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

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In the name of God, the most compassionate the merciful

Specially dedicated to:

My kind parents
Nader and Masoumeh

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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. aisiatica* 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 gibsonni*. 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 dipilh untuk kajian berlanjutan. Asai anti-oksidan yang berlainan digunakan untuk menkaji kesan anti-oksidan manakala kaedah disk diffusion digunakan untuk menkaji kesan anti-bacteria. Kandungan sebatian fenolik bahagian bahagian C. asiatica telah ditentukan oleh kaedah Folin-Ciocalteau. 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 peingkat awal dan peringkat lanjutan. Penghasilan sebatian fenolik oleh tumbuhan yang dirawat juga dikaji dengan mengunakan kaedah HPLC dan dibandingkan dengan tumbuahan yang tidak di rawat. Bakteria endofitik yang terlibat dengan C. asiatica diasingkan dan dikenali dengan mengunakan urutan 16S rDNA. Kesan anti-bakteria dan anti-oksidan supernatant bebas sel daripada kultur bacteria ini juga di kaji. Kaedah multikultur digunakan untuk mengasilkan tisu callus bebas bacteria selepas satu protocol in vitro untuk pengasilan 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 penhasilan bahan fenolic dalam quantity yang tidak menentu. Semua bacteria kecuali Bacillus gibsomni endofitik yang diasingkan berupaya merencat ketumbuhan Pseudomonas aeroginosa. 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

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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_2O	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
et al.	et alia
IAA	indole-3-acetic acid
mg	milligram
$\mu mol\ m^{-2}\ s^{-1}$	micromole per meter square per second
MS	Murashige and Skoog
NAA	a-naphthaleneacetic acid
OD	optical density
PCR	polymerase chain reaction
pH	$-\log [H^+]$

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

LIST OF APPENDICES