2} \text{s}^{-1}$	micromole per meter square per second
MS	Murashige and Skoog
NAA	a-naphthaleneacetic acid
OD	optical density
PCR	polymerase chain reaction
pH	$-\log [\text{H}^+]$

RNA	ribonucleic acid
rpm	revolutions per minute
SOD	superoxide dismutase
sp.	species
subsp.	subspecies
UV	ultraviolet (light)

## LIST OF APPENDICES

<b>Appendix</b>	<b>Page</b>
A – Material and Method	125
B – HPLC Chromatograms	126
C – 16S rRNA Gene Sequences	134
D – ANOVA Tables	140