INTRODUCTION

One of the most challenging problems in the development and use of medicinal plants to produce drugs is the non-economical isolation of their pharmaceutically important compounds due to their low production quantity (Kayser & Quax, 2007). Hence, several biotechnological techniques have been employed to enhance the production of bioactive compounds in plants such as tissue culture and genetic modification methods (Robins 1994; Tripathi & Tripathi, 2003; Kayser & Quax, 2007; Karuppusamy 2009; Chaudhury & Pal, 2010).

Effects of both groups of beneficial and pathogenic bacteria on metabolic pathways in plants have been discussed in several references (Soto et al., 2006; Ding & Friml, 2010). For example, gene-for-gene resistance system of plants in response to pathogenic bacterial infection (Pérez-de-Luque et al., 2004; Poiatti et al., 2009) and plant growth promotion by beneficial bacteria associated with plants (Ali et al., 2009; Hussain & Hasnain, 2009) are some of these interactions.

Although there are a few reports which shows the production of bioactive compounds in plants can be increased by bacteria, application of bacteria to enhance medicinal plant bioactivities by increasing the production of secondary metabolites in the plants has been poorly studied. For example, Ordookhani et al. (2010) showed that lycopene and potassium contents as well as total antioxidant activity were increased in tomatoes treated with plant growth-promoting rhizobacteria.

Although endophytic bacteria show no harm to plants and several studies reported their benefits for plant growth and defense system, they were not clearly classified as beneficial bacteria in scientific literature. There are also lack of studies on the effect of endophytic bacteria present in medicinal plants on the production of plant secondary metabolites and their bioactivities.

Centella asiatica is an important herb which has been used in traditional medicine for several years. It has been mostly used for human wound healing, diarrhea, asthma, inflammations, skin diseases and mental disorders in traditional medicine while treatment of keloids, lupus, leprosy, cellulitis and epilepsy have been also reported for this plant (Samy et al., 2005; Shetty et al., 2006; Bodeker 2009). Several bioactivities of this herb have also been recently investigated such as cytotoxic potential (Ullah et al., 2009), antioxidant capacity (Hussin et al., 2009) and antimicrobial activity (Jagtap et al., 2009).

Some biotechnological methods such as tissue and cell culture (Aziz et al., 2007) and genetic modification (Kim et al., 2007) have been used to increase the production of valuable biocompounds in *C. asiatica*. Although application of chitosan as an elicitor was reported to increase the production of flavonoids in *C. asiatica*, to my knowledge there is no report on the enhancement of the levels of bioactive compounds in this plant using bacteria. There is also no report on bioactivity of endophytic bacteria associated with *C. asiatica* and the effect of this group of bacteria on *C. asiatica* bioactivities.

This study was designed and carried out to investigate if naturally accrued plant pathogenic and beneficial bacteria can increase the production of bioactive compounds in *C. asiatica* and thereby enhance its bioactivities. Antibacterial and antioxidant levels were selected as the test parameters and phenolic compounds have been selected as antimicrobial (Puupponen-Pimiä et al., 2005; Alberto et al., 2006; Nikitina et al., 2007; Maddox & Laur, 2010) and antioxidant (Chun et al., 2005; Kumar et al., 2009; Karamać 2009; Amorati et al., 2010) activities of phenolic compounds have been reported many times. Hence, as the first step, antibacterial and antioxidant activities and total phenolic contents of two commonly used *C. asiatica* subspecies in Malaysia were evaluated to select the most potent subspecies. The selected subspecies was then used for the main investigation that was based on previous reports of different subspecies, cultivars or varieties of a plant species showing different levels of bioactivities (Kedage et al., 2007; Henríquez et al., 2009). Different tissues of a plant may produce different amounts of bioactive compounds and therefore show different bioactivities (Seigler 2002; Jang et al., 2010). Hence, different morphological parts of the selected subspecies of *C. asiatica* were separately studied to evaluate the bioactivities and production of phenolic compounds.

The endophytic bacteria associated with this plant were also isolated, identified and their bioactivities measured. To evaluate the total effect of the endophytic bacteria associated with *C. asiatica* on the bioactivities of the plant, an endophytes-free *C. asiatica* control plant is important for comparison with a normal plant that is already associated with endophytes. Hence, a protocol was also established in this investigation to produce

endophytes-free *C. asiatica* tissue as the first step to produce the endophytes-free plant for any future studies.

Although the key objective of this study was to evaluate the effect of both pathogenic and beneficial bacteria on *C. asiatica*'s antibacterial and antioxidant activities and the production of phenolic compounds in this medicinal plant, the following objectives were also considered:

- To evaluate antibacterial and antioxidant activities and total phenolic contents of two commonly consumed subspecies of Malaysian *C. asiatica* and subsequently select the most potent among them for this study.
- To evaluate the antibacterial and antioxidant activities and total phenolic contents in different morphological parts of *C. asiatica* namely leaf, petiole-stem and root.
- To isolate and identify endophytic bacteria associated with *C. asiatica* from different morphological parts of the plant namely leaf and petiole-stem.
- To evaluate the antibacterial and antioxidant activities of isolated endophytica bacteria associated with *C. asiatica*.
- To optimize an *in vitro* protocol to produce callus tissue from leaf explants of *C. asiatica*.
- To establish a protocol to produce endophytes-free *C. asiatica* callus tissue as the basic step for production of endophytes- free *C. asiatica* plant in future studies.

LITERATURE REVIEW

2.1. Bioactive Compounds Generated by Bioactivities in Plants

The importance of plants as a natural source of pharmaceutical and nutritional compounds is evident (van Wyk & Wink, 2004; Wood 2008). Plants have been examined and used for many years in folk medicine in many countries and, based on this indigenous knowledge, an industry for medicinal plants was established in some eastern countries such as China and India. Based on the research carried out on different medicinal plants in different research institutes, harmless herbal products can be found all over the world (Rates 2001). However, currently the use of medicinal plants is not just confined to the countries of the East as these days a lot of plant-based products with different names can be purchased in western countries. From North America where the market well came the plant-based phytomedicinal products called “health foods” (Calixto 2000) to the Latin American Countries such as Brazil where several well-developed phytomedicinal companies are active (Calixto 2005) and the considerably expanded marketing of phytomedicinal drugs in European countries such as Italy, Spain, Germany and UK (Calixto 2000) all shows the importance of medicinal plants in the western countries.

Plants are not only recommended to be used in a healthy diet (Campbell & Campbell, 2006) but are investigated for the development of plant-based medicines (Newman et al., 2000; Dittmann et al., 2004). Several researches confirmed that plants are the good source of bioactive compounds (Cseke et al., 2006) to support the history of ancient application of herbs in treatments of various diseases.

Anti-inflammatory (Uddin et al., 2010; Lavanya et al, 2010) anti-arthritic (Patil et al., 2009; Rajendran & Krishnakumar, 2010), Antinociceptive (Fernando et al., 2009; Uddin et al., 2010), antiplatelet (Lavanya et al., 2010; Durairaj & Dorai, 2010), anticancer (Soeksmanto et al., 2010; Naama et al., 2010), antioxidant (Patel et al., 2010; Wei et al., 2010), antimalarial (Prachayasittikul et al., 2010; Oliveira et al., 2010), antibacterial (Hema et al., 2010; Jeong et al., 2010), antifungal (Huang et al., 2010; Sule et al., 2010), and antidiabetic (Murugesan et al., 2010; Aina et al., 2010) activities of different plants are some of the examples to prove the medicinal importance of the plants. Several studies have been carried out to identify the bioactive compounds of plants (Polya 2003). Chemical analysis and chromatographic techniques have shown that these bioactive compounds are from various families such as peptides, organic acids and phenolic compounds.

2.1.1. Antibacterial Activity

2.1.1.1. Bacterial infections and natural antibacterials

Antibiotic resistance is the major reason for the need to develop new antibiotics. Humans, animals, plants and even insects are all at risk to bacterial infections. The number of diseases caused by bacteria to humans, animal and plants is high and transmission is variable trough air, food, water, soil and contact. These pathogenic bacteria are from different genera, species and strains and therefore have different antibiotic resistances. Gene transformation between bacteria is the main reason for creation of new kinds of bacteria which contain new antibiotic resistance genes making in multiresistant bacteria (Dzidic & Bedeković, 2003).

Natural antibacterial compounds can be produced by any bio-organism which carry antibacterial resistance gene/s. Discovering antibacterial compounds produced by animals (Conlon & Sonnevend, 2010), insects (Otvos 2000), plants (Castro & Fontes, 2005), fungi (Qi et al., 2009) and bacteria (Eleftherianos 2009) show that screening the antibacterial activity of natural products to find new antibacterial compounds is promising. These antibacterial compounds belong to different phytochemical families such as peptides (Boman 2003), antibacterial phenolic compounds (Maddox & Laur, 2010), and organic acids (Raftari 2009).

2.1.1.2. Methods of antibacterial activity evaluation

Both *in vivo* and *in vitro* methods are applied to determine the antibacterial activity of samples. The *in vivo* evaluation methods of antibacterial compounds usually are carried out on rats and the duration of antibacterial activity is determined (Lee et al., 2003). Several *in vitro* antibacterial assays are developed but agar-well diffusion and disc diffusion methods are the most commonly used techniques to evaluate antimicrobial activity of different kinds of samples (European Committee on Antimicrobial Susceptibility Testing, 2009). Inhibition zone diameters can be measured and antibacterial potential of samples can be stated to compare with a positive control that is usually a commercial antibiotic.

2.1.2. Antioxidant Activity

2.1.2.1. Oxidation and natural antioxidants

Oxygen, the essential element for life (except for anaerobes), generates various types of reactive oxygen species (ROS) which can damage living tissues. The damage caused by

the interaction of ROS with biocompounds increases the risk of many diseases. ROS can be classified as radicals (such as superoxide, hydroxyl, peroxyl and alkoxyl) and non-radicals (such as ozone, peroxynitrite, lipid peroxide and hydrogen peroxide) and can easily be formed in normal and pathological cell metabolisms (Trachootham et al., 2009). The imbalance between pro-oxidants and antioxidants lead to a condition called oxidative stress. Based on high reactivity of free radicals, they can easily damage proteins, lipids and DNA molecules under oxidative stress conditions and are harmful to the intra/extra-cellular systems of organisms. It has the potential to cause numerous kinds of diseases such as cancer, atherosclerosis, vasospasms, stroke, trauma, hyperoxia, Alzheimer, Parkinson and cardiovascular diseases (Halliwell & Gutteridge, 1985; Cross et al., 1992; Ames et al., 1993; Frei 1994; Giasson et al., 2002).

Although the human natural antioxidant defense system is responsible for protection against the destructive action of ROS by production of natural antioxidants, any interruption in their function caused by environmental stresses or a disease can potentially increase the risk of oxidative damage in cells. Furthermore, an increase in the concentration of the oxidizing species can cause oxidative stress in the body and natural antioxidants may not adequately neutralize free radicals. Hence, to reduce the harmful effects of oxidative stress, consuming a diet high in antioxidants is recommended (Packer et al., 1999; Jeep et al., 2008).

There are several strong antioxidants which can be divided into lipid-soluble antioxidants, like vitamin E and butylated hydroxytoluene, and water-soluble antioxidants, like vitamin

C and gluthathione (Donoghue & Donoghue, 1997; McLean et al., 2005). The above mentioned classification of antioxidants may not be a superior categorization as some antioxidants such as phenolics have various phytochemicals of which some are water-soluble and some are lipid-soluble compounds (Steven et al., 1996; Kong et al., 2008). Dividing the antioxidants into enzymatic and non-enzymatic groups is another way of classification of antioxidant compounds. Enzymatic antioxidants including superoxide dismutase, catalase, and glutathione peroxidase are produced endogenously in humans while the non-enzymatic antioixdants like tocopherols, carotenoids, ascorbic acid, flavonoids and tannins are mostly obtained from other sources such as plants (Naskar et al., 2010).

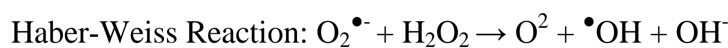
In addition to the above mentioned influence of oxidative reactions on human health, lipid and protein oxidations in foods during food processing and storage are also some of the main reasons for deterioration of flavor, aroma, colour and nutritive value (Stadtman & Levine, 2003; Baron et al., 2007). Usage of antioxidant compounds in foods as preservatives to avoid oxidative reactions has been practiced for many years (Artés et al., 2006).

2.1.2.2. Methods of antioxidant activity evaluation

There are various model systems to determine the antioxidant potential of different samples. However, due to the complex composition of antioxidant compounds, application of different methods of evaluation of the antioxidant capacity which are based on different features of the antioxidant activities is recommended (Wichtl 2001). These assays can be

classified into two groups namely *in vivo* and *in vitro* models. While rats and mice are the usual test animals for *in vivo* evaluation of antioxidant activity techniques, *in vitro* models for evaluation of antioxidant activity of samples include several types of assays such as DPPH (2,2 –diphenyl-1-picrylhydrazil) radical scavenging, super oxide radical scavenging, hydroxyl radical scavenging, nitric oxide radical inhibition, reduction capacity, superoxide dismutase (SOD), oxygen radical absorbance, 2,2-azinobis(3-ethyl benzothiazoline-6-sulfonicacid) diamonium salt (ABTS), N, N-dimethyl-p-phenylene diamine dihydrochloride (DMPD), and erythrocyte technique. These are mostly spectrophotometeric assays. The parameters usually evaluated by *in vivo* models include protein oxidation, lipid peroxidation, reduced glutathione content, superoxide dismutase, and catalase activities (Joharapurkar et al., 2003).

ROS can cause breakdown of the cell membranes through lipid peroxides based on the structure of cell membranes. Therefore, prevention of the cell membrane breakdown by samples can show their antioxidant potential. Hemolysis of erythrocytes is an *in vitro* method based on this action. Hydrogen peroxide (H_2O_2) is the main source of hydroxyl radicals ($^{\bullet}OH$) and therefore is an active oxidizing molecule. H_2O_2 is able to produce $^{\bullet}OH$ in the presence of $O_2^{\bullet -}$ or Fe^{2+} through Haber-Weiss (Koppenol et al., 1978) and Fenton (Fenton 1876) reactions respectively.



Haber-Weiss Reaction: $O_2^{\bullet -} + H_2O_2 \rightarrow O^2 + {}^{\bullet}OH + OH^-$

Fenton Reaction: $H_2O_2 + Fe^{2+} \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$

Application of hydrogen peroxide in an erythrocyte hemolysis model induces the breakdown of the cell membranes and hemoglobins can be released out of the cells. Antioxidants can reduce the hemolysis risk in erythrocytes.

Application of strong antioxidants as a positive control in all types of antioxidant activity assays is necessary. Ascorbic acid (vitamin C) is a strong antioxidant as it donates two electrons from the C-2 and C-3 double bond carbon atoms and is commonly used as a positive control in assays.

2.1.3. Phenolic Compounds

At least one aromatic ring (C6) which bears one or more hydroxyl groups can indicate the phenolic compounds. Phenolics can be called as one of the major groups of plant products within of alkaloids and terpenoids (Ndakidemi & Dakora, 2003). Phenolics are usually classified into various groups based on their structures. In one of the most complete division it is classified into different groups of phytochemicals namely simple phenolics, phenolic acids and aldehydes, acetophenones and phenylacetic acids, cinnamic acids, coumarins, flavonoids, biflavonyls, benzophenones, xanthones, stilbenes, benzoquinones, anthraquinones, naphthaquinones, betacyanins, lignans, lignin, tannis, and phlobaphenes (Vermerris & Nichelson, 2007). Phenolics can also be divided into three groups based on the phenolic phytochemical structures, namely phenolic acids (such as caffeic acid), flavonoids (such as anthocyanins) and non-flavonoid polyphenols (such as Ellagic acid) to make the above mentioned classification simple and easy to use (Basha et al., 2004).

Phenolics are of widely varied range of compounds with low molecular weight found in all higher plants (Makoi & Ndakidemi, 2007). They have been mostly classified as secondary metabolites as they are not directly involved in primary metabolism but involved in different plant processes such as biotic and abiotic stresses (Taylor 1996; Michalak 2006), fruit growth and maturation (Buta & Spaulding, 1997; Ding et al., 2001).

2.1.3.1. Antibacterial activity of phenolic compounds

Antimicrobial activities of many phenolics have been reported in the past. Phenolic compounds are highlighted to adversely affect bacteria through various mechanisms (Puupponen-Pimiä et al., 2005). Review of literature shows that plants induced the production of these compounds as antimicrobial agents against plant pathogens. Hence phenolic compounds produced naturally in plants were studied for their antibacterial and antifungal activities by several researchers. Alberto et al. (2006) reported a direct relationship between the antimicrobial activity of apple skins and their phenolic contents while the antimicrobial potential of polyphenolic compounds isolated from plants of Geraniaceae and Rosaceae families has been shown by Nikitina et al. (2007). Berry phenolics also inhibited the growth of some selective intestinal pathogens Puupponen-Pimiä et al., 2005). The specifically isolated phenolic compounds from the total extract of plants confirmed that several phenolic compounds from different groups have antimicrobial potential. Two phenolic compounds namely 2’,6’,4-trihydroxy-4’-methoxydihydrochalcone and 2’,4’,6-trihydroxydihydrochalcone isolated from *Greyia flanaganii* showed antimicrobial activity against *Propionibacterium acnes* (Mapunya 2009). Inhibition of growth using test bacteria namely *Bacillus subtilis, Staphylococcus*

aureus, Escherichia coli and *Shigella sonnei* by 4-methoxycinnamic acid isolated from root part of *Dendranthema zawadskii* was reported while another phenolic compound isolated from the same source namely caffeic acid methyl ester could only show antibacterial activity against *S. aureus* (Rahman & Moon, 2007). Five isolated phenolic compounds from *Anabasis aphylla* were also shown to have selective antimicrobial activity against both Gram-negative and Gram-positive test bacteria (Du et al., 2009).

2.1.3.2. Antioxidant activity of phenolic compounds

Electron donation ability, termination of radical chain reactions and chelation of metal ions by phenolics cause these compounds to act as strong antioxidants (Kumar et al., 2009; Karamać 2009; Amorati et al., 2010). Their antioxidant potential has a positive relationship with the number of hydroxyl groups on their benzene rings (Packer & Cadenas, 2002). Many studies showed plants containing high amounts of phenolics showed high antioxidant activity. For example, positive correlation between phenolic contents and antioxidant properties in eight *Salvia* species (Tosun et al., 2009) and in some selected Chinese medicinal plants (Song et al., 2010) have been reported. Hence, the total phenolic contents of a plant material can have a positive relationship with its antioxidant capacity. Based on the presence of different phenolic compounds in all types of plants, a diet rich in vegetables and fruits can be a good source of antioxidants and essential for health (Chun et al., 2005).

Simple cell-derived phenolic acids such as 3-hydroxyanthranilic acid are found to be active as co-antioxidants for α – tocopherol to inhibit low density lipoprotein and plasma lipid

peroxidation in humans (Thomas et al., 1996). These studies have discovered the synergistic interactions between phenolic compounds with other antioxidants.

2.1.3.3. Determination of phenolic compounds

The methods which are used to determine the phenolics in samples are divided into spectrophotometric methods and chromatography methods. The total phenolic contents of any sample can be determined colorimetrically using Folin-Ciocalteu or Folin-Denis assays. The reduction of Folin reagent by the test sample in a mixture with alkaline solution can be measured at a wavelength ranging from 725 to a maximum of 765 nm (Waterhouse 2002; Koski et al., 2003; Thiyam et al., 2004).

Chromatography methods usually are applied to separate and identify the phenolic compounds in a sample. The simplest chromatography assay is thin layer chromatography (TLC). Several solvents are used to separate the phenolic compounds from the non hydrolyzed extracts, fatty acids and other contaminants. TLC has the ability to separate individual phenolic compounds as well. For example, Morsch et al. (2002) separated the flavonoids swertisin and 2”-O-rhamnosylswertisin from *Aleurites moluccana*.

Although gas chromatography was reported to be used several times for identification and quantification of phenolic compounds in different samples such as food, plant, soil and wastewater samples (Scaringelli et al., 1980; Zhang et al., 2006), application of high performance liqid chromatography (HPLC) is more common to study the phenolics (Andrade & Seabra, 2005). The HPLC system equipped with a UV detector is a suitable

method to detect the phenolic compounds using the right column and liquid mobile phase. Both normal-phase and reverse phase columns have been reported to identify phenolics of test samples. Li and Rassi (2002) used a normal-phase silica column for determination of betine while Jandera et al. (2005) reported a reverse-phase analysis of phenolics in beverages and plant extracts.

Different HPLC grade phenolic compounds have been applied as the standards in analaysis of plant extracts. Among the phenolics, some compounds such as gallic acid, (+)-catechin, chlorogenic acid, procyanidin B2, p-coumaric acid, (-)-epicatechin, ferulic acid, hperin, rutin, phloridzin, benzoic acid, cafferic acid and tannic acid, which have important bioactivities, have been mostly studied (Nuengchamnong et al., 2004; Chinnici et al., 2004; Chen & Xiao, 2005; Levaj et al., 2010; Singh et al., 2010).

2.2. Biotechnological Techniques to Enhance the Bioactive Compounds Production in Plants

As nearly 80 per cent of the world's population consumes herbal medicines based on the World Health Organization (WHO) report, the importance of medicinal plants are highlighted. Beside the traditional use of medicinal plants, several drugs are produced from the phytochemicals isolated from these and other plants. For example, quinidine is an important chemical extracted from a rainforest tree (*Cinchona ledgeriana*) bark to produce a plant-based cardiac medicine (Bisset 2001). But there are several problems in using medicinal plants to produce herbal based drugs such as low concentrations of pharmaceutically valuable compounds in the plants or their plant extracts and presence of unwanted compounds and toxic constituents. Biotechnological approaches gave the

opportunity to overcome several of these problems. Genetic development via breeding and genetic transformation, metabolic and pathway engineering, micropropagation and cell culture techniques are some of the biotechnological methods to overcome the problems.

Biotechnological tool potential to enhance the production of secondary metabolites in medicinal plants should be well understood as medicinal plants can be the most important source of life saving drugs these days (Tripathi & Tripathi, 2003). Several biotechnological methods were reported to be employed for inducing the production of valuable phytochemicals in medicinal plants such as genetic transformation of plants and *in vitro* plant cell and tissue culture (Barz & Ellis, 1981; Deus & Zenk, 1982; Kayser & Quax, 2007).

2.2.1. Cell and Tissue Culture

The ability of plant cells and tissues to be cultured under sterile condition from their explants eases the mass production of highly valuable plant compounds. Optimization of composition of the culture medium as well as environmental condition of *in vitro* growth is the main strategy to enhance the production of secondary metabolites of interest in medicinal plants (Robins 1994; Rao & Ravishankar, 2002). Different methods of tissue culture were reported to be applied in enhancement of secondary metabolite production in plants. For example, organ culture of *Fritillaria unibracteata* was established with 50 times higher growth rate compared to the control (natural and wild growth) and the concentration of alkaloid in the cultured bulbs of the plant was higher than the wild bulbs (Gao et al., 2004). Kovačević & Grubišić (2005) showed how to enhance the production of

anthraquinon in *Frangula rupestris* via multiple shoot regeneration technique. Root and callus culture were also reported as strong tools to enhance the production of valuable compounds in medicinal plants (Srivastava & Srivastava, 2007). For example, Taniguchi et al. (2000) enhanced the production of gallotannins in *Rhus javanica* through root culture while Salma et al. (2008) reported the production of serpentine in *Rauvolfia serpentina* via callus culture.

Although plant cell cultures had been introduced as a technique which was not able to be used potentially as a method to enhance the production of secondary metabolites and valuable pharmaceutical compounds many years ago (DiCosmo & Misawa, 1985), recent optimized protocols made it one of the most practical plant *in vitro* culture methods to enhance the levels of secondary metabolites these days (Chattopadhyay et al., 2002; Karuppusamy 2009). For example, plant cell cultures which are are referred by Rao and Ravishankar (2002) as chemical factories of secondary metabolites was applied by Jia et al. (2006) to enhance the production of total flavonoids in *Saussurea involucrate*.

2.2.2. Genetic Transformation

The recent approaches in recombinant DNA technology helped the plant genetic improvement programmes. These days the direct and indirect methods of genetic transformation are mostly used rather than the conventional breeding methods as they consume less time (Rafat et al., 2010).

Application of *Agrobacterium rhizogenes* to produce the hairy root plants is a powerful technique to induce the production of secondary metabolites in medicinal plants (Guillon et al., 2006). Production of hairy root cultures for mass production of valuable phytochemicals in plants has been practiced several times and showed a promising potential to the pharmaceutical industry. *A. rhizogenes* which naturally cause the hairy root disease in plants was employed to enhance the yield of important products effectively. Although production of hairy root is used as a biotechnological tool to enhance the secondary metabolite production, there are several reports which show the production of particular compounds in hairy root cells is similar to the plants in nature. For example, hairy root production of *Centaurium erythraea* Rafn could not increase the production of bitter secoiridoid glucosides and xanthones (Subotić et al., 2009). Therefore, the use of tissue culture techniques to enhance the production of bioactive compounds in hairy roots is recommended (Subotić et al., 2009; Chaudhury & Pal, 2010).

Agrobacterium tumefaciens-mediated transformation is the most practical and widely used method to introduce a gene into a plant species especially dicotyledons (Rafat 2008). Spencer et al. (1993) showed that *Agrobacterium tumefaciens*-mediated transformation of *Mentha* species interestingly can enhance the biosynthesis of monoterpenes in shoot cultures. The production kinetics and stoichiometry of alkaloids were increased in different solanaceous plants when treated with *Agrobacterium tumefaciens*-mediated transformation technique (Subroto et al., 1996).

2.2.3. Physical Treatment

Environmental and physical stresses are also reported to increase the production of medicinal plant secondary metabolites (Vanisree et al., 2004; Vasconsuelo & Boland, 2007). Based on this fact, several studies were carried out to increase the production of particular compounds in different plants using physical treatments.

Apart from optimization of the physical conditions during tissue culture such as light period and growth temperature, several other innovative techniques were applied to enhance the production of pharmaceutically and nutritionally important compounds in plants. For example, shikonin production was increased by 60-70% in suspension culture of *Lithospermum erythrorhizon* in response to low energy ultrasound (Lin & Wu, 2002). Production of several isoflavonoids namely genistin, genistein, daidzein, and biochanin A was increased in callus culture of *Genista tinctoria* after UV radiation treatment (Tůmová & Tůma, 2010). Production of saponins in cell culture of *Panax ginseng* was enhanced through osmotic stress induction (Wu et al. 2005).

2.2.4. Chemical Treatment

Presence of different chemicals in the plant growth environment can affect metabolic pathways of the plants (Schauer et al., 2006). Apart from optimization the of plant growth medium in *in-vitro* studies by application of different concentration of hormones and nutrient compounds, adding particular compounds into the growth medium can enhance the production of certain bioactive phytochemicals in plant cells. One of the most applicable groups of compounds which have been used to increase the production of

different phytochemicals is microbial elicitors. Microbial elicitors are prepared from different fungal and bacterial microorganisms. Application of microbial elicitors in the growth medium of plants to enhance the production of secondary metabolites is based on the plant cell response to the microorganism especially pathogenic bacteria and fungi by increasing the production of specific secondary metabolites. For example, based on the plant cell behavior in response to the pathogens, host-pathogen elicitor compounds have been applied to elevate production of secondary metabolites in medicinal plants. Nedêlník (1989) showed the ability of elicitors to induce the production of antimicrobial compounds in callus cultures of *Medicago sativa* treated with an elicitor prepared from the mycelium of *Fusarium oxysporum*. The fungal elicitors obtained from *Aspergillus niger* and *Rhizopus stolonifer* could enhance the production of oleandrin in *Nerium oleander* L. cultures (Ibrahim et al., 2007), while Xu et al. (2007) increased the production of syrigin in suspension cultures of *Saussurea medusa* using yeast elicitors.

Application of fertilizers in cultivation soil to enhance the production of some valuable compounds in plants can also be classified as one type of plant chemical treatment. For example, Palumbo et al. (2007) showed the nitrogen fertilizer effects on the production of secondary metabolites of yaupon.

Chemical treatments are not only limited to the application of chemicals in the tissue culture growth media or soils, but spraying the particular chemicals on the plant tissues were also reported as one type of chemical treatment to enhance the production of valuable

compounds. Eliašová et al. (2004) increased the secondary metabolites production of *Matricaria chamomilla* by spraying the plant leaves with aqueous $CuCl_2$ solution.

Effects of gas concentration on secondary metabolite production in plant cells has been also reported (Linden et al., 2001). Huang and Chou (2000) enhanced the production of ethylene and 3,4-dihydroxyphenylalanine in *Stizolobium hassjoo* cell culture by increasing the concentration of Po_2 to 0.3 atmosphere in early and late stages of cultivation respectively.

2.2.5. Microbial Treatment

Plants are not only borne with microbes from rhizosphere to phyllosphere but also host several microbes inside their tissues which are referred to as endophtes (Elavazhagan et al., 2009). In plant metabolism studies, plant-microbe interactions should be considered as an effective parameter (Nehra 2005). The effects of several beneficial and pathogenic microbes on the plant secondary metabolites have been studied. Based on these effects, application of microbial elicitors was established to improve the plants for secondary metabolite production (as described in 2.2.4.).

Direct application of microbes to promote plant growth is routinely practiced and biofertilizers are the commercial products for these plant growth promoting bacteria. For example, several strains of *rhizobacteria* showed potential in plant growth promotion and therefore used to produce different types of biofertilizers (Vessey 2003). Studies on plant metabolism showed that this group of bacteria also improves the metabolism of the treated

plants. For example, Aseri et al. (2008) showed the improvement of metabolism of *Punica granatum* L. after treatment with nitrogen fixing bacteria and mycorrhiza as the biofertilizers. Studies on defense system of plants also showed several secondary metabolites played a role in defense against insect pests and pathogens. Therefore the production of particular secondary metabolites is increased in the plant cells infected by pathogens (Morimoto & Komai, 2000).

Even though there are several studies which showed the enhancement of secondary metabolites production in plants in response to the beneficial or pathogenic microbes (Yuan et al., 2007), there are few reports on direct application of microbes to enhance the production of valuable compounds in plants. Sheng et al. (2002) showed that *Bacillus mucilaginosus* as a potassium solubilizing bacteria increased the mineral content in plant while Ordookhani et al. (2010) increased the production of lycopene in tomato plants treated with some growth promoting bacteria namely *Pseudomonas putida, Azotobacter chroococcum and Azosprillum lipoferum* and some arbuscular mycorrhiza fungi namely *Glomus intaradics* + *Glomus mossea* + *Glomus etunicatum*.

2.3. Plant-Bacteria Interactions

The constant interactions between plants and a wide range of bacteria is evident. The association created by plant-interacting bacteria has been classified into two groups of pathogenic and mutualistic associations (Soto et al., 2006).

2.3.1. Plant Beneficial Bacteria

The bacteria which have beneficial effects on plant health, growth and development are classified as the group of plant beneficial bacteria (Hayat et al., 2010). Their beneficial effects in terms of plant health is attributed to the production of antimicrobial compounds to protect the plant against pathogenic microbes (Stockwell et al. 2002) or inducing the production of antimicrobial agents by plants (Ramamoorthy et al. 2001). Their benefits in terms of plant growth and development are by providing nutrients (Elmerich & Newton, 2007) and growth factors (Sponsel 2003).

2.3.1.1. Plant-beneficial bacteria interactions

The marked influence of beneficial bacteria on the growth of plants is understood. Soil bacteria affect plant growth in various ways. Several soil beneficial bacteria produce and release growth regulators which promote the plant growth. For example, gibberellins which are engaged with a couple of developmental processes in plants such as seed germination and growth of root, stem and leaf (Crozier et al., 2000; Sponsel 2003) can be produced and released by soil endophytic bacteria and fungi (MacMillan 2002; Bottini et al., 2004). Tsavkelova et al. (2005) reported the production of auxins by bacteria associated with orchid roots while Ali et al. (2009) showed that plant associated bacteria enable auxin production influencing the endogenous auxin content and promote plant growth in *Triticum aestivum* L. Auxins as phytohormones are involved in regulation of plant cell differentiation (Ding & Friml, 2010) and especially root formation (Visser et al., 1995). Cytokinins, which are the main plant growth regulators are engaged in cell division

(Kieber 2002) and can be produced by endophytic bacteria (Lidstrom & Chistoserdova, 2002; Hussain & Hasnain, 2009).

Some of the beneficial bacteria provide absorbable compounds for plants by fixing or solubilizing certain chemicals. For example, nitrogen fixing bacteria are able to fix atmospheric nitrogen and produce nitrates as the available source of nitrogen (Elmerich & Newton, 2007). A lack of phosphorus in alkaline cultivable soil and fixation of phosphatic fertilizers such as tricalcium phosphate which is water-insoluble can cause difficulty in crop growth. Ability of phosphate solubilizing bacteria to convert insoluble forms of phosphorus into an available form results in growth promotion of plants (Chen et al., 2006). *Azospirillum brasilense* helps plants by promoting greater uptake of several compounds such as NO_3^-, K^+, and H_2PO_4 (Saubidet et al. 2000). Shankariah and Hunsigi (2001) and Balandreau (2002) confirmed the beneficial effect of *Azospirillum* due to significant increases of cane and wheat yields respectively in field condition.

Several of plant beneficial bacteria can induce the systemic resistance of plants. Stochwell et al. (2002) showed *Pantoea agglomerans* strain Eh252 produce antibacterial agents against *Erwinia amylovora* which is the pathogenic bacteria causing the fire blight disease in pears. Some beneficial bacteria such as *Pseudomonas spp.,* for which their plant growth promoting potential had been confirmed, are reported to improve the plants defense mechanisms against pathogens, nematodes and insect pests by inducing the plant to synthesise particular proteins and modifying the cell wall structure (Ramamoorthy et al., 2001). Some of these beneficial microbes directly produce antimicrobial compounds. For

example production of different antimicrobial compounds such as amphisin, 2,4-diacetylphoroglucinol, hydrogen cyanide, phenazine, tensin and tropolone by pseudomonads is reported (Nielsen et al., 2002; Nielsen & Sørensen, 2003; de Souza et al., 2003). Although several plant-beneficial (either symbiotic or non-symbiotic) bacterial interactions has been studied, many points still remain unclear (Okon & Kapulnik, 2002).

Biopesticides and biofertilizers are biotechnological products produced by plant-beneficial microbes, especially beneficial bacteria, as a helpful system for biological control of plant diseases and plant growth promotion respectively (Montesions et al., 2002; Hayat et al., 2010). For example, *Acidovorax avenae* subsp. *avenae* AAA99-2, which is a harmless strain of bacteria to watermelon, was used for treatment of watermelon seeds from the infection of *Acidovorax avenae* subsp. *citrulli* pathogenic bacteria (Fessehaie & Walcott, 2005). Medeiros et al. (2009) used *Bacillus sp*. RAB9 as a biocontrol strategy to manage the melon bacterial blotch caused by a pathogenic bacteria namely *Acidovorax citrulli.*

2.3.1.2. Methods of plant inoculation with beneficial bacteria

The methods used for inoculation of beneficial bacteria inoculation varies based on the type of plant and beneficial bacteria. Seed treatment or sometimes called seed bacterization is one of the widely used methods for bacteria inoculation. In this method, the seed of plant are inoculated with the proper bacterial dilution ratio and usually a sticker solution is added to the mixture to ensure attachment of suitable bacterial population to the seeds. For example, Verma et al. (2010) used the seed bacterization method for Rhizobacteria inoculation of chickpea. The chickpea seeds were weighed and separated for each

experimental plot and were mixed with 5ml of 7-day old broth culture of bacteria along with 1 ml of 1% (w/v) gum acacia as the sticker solution. The inoculated seeds were sown after drying. The population of bacteria achieved per seed is within a range of 10^7 to 10^8 cfu using the described method.

Seedling treatment is another method which is reported for beneficial bacteria inoculation studies. For example, Han et al. (2006) used the seedling treatment methods to study the effect of co-inoculation with phosphate and potassium solubilizing bacteria on mineral uptake and growth of plants. Pepper and cucumber seeds were sown, germinated and grown for 20 and 15 days respectively. Then they were transplanted into separated pots. Meanwhile *Bacillus megaterium var. phosphaticum* as the phosphate solubilizing bacteria and *Bacillus mucilaginosus* as the potassium solubilizing bacteria were grown in Tryptone Yeast Medium and sucrose-minimal salts medium respectively for 48 h at 27 °C. The harvested cells from the broth were diluted in sterilized water to a concentration of 10^8 cells/ml ($OD_{620} = 0.08$). Finally, three days after transplanting, seedlings were inoculated with 1 ml of bacterial dilution.

As mostly the beneficial bacteria used for plant treatments are soil bacteria, the soil-beneficial bacteria mixture method was also used in some studies. For example, Son et al. (2006) in a study to evaluate the effect of phosphate solubilizing bacteria application on soybean for achieving better growth yield, mixed the Pseudomonas spp. as the phosphate solubilizing bacteria with a combination of sterile peat and bagasse at 50% moisture to

obtain a population of > 10^9 cells/g. The prepared inoculants were used to fill the holes (2.5 cm in diameter and 2.5-3.0 cm deep) where the seeds were dropped into them.

Another way to treat the plants with beneficial microbes is the method usually used to apply the biofertilizers. Several biofertilizers are diluted with irrigation water and then applied to the plants in the field. The beneficial bacteria can be harvested and re-suspended using buffer/water or directly used as the broth culture. For example, Mayak et al. (2004) centrifuged the bacterial culture and re-suspended the collected cells in distilled water to irrigate plants with 40 ml of bacterial suspension in their study.

Further to the above discussion on the method of plant inoculation with beneficial bacteria, bacteria dilution used in other methods can be either prepared directly from bacterial culture broth (Verma et al., 2010) or from the harvested bacteria from the broth (Han et al., 2006).

2.3.2. Plant Pathogenic Bacteria

Among the groups of bacteria associated with plants, a small group of around 100 species of bacteria are able to cause disease and therefore called pathogenic bacteria (Jackson 2009). Some of them can cause systemic plant diseases such as several species of *Erwinia* (Rojas et al., 2002) or non-systemic plant diseases such as *Agrobacterium tumefaciens* which produce tumors (Rafat 2008).

2.3.2.1. Plant-pathogenic bacteria interactions

The increased defense mechanisms of a plant by any chemical or biological agents are termed systemic acquired resistance (SAR) and it has been confirmed to be an efficient tool for controlling plant pathogens (Percival 2001; Pérez-de-Luque et al., 2004). Several studies on defense mechanisms of plants in response to attack by plant pathogenic bacteria showed the infected plants performed induced mechanisms (Poiatti et al., 2009). Plants recognize the pathogens which attack them to perform their appropriate defense responses, a system that is commonly called gene-for-gene resistance in which one side is the host and the other side is the pathogen (Hammond-Kosack & Jones, 1997). Pathogenic bacteria recognize the biochemical signals which are produced by plants and these signals induce the promoter of specific genes in their genomes therefore the genes are expressed. On the other side, the plants also recognize elicitors which act as motifs and escalate a set of defense responses (Nurnberger & Brunner, 2002). The molecules associated with pathogens and recognized by plant cells named pathogen-associated molecular patterns (PAMPs) and can be derived from different macromolecular structure such as essential components of the pathogen cell wall (Badreddine et al., 2008). Several studies showed that plant-pathogen interactions are moderately arbitrated by plant secondary metabolite production and corresponding pathogen tolerance (Kliebenstein et al., 2005).

In response to the plant pathogen, the plant metabolic pathways get induced and production of some particular compounds was increased. The pathogens resistance performed by plant can be expressed locally at the spot of infection and systemically in uninfected tissues of the plant (Mauch-Mani & Métraux, 1998). Phenolic phytochemicals are one of the most

important compounds in which their production increase as soon as the defense system of a plant is affected by pathogenic bacteria (Nicholson & Hammerschmidt, 1992; Ortega et al., 2005) exactly as pathogenic fungi (Grayer & Kokubun, 2001; Petkovšek et al. 2008). Although Lorenc-Kukula et al. (2005) believe that flavonoids are the largest type of phenolic phytochemicals which shows antimicrobial activities, antimicrobial phenolic compounds belong to other groups of phenolics also reported to be responsible in resistance to attack of pathogens in different kind of plants (Petkovšek et al. 2008; Poiatti et al., 2009). *Arabidopsis thaliana* as a test plant showed a resistance to attack from pathogens by the aromatic compound camalexin (Bednarek et al., 2005). Production of glyceollins in *Glycine max* (L.) Merrill (Abbasi & Graham, 2001), sakuranetin and naringenin in *Oryza sativa* (Jwa et al., 2006), and phytoalexins 3-deoxyanthocyanidin in *Sorghum* sp. (Field et al., 2006) are reported in response to pathogenic infections.

To confirm the antimicrobial activity of phenolics and the correlation between plant resistance and bacterial infection, Lorenc-Kukula et al. (2005) examined the resistance of transgenic lines of potato with ectopic expression of anthocyanin 5-O-Glucosyltransferase (5-UGT) against *Erwinia carotovora* subsp. *carotovora* . The results showed that the resistance of transgenic potato tubers which produce 5-UGT are almost 2-fold higher than for non-transformed control plants.

Induction of resistance to pathogen in plants by using elicitors has been studied (Ferrari et al., 2007). Application of elicitors to modify plant cell metabolism in order to enhance the production of important secondary metabolites in some medicinal plants, based on the

above mentioned plant defense system, has been also reported (Kliebenstein 2004; Jeong & Park, 2005).

Production of rishitin and phytuberin in potato tubers inoculated with *Erwinia carotovora* var. *atroseptica* (Lyon 1972) was one of the earliest reports on the production of phytochemicals using pathogenic bacteria in plants. Production of phenolic compounds was increased in leaves of apple infected with apple scab pathogen (Petkovšek et al., 2008).

The gene transformation ability of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* caused these two pathogenic bacteria, particularly A. *rhizogenes*, find a special place in medicinal plant-pathogen interaction studies to improve the biosynthesis of the secondary metabolites of interest. *A. tumefaciens* is strongly used in genetic transformation of plants. Transferring the T-DNA segment from bacterial tumor-inducing (Ti) plasmid in *A. tumefaciens* caused gall tumor in plants. Therefore, an engineered *A. tumefaciens* is always applied in genetic transformation studies which contain a binary vector to move foreign gene into plants (Slater *et al.,* 2003).

2.3.2.2. Methods of plant inoculation with pathogenic bacteria

In different biological studies, various methods of inoculation of plants with pathogenic bacteria have been employed. The method absolutely depends on the type of study, type of bacteria and the species of plant. For example, in genetic transformation via

Agrobacterium studies, mostly the explants are completely immersed in the bacterial dilution to increase the contact surface of plant tissue with bacteria (Rafat et al., 2010).

For plant pathogenicity studies, miscellaneous methods of inoculation have been optimized. Syringe infiltration technique is one of the reported inoculation methods. In this method, the bacterial inoculums can be injected into the plant tissue using a syringe with a needle. For example, Moretti and Buonaurio (2010) inserted the needle of the syringe at a 30-degree angle to the surface of immature fruits of walnut and infiltrated an area of 1 cm in diameter with the bacterial suspension. In another method of infiltration technique using a syringe the plant tissue mesocarp can be pricked and the inoculation sites can be infilterated by a needle-less syringe. For example, Moretti and Buonaurio (2010) punctured the mesocarp of immature walnut fruits and infiltrated an area of about 0.5 to 0.8 cm in diameter by a syringe without needle. Sometimes the syringe needle can be used for pricking the tissue and facilitating the contact of bacteria cells with subsurface tissue. For example, tomato plant stems were pricked using a 25-gauge syringe needle after depositing the bacterial inoculum on the stems (Guo et al., 2001).

In some types of study in which the whole plant response towards the pathogenic bacteria should be monitored, a whole plant inoculation method must be used. Ruz et al. (2008) described four different whole plant inoculation methods for analyzing the pathogenicity of *Erwinia anylovora* in tomato plant. In all methods leaves were selected for inoculation. Using a scissors contaminated with pathogenic bacteria to bisect the young leaves, pricking the leaf tissue using a sharp calm with double-tooth of 2 cm length contaminated with

pathogenic bacteria, infiltration of leaf tissue with bacterial suspension using a needle and painting the wounded tips of leaves using a paintbrush contaminated with pathogenic bacteria were the four used methods in their study.

For preparation of bacterial inoculums, usually general bacterial growth media are used. For example, brain heart infusion (Guo et al., 2001), Luria Bertani (Ruz et al.,2008), and nutrient agar (Moretti & Buonaurio, 2010) were used for *Erwinia amylovora, Salmonella enteric* and *Brenneria nigrifluens* respectively. The bacterial solution is mostly adjusted to 10^8-10^9 cfu/ml using deionised sterile water or sterile phosphate buffered saline (Moretti & Buonaurio, 2010; Guo et al., 2001).

2.3.3. Endophytic Bacteria Associated with Plants

All plant species grown in nature are hosts of different endophytic bacteria (Long et al., 2008). The most important characteristic of this group of bacteria is that they are not harmful for their host plants and the phytopathogenic bacteria are not classified as the endophytic bacteria (Araújo et al., 2002). Although most of the earlier literature did not point to any special activities of them, the current studies showed some of these bacteria play beneficial roles for their host plants (Lodewyckx et al., 2002; Weyens et al., 2009)**.**

Several factors are reported to affect the diversity of endophytic bacteri associated with plants such as plant species and cultivation conditions (Ulrich et al., 2008). Reiter et al. (2002) showed the endophytic bacteria communities can be affected by infections. They have reported that the population of endophytic bacteria was increased after infection of

potato plants with *Erwinia carotovora*. Rasche et al. (2006) demonstrated that chilling has an essential effect on the diversity of endophytic bacteria colonizing sweet pepper using a climate chamber experiment. They have also showed the relationship between endophytic bacteria diversity and the cultivar type of sweet pepper.

There is a belief which many of endophytic bacteria associated with plants are from the bacterial sources colonizing in the root zone of their hosts (Sturz et al., 2000; Welbaum et al., 2004). Although many of endophytic bacteria are reported to be closely related to the genera of soil bacteria such as *Enterobacter, Pseudomonas*, *Burkholderia*, *Bacillus*, and *Azospirillum* (Lodewyckx et al., 2002), endophytic bacteria from other genera are also isolated from different plants (Weyens et al., 2009) which can originate from the phyllosphere, the spermosphere, or the anthosphere (Hallmann et al., 1997; Compant et al., 2005).

2.3.3.1. Plant-endophytic bacteria interactions

Various types of bacteria from different families have been reported as endophytic bacteria from different plants and different tissues. Elavazhagan et al. (2009) believe that these bacteria are protected from environmental stresses by the host plant and are secure from outside microbial competition. Several of endophytic bacteria benefits have been reported. Plant growth promotion is reported frequently as one of the potential benefits of endophytic bacteria isolated from different plant species. Plant growth promoting potentials of endophtic bacteria isolated from some sand dune plants were reported by Shin

et al. (2007), while Vendan et al. (2010) showed the stimulation of plant growth potential of endophytic bacteria from ginseng.

Antimicrobial activity of several isolated endophytic bacteria has been investigated. Long et al. (2003) showed the antibacterial activity of the isolated endophytic bacteria from *Solanum sp.* against plant pathogenic bacteria while antigungal activity of endophytic bacteria isolated from ginseng against the fungal disease pathogens was reported by Cho et al. (2007). The antimicrobial activity of endophytic bacteria helped the discovery of their role as the plant resistance inducer against pathogens. Based on this benefit, Harish et al. (2009) used the endophytic bacteria to enhance the systemic resistance in banana against banana bunchy top virus (BBTV) which is one of the serious diseases of this plant. The banana plants treated with the endophytic bacteria showed minimum infection. Their study showed how the endophytic bacteria can be applied in plant disease management and bio-control programmes.

Munif et al. (2001) showed how endophytic bacteria can induce the systematic resistance of plants against nematodes by study the effect of selected endophytic bacteria against *Meloidogyne incognita* on tomato. Endophytic bacteria associated with plants have not only the capacity to control plant pathogens and nematodes but are reported to increase the resistance of plants against the insect pests (Azevedo et al., 2000; Saravanakumar et al., 2008).

The role of endophtic bacteria to increase the resistance of plants against environmental stress has been also reported. Forchetti et al. (2010) showed how endophytic bacteria improve seedling growth of sunflower under water stress condition by production of salicylic acid as well as inhibition of the growth of pathogenic fungi. The reduction of cadmium phytotoxicity by tobacco plant seeds associated endophytic bacteria has been also reported (Mastretta et al., 2009).

Several of the mechanisms in which endophytic bacteria were applied to promote plant growth and control phytopathogens are similar to the mechanisms involving beneficial rhizobacteria which involve the production of inhibitory compounds, competition for a substrate or an ecological niche, and induction of host plant systemic resistance (Lugtenberg et al., 2001; Bloemberg & Lugtenberg, 2001; Lodewyckx et al., 2002; Haas et al., 2002; Dobbelaere et al., 2003; Hass & Keel, 2003; Ryu et al., 2004).

2.3.3.2. Methods of isolation of endophytic bacteria

Endophytic bacteria have been isolated from various plant tissues such as seeds, fruits, flowers, leaves, stems, and roots in different plant species (Elavazhagan et al., 2009). Different methods of isolation of endophytic bacteria are reported. One of the most straightforward ways of endophytic bacteria isolation is the direct application of plant pieces. The small pieces of surface sterilized plant tissues are placed on a bacterial growth medium and incubated to allow the growth of endophytic bacteria from the cut sided of the plant piece. For example, Elavazhagan et al. (2009) directly isolated the endophytic bacteria from small segments (1-2 cm) of petioles, stems and roots of *Mikania micrantha*.

In other methods, mostly the surface sterilized plant tissues were macerated using a sterile tool such as homogenizer (Zinniel et al., 2002), mortar and pestle (Figueiredo et al., 2009) or a blade (Long et al., 2003). Then, some sterile water (Hung and Annapurna 2004), sterile potassium phosphate buffer (Vetrivelkalai et al. 2010) or sterile magnesium sulfate solution (Wang et al. 2006) were added to the macerated plant tissues to prepare a mixture. The mixture is finally streaked onto a bacterial growth medium and incubated.

The initial bacterial growth media which are used for isolation of endophytic bacteria are usually selected general and rich media. For example, yeast peptone dextrose agar was employed by Long et al. (2003) to isolate *Solanum* sp. endophytic bacteria while nutrient broth yeast extract medium was used by Zinniel et al. (2002) to isolate different agronomic crops endophytic bacteria. Nutrient broth (Tiwari et al., 2010), brain heart infusion (Thomas et al., 2007) and tryptic soy broth (Hung & Annapurna, 2004) are some of the commonly used media to isolate the endophytic bacteria from different plant sources.

The incubation temperature is almost close to the natural plant host growth temperature at around 24-28 °C (Zinniel et al., 2002; Arunachalam & Gayathri, 2010) but in a few cases a higher incubation temperature is reported. For example, Long et al. (2003), Hung et al. (2007) and koomnok et al. (2007) adjusted the incubation temperature at 30 °C for isolation of plant enodphytic bacteria. The incubation period is reported from a few hours to several days (Zinniel et al., 2002; Long et al., 2003; Hung et al., 2007; Arunachalam & Gayathri, 2010).

2.3.3.3. Methods of endophytic bacteria identification

Although there are different ways of classification of bacterial identification techniques, in general it can be divided into three major groups namely morphological characterization, physiological and biochemical analysis and molecular biology methods (Bridge et al., 1993). Otherwise it can be divided into traditional microbiological identification and molecular identification methods (Spratt 2004, Naz et al., 2009). Dividing the identification methods into two groups namely traditional and molecular methods is based on the application of molecular biology techniques in recent studies. For example, Chester and Cooper (1979) only used the morphological and biochemical characterization techniques to identify the Achromobacter species while the same species have been recently identified using molecular techniques by Gomila et al. (2009). The shape, size and color of the bacteria colonies as well as some microscopic studies can be all classified as the morphological characterization methods. The morphological characterization usually can only give some wide-ranging ideas about the bacteria and mostly needs to be combined with other methods for reliable identification of bacteria (Singleton 2004). Several physiological and biochemical tests such as Gram reaction, oxidase reaction, catalase reaction, pectinase activity test, growth in nitrogen-free medium, antibiotic resistance and acidification of glucose under aerobic and anaerobic conditions have been reported. Although, these biochemical tests can be carried out manually there are some commercially available biochemical test kits which facilitate the procedure of identification (Ng et al., 2007).

Molecular methods which are widely used in recent studies for identification of the bacteria are known as one of the reliable systems of bacterial identification (Siqueira & Rôças, 2005). Although there are different molecular biology techniques of bacterial identification such as fatty acid chromatographic analysis (Teska et al. 2001) and MALDI-TOF MS-based bacterial identification (Hotta et al., 2010), DNA molecule-base techniques are more commonly used (Oliveira et al., 2007). Polymerase chain reaction (PCR) amplification of conserved regions of bacterial DNA molecules followed by sequencing of the amplified segments has been reported more than other molecular techniques to identify different bacteria (Relamn et al., 2002; Zoetendal et al., 2006; Mitra & Roy, 2010). 16S rRNA gene segment is a conserved region in bacteria genomes which routinely is amplified in PCR based identification of bacteria (Mitra & Roy, 2010).

Based on the available reports on identification of endophytic bacteria associated with plants, researchers use one of the identification methods or a combination of them. For example, Koomnok et al. (2007) selected only the biochemical methods to identify the endophytic bacteria isolated from various types of rice while Thomas et al. (2007) employed only the 16S rDNA sequencing technique as a molecular technique to identify some endophytic bacteria. There are several reports involving a combination of identification methods has been used. For example, Hung and Annapurna (2004) and Xu et al. (2007), used a combination of biochemical and molecular techniques to identify the endophytic bacteria isolated from soybean and wild blueberry plants respectively.

The first step in DNA-base molecular method of identification is the genomic DNA extraction of endophytic bacteria. Although there are several manual methods of extraction of bacterial genomic DNA, in most of the recent studies, DNA extraction kits have been used to extract the DNA. In manual protocols of bacterial DNA extraction, there are very simple methods of extractions such as boiling the cells in water which may produce unreliable results after polymerase chain reaction (PCR) study (Chachaty & Saulnier, 2000). Many bacterial DNA extraction methods have been optimized to obtain fine and purified DNA which is a precondition for molecular studies (Rapley 2000). For example, Chachaty and Saulnier (2000) optimized a protocol for bacterial DNA extraction containing three main buffer mainly Tris-EDTA buffer, Lysis buffer and Digestion buffer. The extracted DNA can be used in PCR mixture to amplify the region of interest. Although the primers can be designed based on the sequence, several universal primers have been designed to amplify the specific regions. For example, several universal primers have been designed for the 16S rDNA region which is commonly amplified for identification of bacteria (Sauer et al., 2005). The sequenced 16S rDNA segment of each bacteria are compared to the available GeneBank databases and the identification is carried out based on gene sequence similarity (Benson et al., 2002; Halda-Alija, 2003, 2004; Taghavi et al., 2009; Magnani et al., 2010).

2.3.3.4. Importance of plant axenic culture to study the plant-endophytic bacteria interactions

In some cases, obtaining a plant which is entirely free of microorganisms is important. For example, in asexual propagation of plants, if the main plant source is infected with any pathogen, the pathogen can be transferred during the propagation. The pathogens can also

be transmitted though plant seeds. In plant conservation programmes, especially in a case of a rare plant threatened by a pathogen, obtaining a pathogen-free plant to survive the endangered species is important (Sivasithamparam et al. 2002). In some biological studies, axenic cultures play an important role as the study control. To evaluate the total effect of microorganism associated with a plant on the plants metabolic pathways, a microorganism-free plant is a perfect control to be compared to the plant in association with microorganisms.

Tissue culture has been introduced as one of the most effective techniques to produce plants free of virus, fungi and bacteria (Pierik 1987; Previati et al., 2008). The first step in plant tissue culture is the surface sterilization of the plant material to ensure the absence of fungal and bacterial microorganisms. The explants should be removed from any remaining soil, dust, and dead parts followed by washing in water. The explants should be selected from the source with no symptoms of any viral, fungal or bacterial diseases. A thorough surface sterilization usually can be carried out by dipping the explants for few seconds in 70% ethanol followed by sterilization using 1% NaClO containing a few drops of Tween 20 or 80 for 10 to 30 minutes. The explants are lastly rinsed several times (not usually less than three times) in sterile water before they are ready for culturing (Mineo 1990; Giri & Giri, 2007). Although the plant surface sterilization eliminates the majority of bacteria and fungi present on the explants surface, the endophytic bacteria which live within plant tissues may still be alive and active after surface sterilization of the explants (Pierik 1987; Reed et al., 1996). Therefore, some tissue culture techniques are applied to eliminate the growth of those microorganisms which live within plant tissues. For example, one of the

most reported technique is the meristem culture or also called specifically meristem tip culture (Stewart 2008). Based on ability of this technique for production of bacteria-, fungi- and virus-free plants (Grout 1999; Stewart 2008), it has been widely used to produce pathogen-free plants. For example, Darvishi et al. (2006) produced pathogen-free plantlets of saffron via meristem culture technique while pathogen-free plantlets of *Cucurbita pepo* L. were obtained using the same method by Kabir et al. (2010).

Callus induction and culturing has been also reported as an *in vitro* tool to eliminate the growth of microorganisms within the plant tissue and production of bacteria-, fungi- and virus- free plant tissue (Pierik 1987; Pushkar 2009). Bacteria-free calli were produced though subculturing the callus tissues of *Ginkgo biloba* (Ayadi et al., 2003) while Kumar et al. (2010) also produced pathogen-free calli of *Gymnema sylvestre* by callus induction and subculturing technique.

In some reports, other methods have been added to the tissue culture techniques to assist the production of a virus-, bacteria- or fungi-free plant. For example, heat treatment (thermotherapy) of explants is shown to be helpful for elimination of microorganisms present within the plant tissues (Agrios 2005; Shivankar & Singh, 2007). Chemical treatment has been also reported as another method routinely combined with plant tissue culture techniques to obtain microorganisms-free plant (Taji et al., 2002; Shivankar & Singh, 2007). Thermotherapy and chemotherapy have been mostly applied for the production of virus-free plants. For example, El Far and Ashoub (2009) employed the combination of thermotherapy technique with mersitem culture to produce virus-free

sweetpotato plant while thermotherapy was used to obtain virus-free horseradish plants by Uchanski et al. (2004). Weiland et al. (2004) showed that chemotherapy technique was able to eliminate grapevine ganleaf virus by production of virus-free plants from infected *Vitis vinifera* plants. Paunovic et al. (2007) have also produced virus-free *Prunus domestica* plants via chemotherapy.

2.4. *Centella asiatica*

2.4.1. Botanical Information

Centella asiatica L. Urban belongs to the family of Umbelliferae (Apiaceae) and although it is native to India (Samy et al., 2005) can be found in marshy lands of many countries including India, Sri Lanka, Madagascar, Nepal, China and Malaysia (Somchit et al., 2004; Panthi & Chaudhary, 2006) and also Middle Eastern Countries (Abdulla et al., 2010). This wide distribution from subtropical to tropical areas is based on the strange growth habit of *C. asiatica* as it is able to grow from shady conditions to open sunny places in different soils and even in shallow waters (Samy et al., 2005). It is herbaceous perennial with rhizomes growing into the soil at their node sides. The usual propagation of plant is by producing stolons (Zheng & Qin, 2007). Their upright petioles have a length of 10 to 20 cm with only one single leaf (Samy et al., 2005). Flower and fruits of *C. asiatica* are very small and are produced near the soil surface. International Union for Conservation of Nature and National Resource has placed the *C. asiatica* in the list of threatened species (Zheng & Qin, 2007).

2.4.2. *C. asiatica* in Malaysia

Although in Malaysia the Malay dish called "nasi ulam" (vegetables and rice) constitutes rice and a mixture of some vegetables such as *Vitex negundo* and *Centella asiatica* (Samy et al., 2005), *C. asiatica* is commonly consumed fresh and uncooked as a salad vegetable (Somchit et al., 2004). Consuming this plant as a side dish is based on its ability to stimulate the appetite as well as its effect on digestion of foods. It is locally called pegaga or daun pegaga and can be easily purchased in fresh markets as well as hyper markets such as Carrefour. Malays produce a drink called pegaga tea (gota kola) from this herbal plant which is recommended to be taken as a pre- or post-treatment tea in most Malay spas. They believe this drink can cure indigestion, nervousness and dysentery by cleaning the blood (Bodeker 2009). The cosmetic value of this herbal plant to conserve a youthful appearance caused it to be named as "*awet muda*" among Malay communities (Samy et al., 2005). For example, a soft herbal paste made of a mixture of herbal plants including cinnamon, nutmeg, white turmeric, and pegaga is produced that has emollient properties and help reduce redness and soreness of skin (Bodeker 2009). Three sub-species of *C. asiatica* in Malaysia have been identified which can be differentiated based on their general appearance such as the shapes and sizes of leaves, and petiole length (Samy et al., 2005).

2.4.3. Nutrient and Chemical Constituents

Several valuable compounds are isolated from *C. asiatica* such as asiaticoside, madecassoside, asiatic acid and madecassic acid (Rafamantanana et al., 2009). Table 2.1 shows the nutrient composition of 100 g of edible portion of the herb (Samy et al., 2005).

Table 2.1. Nutrient table of 100 g edible portion of *Centella asiatica*

Nutrient	Amount (mg)
Water	87700
Protein	2000
Fat	200
Carbohydrate	6700
Fiber	1600
Calcium	171
Phosphorus	32
Iron	5.6
Sodium	21.0
Potassium	391
Magnesium	42.0
Copper	0.3
Zinc	2.0
Carotenes	2.649
Vitamin B1	0.09
Vitamin B2	0.19
Niacin	0.1
Vitamin C	48.5

Data was obtained from Samy et al. (2005).

2.4.4. Bioactive Compounds and Medicinal Value

Consumption of *C. asiatica* as an important folk medicinal plant has a very long history (Zheng & Qin, 2007). The plant has been mostly used for human wound healing, diarrhea, asthma, inflammations, skin diseases and mental disorder treatment in traditional medicine. Treatment of keloids, lupus, leprosy, cellulitis and epilepsy were also done using this plant (Samy et al., 2005; Zheng & Qin, 2007). Several pharmacological properties of this plant were also reported recently such as antibacterial, fungicidal, antioxidant and cytotoxic activities. Suguna et al. (1996) and Shetty et al. (2006) showed that the degree of epithelialization was increased and a more rapid rate of contracture was obtained when the rat wounds were treated with *C. asiatica*. A quick recovery of axons was reported in the

rats with nerve damages and fed with *C. asiatica* extract compared to the control (Soumyanath et al., 2005). There are a few studies showing the effect of *C. asiatica* to protect the gastric lining against aspirin and ethanol induced gastric ulcers by inducing gastric mucin secrection and mucosal cell glycoprotein (Cheng et al., 2000; Sairam et al., 2001; Abdulla et al., 2010). Cytotoxic potential of this herb (Ullah et al., 2009; Pittella et al., 2009) was shown while anticancer activity of asiaticoside (Huang et al., 2004) and asiatic acid (Park et al., 2005; Hsu et al., 2005), two important compounds produced by *C. asiatica,* has also been reported. These recent anticancer studies were carried out while the anti-tumor activity of the plant had been reported earlier (Babu et al., 1995). Babu et al. (2004) showed the chemopreventive effect of water extract of *C. asiatica* on colon tumorigenesis. The potent antioxidant activity of this plant was also reported many times (Huda-Faujan et al., 2003; Ullah et al., 2009; Pittella et al., 2009; Huda-Faujan et al., 2009; Hussin et al., 2009). Guo et al. (2004) and Somchit et al. (2004) reported the anti-inflammatory activity of this medicinal plant while several reports on antimicrobial activities of *C. asiatica* are available. For example, Mamtha et al. (2004) showed the antibacterial activity of ethanolic extract of *C. asiatica* against a wide range of enteric pathogenic bacteria. Methanolic extract of entire *C. asiatica* plant also inhibited the growth of some pathogenic bacteria (Panthi & Chaudhary, 2006). Ethanolic extract of this herb showed a higher antimicrobial activity compared to petroleum ether and water extracts (Jagtap et al., 2009). A novel ursane triterpenoid isolated from *C. asiatica* by Shukla et al. (2000) could inhibit the growth of *Spilarctia oblique* larvae. Terpenoids extracted from C. asiatica such as centelloids, asiatic, brahmic, centellic, and madecassic acids have been

reported as one of the most important bioactive groups in this plant (James & Dubery, 2009; Gohil et al., 2010; Loc & An, 2010).

2.4.5. Phenolic Contents of *C. asiatica*

The importance of *C. asiatica* as a herbal plant containing valuable phenolic compounds has been clarified (Mohd Zainol 2004; Zainol et al., 2008). The total phenolic contents of aqueous extract of *C. asiatica* leaves, collected from the plants grown at Universidade Federal de Juiz de Fora (Minas Gerais, Brazil), was 2.86 g/100 g (Pittella et al., 2009) while 7.79 mg TAE/100 g fresh weight of *C. asiatica* was reported as whole plant methanolic extract of total phenolic contents (Huda-Faujan et al., 2003). Although several bioactive compounds from *C. asiatica* have been reported (Gohil et al., 2010), to the knowledge of this investigation and based on the available reports, only a few of them belongs to the phenolics group. Catechin, quercetin and rutin from flavonoids group has been detected in leaves of *C. asiatica* (Mohd Zainol 2004). Subban et al. (2008) reported two new flavonoids named castilliferol and castillicetin and confirmed the presence of isochlorogenic acid as a phenolic acid in *C. asiatica*.

METHODOLOGY

The experiments were carried out in the Fermentation Laboratory of the Division of Microbiology as well as the Microbial Biotechnology Laboratory of the Division of Biotechnology, Institute of Biological Sciences, University of Malaya, Kuala Lumpur. The green house experiments were conducted at the Green House, Botanical Garden (Rimba Ilmu) of Institute of Biological Sciences, University of Malaya, Kuala Lumpur.

3.1. Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of Two Different Subspecies of *Centella asiatica*

3.1.1. Plant Material

Two fresh *Centella asiatica* subspecies, consumed widely in Malaysia, were selected for the first part of this study to evaluate their antioxidant and antimicrobial activities. The subspecies which showed stronger activities were selected for the rest of the study. The first subspecies had big edged leaves with maximum diameter of 7.5 cm (University of Malaya Herbarium Voucher Specimen: KLU047364) named as subspecies A (Figure 2.1a). The second subspecies had small round and near smooth leaves with a maximum diameter of 4.0 cm (University of Malaya Herbarium Voucher Specimen: KLU047552) named as subspecies B (Figure 2.1b).

Figure 3.1. *Centella asiatica* (pegaga). a- Subspecies A (University of Malaya Herbarium Voucher Specimen: KLU047364). b- Subspecies B (University of Malaya Herbarium Voucher Specimen: KLU047552).

3.1.2. Sample Preparation and Extraction

Samples of the whole plant were rinsed under running tap water for 15 minutes. The plants were separated into three different morphological parts namely leaf, petiole and stem and root for extraction. The plant parts were dried in an incubator at 40 ºC in the dark. Dried parts of the plants were ground to produce fine homogenous powders using an electrical blender (Super Blender National®, Japan). The fine plant powder (3 g) was soaked in 40 ml of 95% ethanol at room temperature for 72 h in the dark. The solution was then filtered through Whatman® 1 filter paper (Whatman International, Maidstone) and evaporated to dryness using a water-bath at 40º C. Two working solutions of the each plant extract at concentrations of 10 and 100 mg/ml were prepared in 5% Tween 80 dissolved in phosphate buffered saline (PBS), pH 7.4 (preparation of PBS is described in Appendix A-1) for antioxidant and antimicrobial assays respectively. The solutions were kept at 4ºC until required for the experiments.

3.1.3. Antibacterial Activity Assay

The antibacterial potential of ethanolic extracts was studied using the paper disc diffusion method of Kil et al. (2009). Two Gram-negative pathogenic bacteria (*Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) and two Gram-positive pathogenic bacteria (*Staphylococcus aureus* ATCC 29213 and *Bacillus cereus* ATCC 14579) were obtained from the microbial depository maintained at the Fermentation Laboratory, Microbiology Division of Institute of Biological Sciences, University of Malaya. These bacterial species were propagated in Nutrient Broth medium to yield a final concentration of 10^7 CFU/ml. The test bacteria (0.1 ml) were streaked on Mueller Hinton agar plates using sterile cotton swab. Sterilized filter paper discs impregnated with 40µl of the extracts (100 mg/ml) and were then placed in the center of the test bacteria agar plates. The plates were incubated for 24 h and diameters of the inhibition zones produced were measured. Tetracycline disc (30 µg), sterile distilled water, and 5% Tween 80 dissolved in PBS were used as the positive control, negative control, and blank respectively.

3.1.4. Antioxidant Activity Evaluation Assays

3.1.4.1. Erythrocytes hemolysis assay

The erythrocytes hemolysis assay protocol is adapted from Reddy et al. (2007).

3.1.4.1.1 Blood sampling

Healthy normal New Zealand white rabbits were selected for blood sampling. The fur on the ear proximal to the marginal vein was removed. The skin was sterilized with 95% ethanol. The ear was dilated by rapid and gentle massage. A needle (26Gx1/2"

TERUMO®, Belgium) was carefully inserted into the vein and blood slowly withdrawn using a 5 ml disposable syringe. The collected blood was quickly aspirated into Silicone Coated Blood Collection Tube (Vacutainer®, Becton Dickinson, Franklin Lakes) to avoid the formation of blood clots.

3.1.4.1.2. Preparation of erythrocyte suspension

Rabbit erythrocytes were isolated by centrifugation of collected blood at 1000 × g at 4 °C for 20 minutes. After removal of the buffy coat produced on the top of centrifuged blood and the blood plasma using a micropipette, the cells were washed three times with PBS at pH 7.4. The final suspension of erythrocytes was prepared by adding an equal volume of PBS to the washed erythrocytes.

3.1.4.1.3. Erythrocytes pretreatment procedure

Before induction of oxidative stress by hydrogen peroxide (H_2O_2), 500 µl of erythrocyte suspension was pretreated with 1 ml of sample (10 mg/ml) for 40 minutes. 500 µl of erythrocyte suspension was pretreated with vitamin C (1ml of 10 mg/ml solution in PBS) as the positive control.

3.1.4.1.4. Oxidative stress induction and hemolysis assay

Oxidative stress was induced in the pretreated erythrocyte suspension by using H_2O_2. The volume of pretreated erythrocyte suspension was adjusted to 9 ml by adding PBS. Then 1 ml of 10 mM H_2O_2 was mixed and the suspension was gently shaken. Oxidative stress was induced for a non-pretreated erythrocyte suspension as the negative control. The released

hemoglobin in the supernatant of the mixture was measured in a spectrophotometer at 540 nm after 150 minutes inoculation of the induced samples at 37 °C. Erythrocyte hemolysis in pure water was considered complete erythrocytes hemolysis (100%) while hemolysis of the pretreated erythrocytes was expressed as a percentage of this value.

3.1.4.2. Free radical scavenging activity assay

The method described by Bozin et al. (2006) was applied with some modification. The free radical scavenging capacities of samples were measured by using 2,2 –diphenyl-1-picrylhydrazil (DPPH*). Briefly, 50 µl of each sample (10 mg/ml) was added to 950 µl of 90 µM DPPH* solution and made up to the final volume of 4 ml with 95% ethanol. The mixtures were vigorously shaken and incubated for 2 hours at room temperature in the dark before their absorbance were measured at 515 nm. Tert-butylated hydroxytoluene (BHT) at concentrations of 1 and 10 mg/ml of PBS was used as the positive controls. The capability of samples to scavenge DPPH* was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

3.1.4.3. Superoxide dismutase (SOD) activity assay

SOD Assay Kit (19160) from Sigma-Aldrich (Japan) was used to determine the superoxide dismutase (SOD) activity of prepared extracts based on the Assay Kit protocol. Plant extracts (20 µl) at the concentration of 10 mg/ml were added to 200 µl of the Working Solution as described in the Assay Kit. The mixture, after five minutes of gentle shaking was incubated at 37 °C for 20 min after adding 20 µl of the Enzyme Working Solution as

contained in the Kit. The absorbance of the mixtures was measured at 450 nm using a microplate reader to measure the inhibition activity of SOD (inhibition rate expressed as % inhibition) using the following equation:

Percentage of Inhibition (SOD Activity) = {[(blank 1 – blank 3) – (Sample A – Sample A's blank 2)] / (blank 1 – blank 3)} × 100

where blank 1 was a mixture of the Working Solution (200 µl) and Enzyme Working Solution (20 µl) containing 20 µl double distilled water (ddH_2O). Blank 2 contained the plant extract (20 µl) with Working Solution (200 µl) and Dilution Buffer (20 µl) while ddH_2O (20 µl) was added to the plant extract in the blank 3. Ascorbic acid at concentrations of 1 and 10 mg/ml were employed as the positive controls in this study.

3.1.5. Total Phenolic Content Determination

A Folin-Ciocalteau method based on Slinkard and Singleton (1977) report was applied to determine the total amount of phenolic compounds in different parts of *C. asiatica*. Plant samples (20 µl) at concentration of 10 mg/ml were added to 100 µl of 2N Folin-Ciocalteu reagents. The mixture was made up to a final volume of 1600 µl using distilled water. Lastly 300 µl of sodium carbonate solution (0.2 mg/ml) was added and incubated at 37 °C for 45 minutes. Absorbances of the solutions were measured at 760 nm. Total phenolic contents were determined as a gallic acid equivalent (GAE) based on Folin-Ciocalteau calibration curve using gallic acid (ranging from 50 to 1000 mg/ml) as the standard and expressed as mg gallic acid per gram of dry sample.

3.2. Evaluation of Antibacterial and Antioxidant Activities and Total Phenolic Contents of *Centella asiatica* after Inoculation with Pathogenic Bacteria

3.2.1. Preparation of Bacterial Suspension

Enterobacter sp. as a vegetable pathogenic bacterium was obtained from the Department of Plant Pathology, Faculty of Agriculture, University Putra Malaysia. The bacteria were cultured at 25 °C for 16-18 hours and then centrifuged at 6000 rpm for 10 minutes to harvest the bacteria and lastly resuspended with sterilized 0.85% saline to attain a population of 10^8 cfu / ml for use as inoculum.

3.2.2. Inoculation Method and Growth Condition

A whole-plant inoculation method was obtained from Ruz et al. (2008) and modified for this study. The leaves of *C. asiatica* plant are induced with slight injury at the edge using a sterile surgical blade (Wuxi Xinda Medical Device, China). The induced wounds were gently rubbed with a piece of autoclaved cotton dipped into the bacterial suspension two hours after wounding the leaves. Inoculated plants were placed into wet plastic bags and sealed. The inoculated plants were divided into two groups namely inoculated and infected. The inoculated group comprised of the plants which were harvested three days after inoculation with no visible sign of disease on plant tissues. The infected group comprised of the plants which were harvested seven days after inoculation and the disease symptom could be seen on these plants. Control plants were also injured using a sterile blade but rubbed with a piece of cotton dipped in sterilized 0.85% saline solution. Control plants were also sealed in wet plastic bags for incubation in the same environmental condition.

Inoculation procedure was carried out inside a class II laminar air flow cabinet (Labcaire System Ltd, England). The plants were kept under quarantine condition using the wet plastic bags (Figure 3.2) at 25 ± 2 °C, 16h (light) / 8h (dark) photoperiod. The plastic bags were opened under the laminar air flow every two days to allow air exchange with the environment. The pots were arranged in a completely randomized design (CRD). Each treatment contained ten pots and it was replicated three times.

Figure 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.

3.2.3. Sample Preparation and Extraction

Three groups of plants were harvested in this study. The first group belonged to the inoculated plants which were harvested three days after inoculation with bacteria and it

was named inoculated plants. The second group belonged to the plants harvested seven days after inoculation which was named infected plants. The third group was the control plants. The plants were separated into three different parts namely leaf, petiole and stem, and root before extraction The different plant parts were dried, powdered and their ethanolic extracts were prepared based on the method described in Study 3.1 (Section 3.1.2).

3.2.4. Evaluation of Antibacterial Activity of Plants Inoculated with Pathogenic Bacteria

Antibacterial activity of three groups of *C. asiatica* ethanolic extracts (100 mg/ml) in this experiment was evaluated using disc diffusion method as described in section 3.1.3.

3.2.5. Evaluation of Antioxidant Activity of Plants Inoculated with Pathogenic Bacteria

Antioxidant activity of three groups of *C. asiatica* ethanolic extracts (10 mg/ml) in this experiment was evaluated using free radical scavenging activity and SOD activity assays as described in sections 3.1.4.2 and 3.1.4.3 respectively.

3.2.6. Determination of Phenolic Compounds

3.2.6.1. Total phenolic content determination

A Folin-Ciocalteau method as described in 3.1.5 section was employed to determine the total phenolic contents of the plants extracts (10 mg/ml) in this experiment.

3.2.6.2. Phenolic compounds determination using high performance liquid chromatography

High performance liquid chromatography (HPLC) analysis was carried out to determine the phenolic compounds present in ethanolic extracts of plant samples (10 mg/ml) in this experiment. *Waters* (USA) HPLC equipment, including *Waters TM 717 Plus Autosampler and Waters TH 486 UV Detector,* was used. A Chromolith® Performance Reversed-Phase-18 endcapped (100-4.6 mm) column (Cat. No. 1.02129) from Merck (Darmstadt, Germany) was employed at 25 °C to separate the phenolic compounds in samples. Chromotographic conditions were obtained from Chinnici et al. (2004). Methanol (0.5%) from Merck (Darmstadt**,** Germany) 0.01 M phosphoric acid (Cat. No. 79606 from Fluka, Germany) was used as *solvent A* while *solvent B* was 100% acetonitrile (Merck KGaA, Darmstadt**,** Germany). A gradient elution condition (Table 3.1) was adjusted at flow rate of 2.5 mL/min to detect the phenolic compounds at 280 nm.

The solvents applied in this experiment were of HPLC grade and six HPLC grade phenolic compounds were used as the standard namely gallic acid monohydrate puriss (Cat. No. 27645, Sigma), (+)-catechin hydrate (Cat. No. C1251, Sigma), p-coumaric acid (Cat. No. C9008), phlorizin dihydrate (Cat. No. 79320, Fluka) and quercetin (Q4951, Sigma).

Table 3.1. Gradient elution condition

Time (min)	Solvent A (%)	Solvent B (%)	Elution
0	96.50	3.50	
11	90.00	10.00	Linear Gradient
22.5	80.00	20.00	Linear Gradient
23.5	96.50	3.50	Linear Gradient
24.5	96.50	3.50	Isocratic

Solvent A, 0.5% Methanol in 0.01 M phosphoric Acid; Solvent B, 100% Acetonitrile

3.3. Evaluation of Antibacterial and Antioxidant Activities and Total Phenolic Contents of *Centella asiatica* after Inoculation with Beneficial Bacteria

3.3.1. Bacterial Suspension Preparation

A strain of phosphate solubilizing bacteria namely *Pseudomonas sp.* was obtained from the Department of Agriculture Technology, Faculty of Agriculture, University Putra Malaysia. The bacteria were cultured at 25 °C for 16-18 hours and then collected by centrifugation at 6000 rpm for 10 minutes. The bacteria were resuspended with sterilized 0.85% saline to attain a population of 10^8 cfu/ml.

3.3.2. Inoculation Method and Growth Condition

An inoculation method was obtained from Mayak et al. (2004) and modified for this study. The sterilized soil (1.0 kg) was transferred into pots and one test plant was placed in each pot after their roots were rinsed by running tap water. The plants were divided into two groups of control and treated plants. The treated plants were irrigated with freshly prepared bacterial suspension (50 ml per pot) every two days while control plants were only

irrigated with sterilized 0.85% saline. The plants were harvested after 14 days. The pots were arranged in a completely randomized design (CRD) and each treatment was replicated three times.

3.3.3. Sample Preparation and Extraction

Two groups of plants namely treated and control were harvested 14 days after first inoculation. The plants were separated into three different morphological parts namely leaf, petiole and stem, and root before extraction. The plants parts were dried and ethanolic extracts of them were prepared based on the method described in Study 3.1 (Section 3.1.2).

3.3.4. Evaluation of Antibacterial Activity of Plants Inoculated with Beneficial Bacteria

Antibacterial activity of both groups of *C. asiatica* ethanolic extracts (100 mg/ml) in this experiment was evaluated using disc diffusion method as described in section 3.1.3.

3.3.5. Evaluation of Antioxidant Activity of Plants Inoculated with Beneficial Bacteria

Antibacterial activity of both groups of *C. asiatica* ethanolic extracts (10 mg/ml) in this experiment was evaluated using free radical scavenging activity and SOD activity assays as described in sections 3.1.4.2 and 3.1.4.3 respectively.

3.3.6. Determination of Phenolic Compounds

3.3.6.1. Total phenolic content determination

A Folin-Ciocalteau method as described in 3.1.5 section was employed to determine the total phenolic contents of the plants extracts (10 mg/ml) in this experiment.

3.3.6.2. Phenolic compounds determination using high performance liquid chromatography

HPLC analysis was carried out to determine the phenolic compounds present in ethanolic extracts of plant samples (10 mg/ml) in this experiment as described in section 3.2.6.2.

3.4. *C. asiatica* Associated Endophytic Bacteria

3.4.1. Isolation of Bacteria

3.4.1.1. Surface sterilization procedure

Aerial parts of healthy *Centtela asiatica* plants of 2-3 months age were cut using a sterile surgical blade (Wuxi Xinda Medical Device, China). The stems and leaves were excised and surface sterilized separately by first placing under running tap water for 30 minutes, immersed in 70% ethanol for 3 minutes and rinsed once with sterile distilled water. The stems and leaves were then immersed in 20% Clorox® added with 1-2 drop(s) of Tween 20 for 20 minutes, rinsed with sterile distilled water thrice. The last used sterile distilled water (50 µl) was spread on nutrient broth plate to confirm the success of surface sterilization protocol.

3.4.1.2. Preparation of plant solution and incubation

The surface sterilized stems and petioles were separated from the leaves into two groups namely petiole-stem and leaf. Both groups of tissues were macerated with a sterile mortar and pestle and diluted in sterile PBS. Each diluted sample was plated on nutrient broth agar and incubated at 25 °C for 5 days.

3.4.1.3. Sub-culturing and preparation of bacterial glycerol stock

Based on the morphological appearance of the bacterial colonies, the colonies were randomly picked and subcultured separately. A few subcultures were performed to purify the bacteria. Twenty-four distinct colony morphotypes were selected and glycerol stocks from cultured single colonies were prepared (preparation method is described in Appendix A-2) and stored at -80 °C.

3.4.2. Identification of Isolated Bacteria

3.4.2.1. Gram-staining

The antagonistic strains were subjected to Gram-stain for primary identification. Few drops of sterile distilled water were placed on glass slides and a bacterial colony was picked up and emulsified on the slide to produce a smear. Crystal violet was added to the smear after fixing the smear by heating over a flame for a second. The glass slide was rinsed with water 30 seconds after adding crystal violet. Then, the smear was covered with iodine for 1 minute. After rinsing the glass slide, ethanol was used for 10-15 seconds and the slide was washed again. Lastly secondary dyeing was carried out using safranin for 30 seconds and bacteria were observed under microscope to distinguish the purple or pink color of their cell walls after rinsing and blot drying the glass slide.

3.4.2.2. Molecular identification

Genomic DNA of isolated bacteria were extracted using *i-genomic DNA Extraction Mini Kit* (iNtRON Biotechnology, Korea) based on the kit protocol. The 16S ribosomal RNA gene was amplified with universal forward primer F29 (AGAGTTTGATCATGGCTCAG)

and universal reverse primer R1492 (TAC GGC TAC CTT GTT ACG ACTT). PCR reaction mixtures contained 2 µl of 10X PCR buffer, 2 µl of dNTP mixture (2.5 mM each), 1µl of each primer (10 pmoles), 5 µl of the extracted genomic DNA, 0.5 µl of *Taq* DNA polymerase (5U/µl) from *iNtRON Biotechnology* (Korea) and finally were made up to 20 µl with double distilled water (ddH_2O). PCR condition comprised an initial denaturation at 94 °C for 5 min, continued by 32 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 30 sec, extension at 72 °C for 1 min and ended with a final extension at 72 °C for 5 min. The PCR products sizes were confirmed to be 16S rDNA by electrophoresis on a 1.5% agarose gel and then were purified using *PCR Quickspin Kit* (iNtRON Biotechnology, Korea). The result of DNA sequencing by Macrogen Inc. (Korea) were matched with NCBI nucleotide database using alignment search tool of BlastN and the bacteria were identified on the basis of at least 98% similarity to the 16S rRNA gene sequences.

3.4.3. Evaluation of Antibacterial and Antioxidant Activities of Isolated Endophytic Bacteria

3.4.3.1. Preparation of bacterial products solution

The identified bacteria were cultured in nutrient broth at 25 °C for 96 h and then were centrifuged at 10000 Xg for 10 min. The supernatant of each bacterial culture was collected and filter sterilized using a 0.20 µm *Minisart*® sterile syringe filter (Sartorius Stedim Biotech, Germany) and refrigerated at 4 °C.

3.4.3.2. Evaluation of antibacterial activity of isolated endophytic bacteria

Antibacterial activity of endophytic bacterial product solutions in this experiment was evaluated using disc diffusion method as described in section 3.1.3.

3.4.3.3. Evaluation of antioxidant activity of isolated endophytic bacteria

Antioxidant activity of endophytic bacterial product solutions in this experiment was evaluated using erythrocytes hemolysis, free radical scavenging activity and SOD activity assays as described in sections 3.1.4.1, 3.1.4.2 and 3.1.4.3 respectively.

3.5. Production of Bacteria-Free *C. asiatica* Callus

3.5.1. Optimization of *C. asiatica* Callus Induction Protocol Using BAP and NAA Growth Regulators

3.5.1.1. Explant preparation

Young and healthy leaves of *C. asiatica* were selected as the explants for production of callus. The leaves were surface sterilized using the method of sterilization described in Section 3.4.1.1. The petioles of surface sterilized leaves were completely removed using a sterile blade and explants were placed on media horizontally so that the leaf blade completely touched the media surface.

3.5.1.2. Growth media and treatments

Murashige and Skoog (MS) media containing Gamborg's vitamins (Cat. No. 0404 Sigma, Germany), 30 g/L sucrose, 2.5 g/L phytagel (Cat. No. P8169, Sigma, Germany) with combinations of different concentrations of 6-benzylaminopurine (BAP) from Sigma (Cat. No. N0640) and 1-naphthaleneacetic acid (NAA) from Sigma (Cat. No. B3408) were

prepared and pH adjusted to 5.8 for optimization of callus induction from leaf explants of *C. asiatica*. Table 3.2 shows the treatments applied in this experiment.

Table 3.2. The combination of different concentrations of BAP and NAA for induction of callus from leaf explants of *C. asiatica*

BAP + NAA (mg/L)	**Treatment Code**
0 + 0	T0
1 + 1	T1
3 + 3	T3
5 + 5	T5

3.5.1.3. Growth condition

Cultures were incubated at 25 ± 2° C, 16h (light) /8h (dark) photoperiod, and a light intensity of 60 µmol m^{-2} s^{-1}. The explants were sub-cultured on the same medium after four weeks of culture.

3.5.1.4. Experimental design

The treatments were replicated three times and each treatment per replication contained 10 explants. All the experiments were arranged in a Completely Randomized Design (CRD). Parameters observed after six weeks of tissue culturing the plants were based on the percentage of explants producing callus and mean weights of calli produced per explant.

3.5.2. Callus Multi-Subculture Method to Obtain Bacteria-Free *C. asiatica* Callus

In this study, the callus tissues were first induced from leaves of *C. asiatica* using the best result of combinations of plant growth regulators obtained from Study 3.5.1. The generated calli were cultured on the same medium. Three subcultures were performed and period of each culture took four weeks. After three subcultures, the calli were cultured on a rich MS medium supplemented with 5 g/L peptone to screen the bacteria-free calli.

3.6. Statistical Analysis

Data were analyzed using the analysis of variance (ANOVA) and Duncan New Multiple Range Test (DNMRT) at p = 5% for comparison between treatment means using SPSS (Version 17) programme.

RESULTS

4.1. Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of Two Different Subspecies of *Centella asiatica*

Antibacterial and antioxidant activities of selected parts of two commonly used subspecies of indigenous *C. asiatica* named subsp. A and subsp. B (University of Malaya Herbarium Voucher Specimens of KLU047364 and KLU047552 respectively) were evaluated in this study. Total phenolic contents of these two subspecies were also measured.

4.1.1. Antibacterial Activity

The antibacterial activity result (Table 4.1.) showed that leaf extracts of both subspecies have no inhibition against any of the test bacterial growth while both petiole-stem and root extracts of these subspecies inhibited the growth of *Pseudomonas aeruginosa.* Although the antibacterial activity of petiole-stem parts of subspecies A is higher than subspecies B against *P. aeruginosa*, the difference is not significant. Meanwhile the antibacterial activity of the root part of subspecies A is significantly higher than subspecies B against *P. aeruginosa.* The root extract of subspecies A also inhibited the growth of *Bacillus cereus* while no antibacterial activity against *B. cereus* was observed with the root extract of subspecies B. None of the samples' antibacterial activity was higher than positive control (30 µg of tetracycline).

Table. 4.1. Antibacterial activity of extracts and controls against bacterial species tested by disc diffusion assay.

Sample	Inhibition Zone (mm)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus cereus*	*Staphylococcus aureus*
Subsp A (Leaf)	NI^{b}	NI^{d}	NI^{c}	NI^{b}
Subsp B (Leaf)	NI^{b}	NI^{d}	NI^{c}	NI^{b}
Subsp A (Petiole-Stem)	NI^{b}	$11^{b} \pm 1.34$	NI^{c}	NI^{b}
Subsp B (Petiole-Stem)	NI^{b}	$12^{b} \pm 1.18$	NI^{c}	NI^{b}
Subsp A (Root)	NI^{b}	$12^{b} \pm 0.44$	$8^{b} \pm 0.89$	NI^{b}
Subsp B (Root)	NI^{b}	$9^{c} \pm 1.18$	NI^{c}	NI^{b}
Tetracycline (30 μg)	$25^{a} \pm 1.54$	$17^{a} \pm 0.89$	$21^{a} \pm 0.44$	$30^{a} \pm 0.89$
Sterilized dH_2O	NI^{b}	NI^{d}	NI^{c}	NI^{b}

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$; NI: No Inhibition

4.1.2. Antioxidant Activity

4.1.2.1. Hemolysis assay using rabbit erythrocyte cells

Based on the hemolysis results (Figure 4.1.), ethanolic extracts of all parts of both subspecies of *C. asiatica* significantly reduced the percentage of hemolysis compared to the non-pretreated sample. Erythrocyte hemolysis reduction resulted by the root extract of the subspecies A was not significantly different with the positive control (1 mg/ml vitamin C). Comparison between two subspecies showed all parts of subspecies A reduced the percentage of erythrocyte hemolysis more than that of subspecies B. While the reduction made by the leaf and root parts of subspecies A is significantly higher than the leaf and root parts of subspecies B, there is no significant difference between stem extracts of both subspecies.

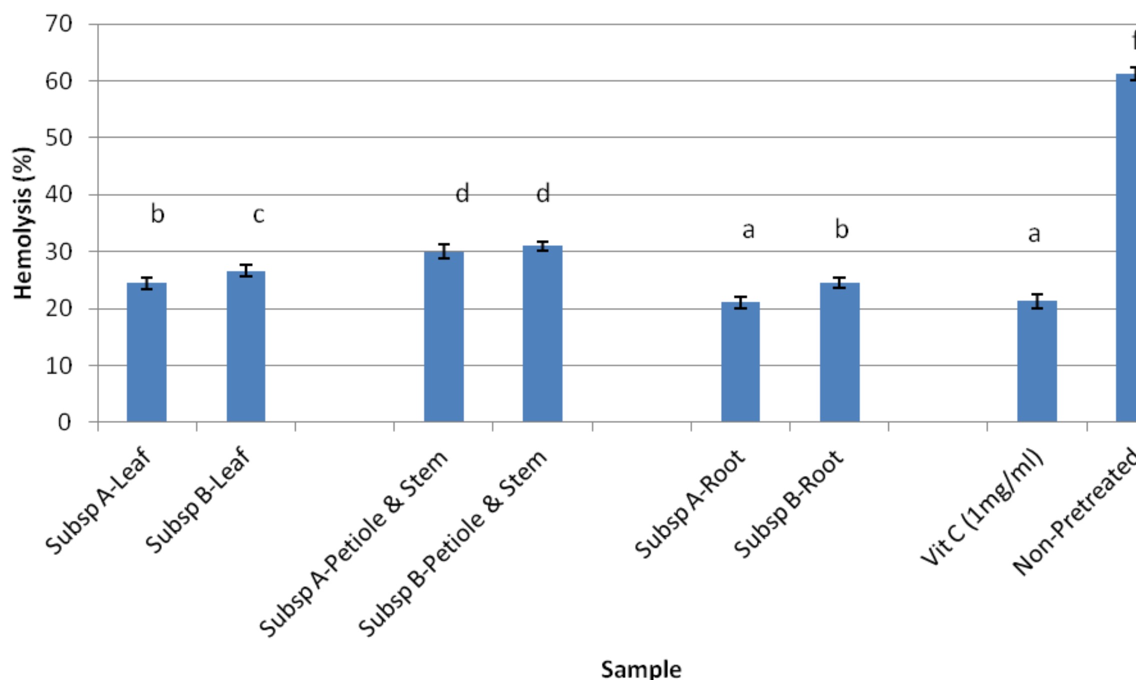


Figure 4.1. Hemolysis of rabbit erythrocytes is expressed as percentage values. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.1.2.2. Free radical scavenging activity

Figure 4.2 shows the free radical scavenging activity of different parts of both examined subspecies of *C. asiatica* and the controls. Free radical scavenging activity of all parts of both subspecies was significantly higher and lower than 1 mg/ml and 10 mg/ml of BHT respectively. Although there was no difference between the activities of both subspecies root extracts, leaf and petiole-stem extracts of subspecies A has a higher radical scavenging activity compared to the subspecies B leaf and petiole-stem extracts. The radical scavenging activity of petiole-stem extract of the subspecies A was only significantly higher than subspecies B.

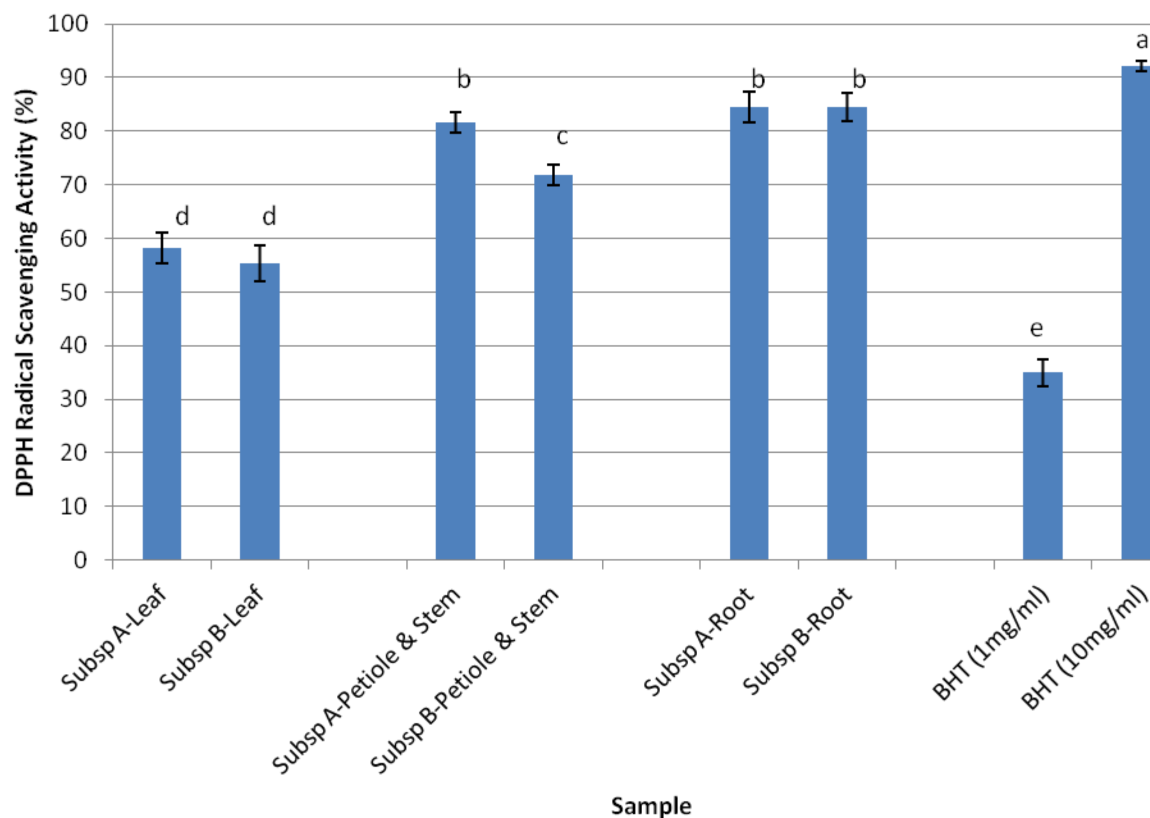


Figure 4.2. DPPH assay of samples. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.1.2.3. Superoxide dismutase (SOD) activity assay

The result of SOD activity assay is shown in Figure 4.3. The SOD activity of all parts of both examined subspecies of *C. asiatica* was significantly lower than 10 mg/ml of vitamin C. The root extract of the subspecies A which had a significantly higher SOD activity compared to vitamin C at a concentration of 1 mg/ml. Petiole-stem extract of the subspecies A showed a higher SOD activity than vitamin C (1 mg/ml) but it was not significant. The SOD activities of all parts of subspecies A were higher than subspecies B. The SOD activities of the petiole-stem and root parts of subspecies A were significantly higher than the petiole-stem and root extracts of the subspecies B.

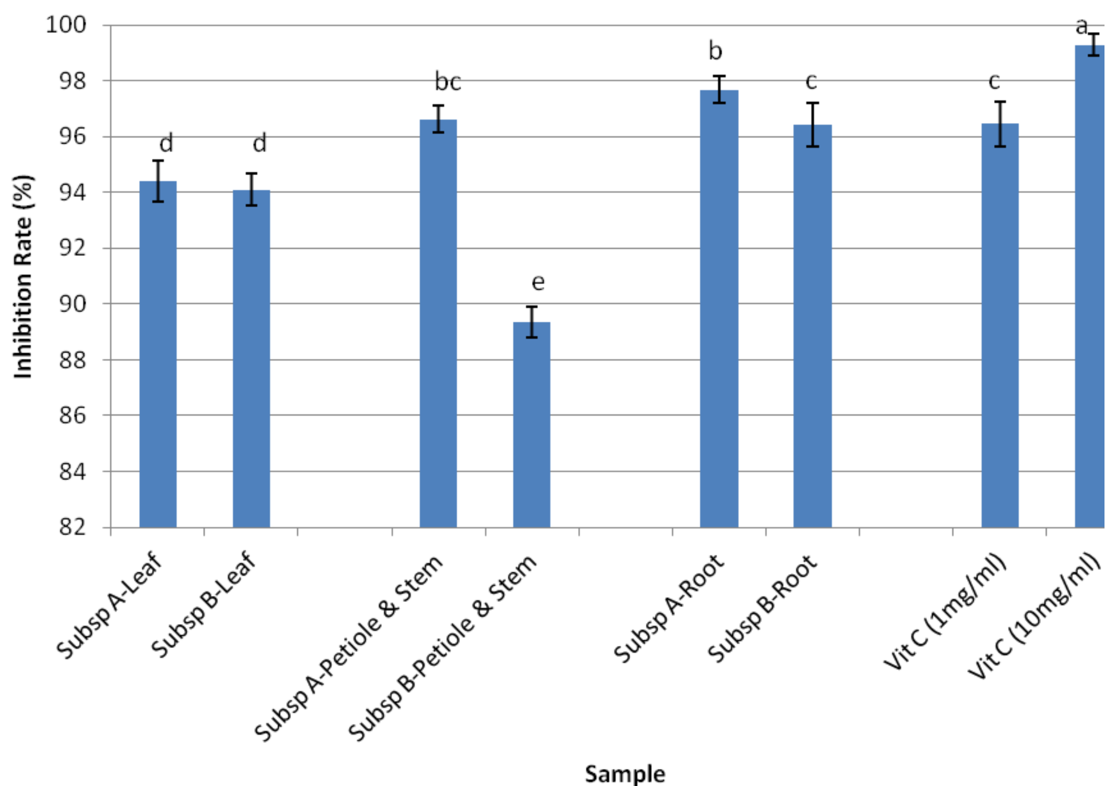


Figure 4.3. SOD activities of the examined samples are presented as inhibition rates. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.1.3. Determination of Total Phenolic Content

The total phenolic content determination results (Table 4.2.) showed that the phenolic contents of leaf and root extracts of subspecies A were higher than subspecies B but the difference was not significant in leaf extract. The subspecies B's petiole-stem extract had significantly higher amount of phenolic compounds compared to the petiole-stem extract of subspecies A. The highest amount of phenolic compounds in *C. asiatica* subspecies A was obtained from root part while it was not significantly different with the phenolic

content of its leaf part. In subspecies B, the highest phenolic content was detected in the leaf although not significantly differing from that of petiole-stem part.

Table 4.2. Total phenolic content of samples presented as gallic acid equivalent.

Sample	Gallic Acid Equivalents (g/100g)
Subsp A (Leaf)	5.02 ± 0.179 [a]
Subsp B (Leaf)	4.98 ± 0.101 [a]
Subsp A (Petiole-Stem)	3.99 ± 0.122 [b]
Subsp B (Petiole-Stem)	4.85 ± 0.095 [a]
Subsp A (Root)	5.12 ± 0.23 [a]
Subsp B (Root)	3.36 ± 0.096 [c]

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.2. Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents of *Centella asiatica* after Inoculation with Pathogenic Bacteria

The subspecies A of *C. asiatica* which were selected from previous study (4.2.) based on its higher antibacterial and antioxidant activities and production of phenolic compounds was inoculated with *Enterobacter sp.,* a pathogenic bacteria. Antibacterial and antioxidant activities of selected parts of the inoculated plant in the early (Figure 4.4b) and late (Figure 4.4.c) stages of infection were compared to the non-inoculated plant (Figure 4.4a) as the control while total phenolic contents of selected parts of inoculated plant were measured and the production of phenolic compounds were studied through HPLC analysis.

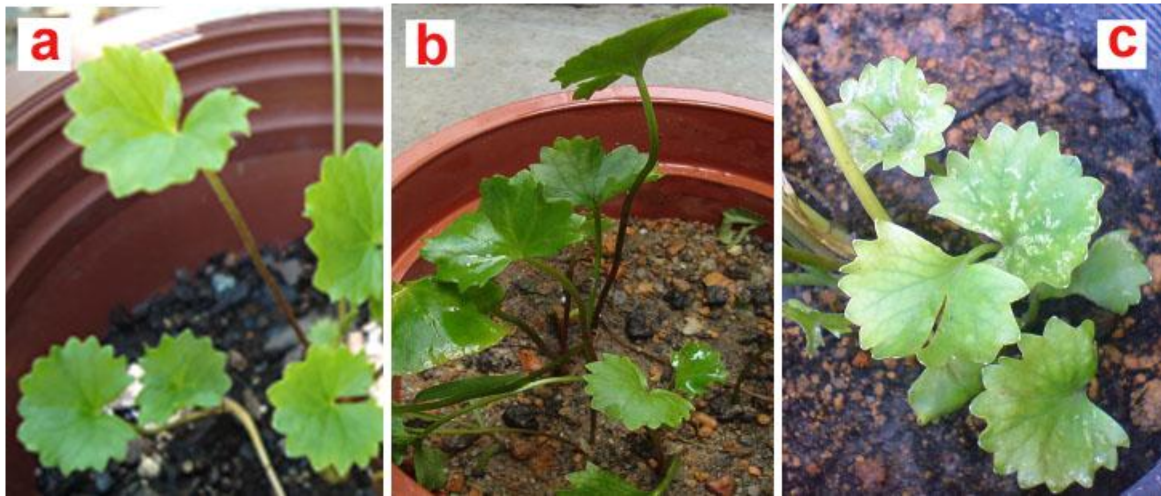


Figure 4.4. *Centella asiatica* inoculation with *Enterobacter sp.* as the pathogenic bacteria. a- Non-inoculated *C. asiatica* as the control plant; b- *C. asiatica* three days after inoculation with the pathogenic bacteria; c- *C. asiatica* seven days after inoculation with the pathogenic bacteria.

4.2.1. Antibacterial Activity

Antimicrobial assay results (Table 4.3) showed that antibacterial activity of plant increased after the inoculation with *Enterobacter sp.*. Leaves of *C. asiatica* inoculated with pathogenic *Enterobacter sp.* could inhibit the growth of *P. aeruginosa* while the leaf extract of non-inoculated plant (control) showed no inhibition against any of the test bacteria. No significant difference was observed between the antibacterial activity of leaf explant in early and late stages of infection. Although the petiole-stem part of *C. asiatica* could only inhibit the growth of *P. aeruginosa* among the test bacteria, the petiole-stem of inoculated plant could also inhibit the growth of *B. cereus*. The antibacterial activity of petiole-stem part of plant against *P. aeruginosa* was also significantly enhanced after inoculation of *C. asiatica* with plant pathogenic bacteria in both early and late stages of infection. Meanwhile, the antibacterial activity of plant root was also increased after inoculation. Though the non-inoculated plant root could not inhibit the growth of *E. coli*, root extract from inoculated *C. asiatica* showed antibacterial activity against this test

bacterium. The antibacterial activity of root part of *C. asiatica* against *P. aeruginosa* was also increased but the diference was not significant. No significant changes were observed in antibacterial activity of plant root after inoculation. The antibacterial activity of plant root extract three days after inoculation was not significantly different with the plant root extract seven days after inoculation against all test bacteria.

Table 4.3. Antibacterial activity of extracts of *C. asiatica* inoculated with pathogenic bacteria and controls against bacterial species tested by disc diffusion assay.

Sample	Inhibition Zone (mm)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus cereus*	*Staphylococcus aureus*
Control (Leaf)	NI^{c}	NI^{f}	NI^{c}	NI^{b}
3-day Inoculated (Leaf)	NI^{c}	$9^{e} \pm 0.44$	NI^{c}	NI^{b}
Infected (Leaf)	NI^{c}	$9^{e} \pm 0.77$	NI^{c}	NI^{b}
Control (Petiole-Stem)	NI^{c}	$10^{de} \pm 1.18$	NI^{c}	NI^{b}
3-day Inoculated (Petiole-Stem)	NI^{c}	$12^{bc} \pm 1.18$	$8^{b} \pm 0.44$	NI^{b}
Infected (Petiole-Stem)	NI^{c}	$13^{b} \pm 0.89$	$8^{b} \pm 1.18$	NI^{b}
Control (Root)	NI^{c}	$11^{cd} \pm 0.44$	$9^{b} \pm 0.44$	NI^{b}
3-day Inoculated (Root)	$9^{b} \pm 1.36$	$12^{bc} \pm 0.77$	$9^{b} \pm 0.89$	NI^{b}
Infected (Root)	$9^{b} \pm 0.89$	$12^{bc} \pm 0.89$	$9^{b} \pm 1.61$	NI^{b}
Tetracycline (30 µg)	$27^{a} \pm 0.77$	$18^{a} \pm 1.18$	$22^{a} \pm 1.18$	$31^{a} \pm 0.77$
Sterilized dH_2O	NI^{c}	NI^{f}	NI^{c}	NI^{b}

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$; NI: No Inhibition

4.2.2. Antioxidant Activity

4.2.2.1. Free radical scavenging activity

Based on the results of DPPH free radical scavenging assay (Figure 4.5), 3-day inoculated leaf extract has significantly higher radical scavenging activity compared to the control and infected leaf extracts. The root extract of 3-day inoculated *C. asiatica* with the pathogenic

bacteria has significantly higher radical scavenging activity compared to the control and infected leaf extracts. There was no significant difference between control and infected plant extracts in all leaf, petiole-stem and root extracts in terms of DPPH radical scavenging capacity.

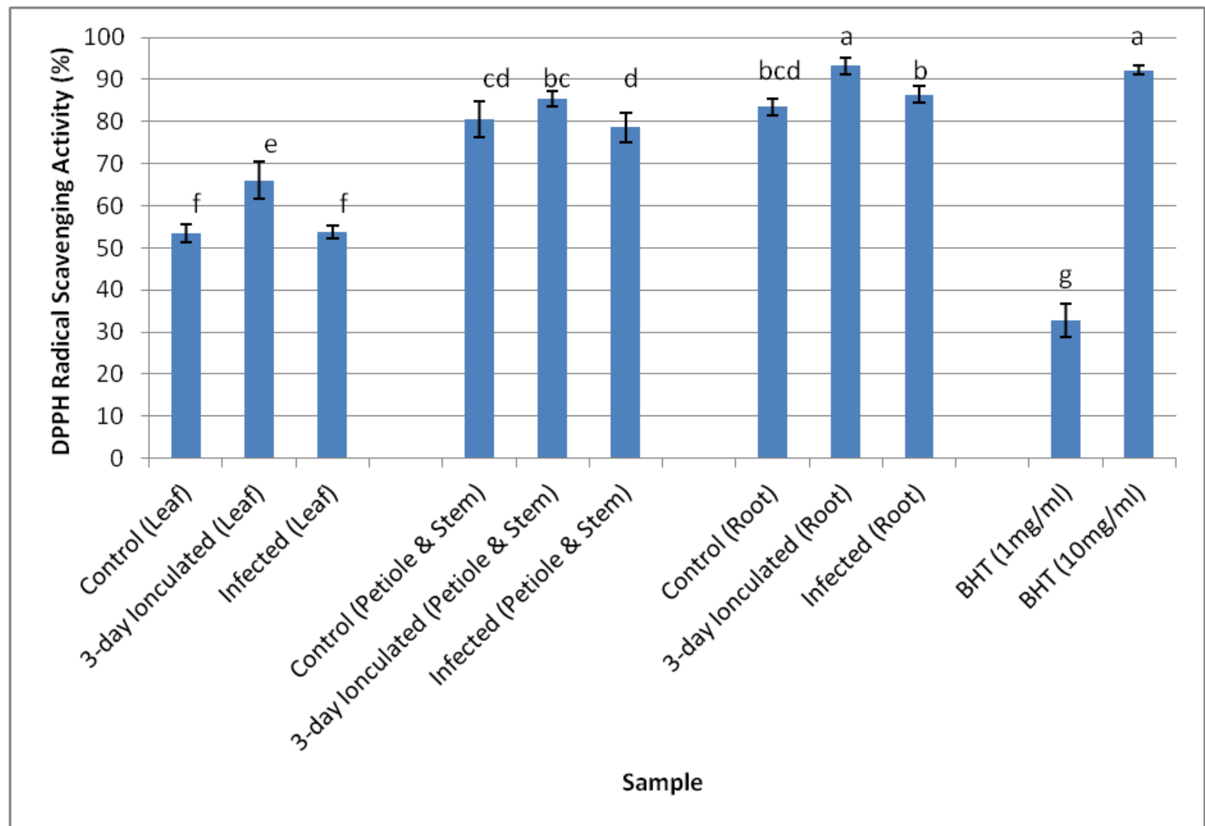


Figure 4.5. DPPH assay of *C. asiatica* inoculated with the pathogenic bacteria. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.2.2.2. Superoxide dismutase (SOD) activity assay

Based on the results obtained from the SOD activity assay (Figure 4.6), leaf and petiole-stem extracts of 3-day inoculated samples had significantly higher SOD activity compared to the control samples and infected leaf extracts. Although the root extract of the 3-day

inoculated sample has a higher SOD activity compared to the root of control and infected samples but this difference was not significant. There was no significant difference between control plant extracts and infected plant extracts in all leaf, petiole-stem and root extracts in terms of DPPH radical scavenging capacity.

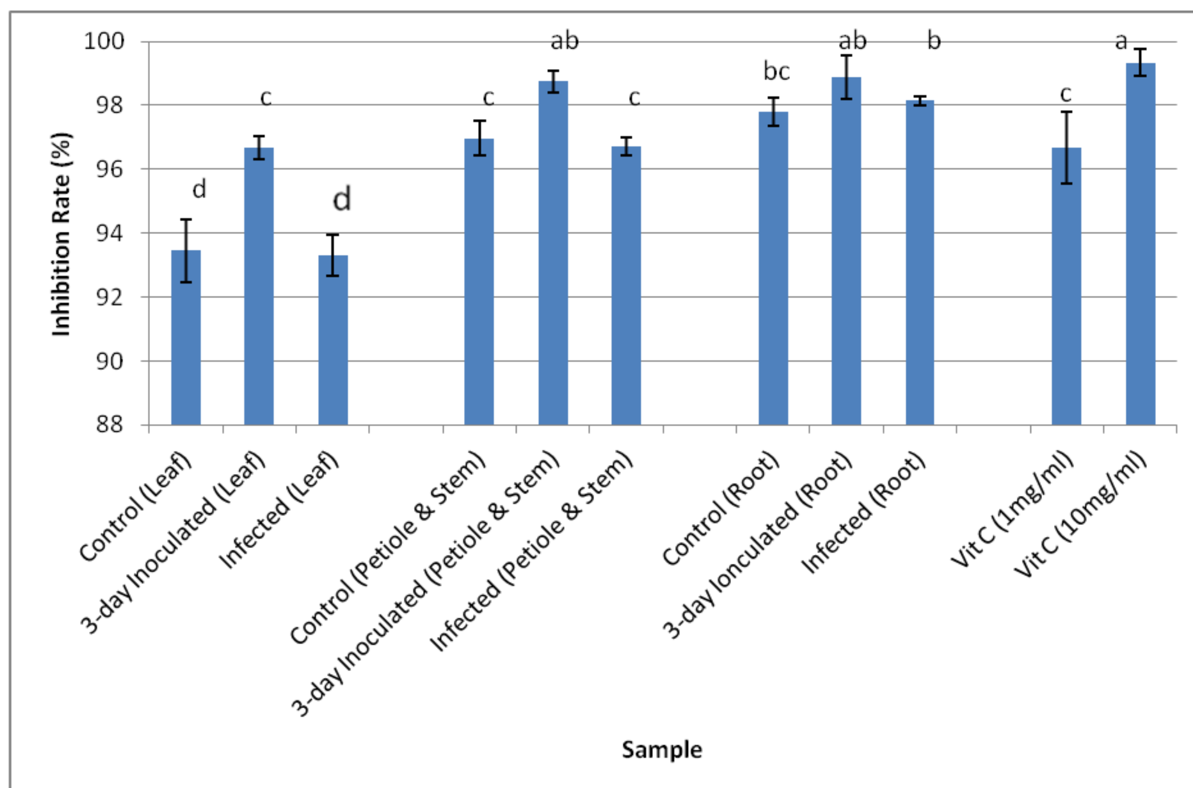


Figure 4.6. SOD activities of *C. asiatica* inoculated with the pathogenic bacteria are presented as inhibition rate. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.2.3. Determination of Phenolic Compounds

4.2.3.1. Total phenolic content determination

Table 4.4 shows that leaf, petiole-stem and root extracts of infected *C. asiatica* contained significantly lower phenolic content compared to the control and 3-day inoculated leaf, petiole-stem and root extracts respectively. The total phenolic contents increased 3 days

after treatment of plants with pathogenic bacteria in all the examined morphological parts. Although this increase was not significant in leaf and petiole-stem parts but it was significant in root sample.

Table 4.4. Total phenolic content of *C. asiatica* inoculated with pathogenic bacteria and the control plants presented as gallic acid equivalent.

Sample	Gallic Acid Equivalents (g/100g)
Control (Leaf)	5.01 ± 0.205 [b]
3-day Inoculated (Leaf)	5.12 ± 0.300 [b]
Infected (Leaf)	4.43 ± 0.072 [c]
Control (Petiole & Stem)	4.00 ± 0.094 [d]
3-day Inoculated (Petiole & Stem)	4.21 ± 0.190 [cd]
Infected (Petiole & Stem)	3.51 ± 0.108 [e]
Control (Root)	5.08 ± 0.259 [b]
3-day Inoculated (Root)	5.74 ± 0.072 [a]
Infected (Root)	4.27 ± 0.156 [cd]

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.2.3.2. Detection of phenolic compounds using high performance liquid chromatography

Table 4.5. shows the retention times of the five selected phenolic compound standards namely, gallic acid, catechin, p-coumaric acid, chloramphenical, phorozin and quercetin in the HPLC study. (The HPLC chromatogram is shown in Appendix B-1). All the HPLC results of plant extracts have been compared to these standards to detect if any of them is present in samples. The quantitative analysis was downloaded using *Empower2* software based on the standard calibration curves.

Table 4.5. Retention times for standard phenolic compounds.

	Compound	t_R (min) ± SD
1	gallic acid	2.261 ± 0.101
2	(+)-catechin	8.051 ± 0.133
3	p-coumaric acid	10.996 ± 0.357
4	phlorizin	22.344 ± 0.223
5	quercetin	25.432 ± 0.360

The HPLC analysis result of this study is summarized in Table 4.6. The result clearly showed that the total number of detected phenolic compounds produced in *C. asiatica* increased in early stage of infection (three days after inoculation) while this number was reduced in late stage of infection (seven days after inoculation). P-coumaric acid is one of the detected phenolic compounds which showed a slight increase in petiole-stem and root parts of *C. asiatica* after inoculation of plant with pathogenic bacteria in early stage of infection while there is no change in its amount in leaf of inoculated plants at the same time. Although the amount of (+)-catechin in petiole-stem part was reduced in early stage of infection, its production was enhanced in both leaf and root parts of inoculated *C. asiatica*. Quercetin, which was produced in non-inoculated (control) plants, could not be detected any more in all parts of plant in late stage of infection. The HPLC chromatograms of this study are shown in Appendix B-2 to B-10.

Table 4.6. Number of detected phenolic compounds and the amount of the detected phenolic compounds based on standard phenolic compounds in different parts of *C. asiatica* inoculated with the pathogenic bacteria and non-inoculated *C. asiatica* (Control).

Sample	Content (mg/10g of dry weight)					Number of Total Detected Phenolic Compounds
	gallic acid	(+)-catechin	p-coumaric acid	phlorizin	quercetin	
Control (Leaf)	ND	4 ± 1	1 ± 0.0	10 ± 1	1 ± 0.0	33
3-day Inoculated (Leaf)	ND	5 ± 1	1 ± 0.0	< 1	1 ± 0.0	37
Infected (Leaf)	ND	1 ± 0.0	< 1	1 ± 0.0	ND	28
Control (Petiole & Stem)	ND	3 ± 0.0	ND	1 ± 0.0	1 ± 0.0	27
3-day Inoculated (Petiole & Stem)	ND	1 ± 0.0	< 1	2 ± 0.0	1 ± 0.0	28
Infected (Petiole & Stem)	ND	5 ± 2	< 1	4 ± 1	ND	25
Control (Root)	ND	5 ± 0.0	< 1	4 ± 1	10 ± 2	34
3-day Inoculated (Root)	ND	7 ± 0.0	1 ± 0.0	< 1	3 ± 0.0	39
Infected (Root)	ND	5 ± 0.0	< 1	1 ± 0.0	ND	27
Blank (methanol)	ND	ND	ND	ND	ND	0

ND: Not Detected

4.3. Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents of *Centella asiatica* after Inoculation with Beneficial Bacteria

The study was performed by treating *C. asiatica* plants with a phosphate solubilizing bacteria (PSB) strain of *Pseudomonas sp.* as beneficial bacteria to evaluate the changes in production of bioactive compounds in the treated plants. Hence, the antibacterial and antioxidant activities of treated *C. asiatica* were measured and compared to the control plants. The study was focused on the phenolic compounds as the potential antibacterial and antioxidant compounds by measuring the total phenolic contents as well as detecting the phenolic compounds through HPLC analysis.

4.3.1. Antibacterial Activity

The antibacterial results (Table 4.7.) showed an increase in antibacterial potential of both petiole-stem and root parts of treated *C. asiatica* but no change in the antibacterial capacity of the leaf part of plant. While the leaf of non-treated *C. asiatica* had no antibacterial activity against all test bacteria, the leaf extract of treated plants could not also show any antibacterial activity. Meanwhile, the antibacterial capacity of petiole-stem and root of plant against *P. aeruginosa* was significantly enhanced by treating the plant with beneficial bacteria. The same significant enhancement was observed from the root of plant against *B. cereus.* None of the detected inhibition against the test bacteria from plant extracts was significantly higher than the used positive control (30 µg of tetracycline) in this study.

Table 4.7. Antibacterial activity of *C. asiatica* treated with beneficial bacteria and controls against bacterial species tested by disc diffusion assay.

Sample	Inhibition Zone (mm)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus cereus*	*Staphylococcus aureus*
Control (Leaf)	NI^{b}	NI^{e}	NI^{d}	NI^{b}
PSB-Treated (Leaf)	NI^{b}	NI^{e}	NI^{d}	NI^{b}
Control (Petiole-Stem)	NI^{b}	$10^{d} \pm 0.77$	NI^{d}	NI^{b}
PSB-Treated (Petiole-Stem)	NI^{b}	$12^{c} \pm 0.89$	NI^{d}	NI^{b}
Control (Root)	NI^{b}	$11^{cd} \pm 1.18$	$8^{c} \pm 0.89$	NI^{b}
PSB-Treated (Root)	NI^{b}	$14^{b} \pm 0.77$	$10^{b} \pm 0.89$	NI^{b}
Tetracycline (30 µg)	$26^{a} \pm 1.61$	$18^{a} \pm 0.44$	$22^{a} \pm 0.77$	$31^{a} \pm 2.04$
Sterilized dH_2O	NI^{b}	NI^{e}	NI^{d}	NI^{b}

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$; NI: No Inhibition

4.3.2. Antioxidant Activity

4.3.2.1. Free radical scavenging activity

The result of this study (Figure 4.7) shows that although radical scavenging capacity of all parts of *C. asiatica* was increased after treatment with phosphorus solubilizing bacteria, the increase was not significantly different compared to the control samples. None of the plant samples showed a higher activity compared to the 10 mg/ml BHT but their activities were significantly higher than 1 mg/ml BHT as one of the positive controls.

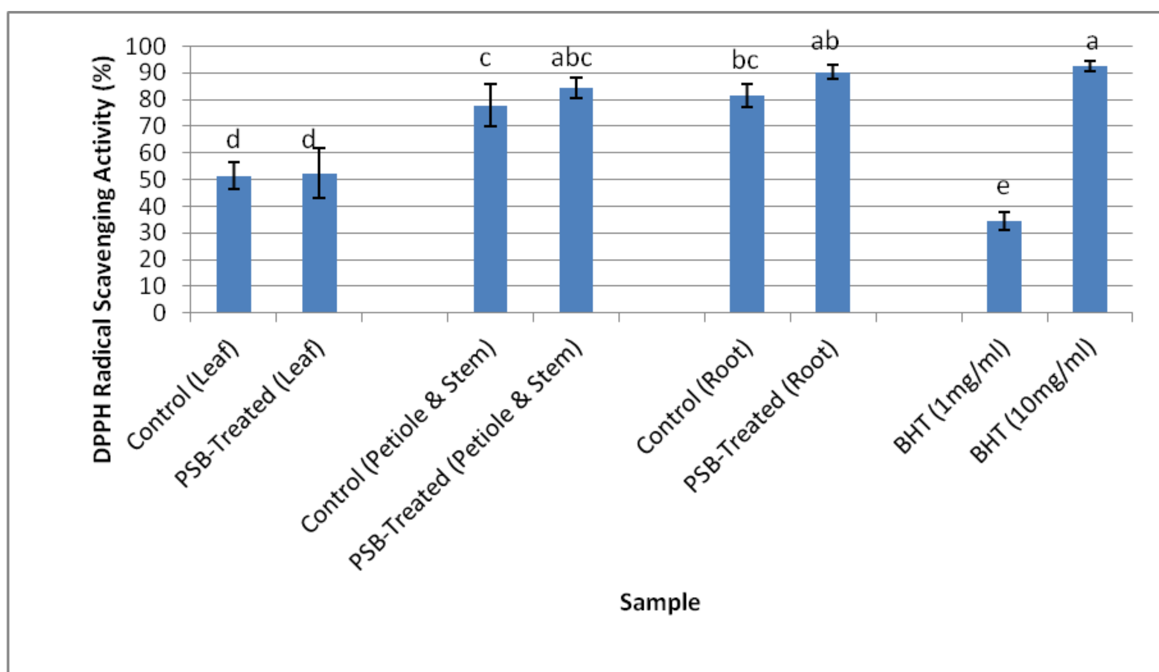


Figure 4.7. DPPH assay of *C. asiatica* treated with the beneficial bacteria. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.3.2.2. Superoxide dismutase (SOD) activity assay

Based on the results shown in Figure 4.8, SOD activity of all parts of the plants treated with phosphorus solubilizing bacteria was significantly increased. All the plant parts showed a SOD activity as potent as the positive controls. The SOD activity of petiole-stem and root of treated *C. asiatica* was not significantly different from that of vitamin C (10mg/ml).

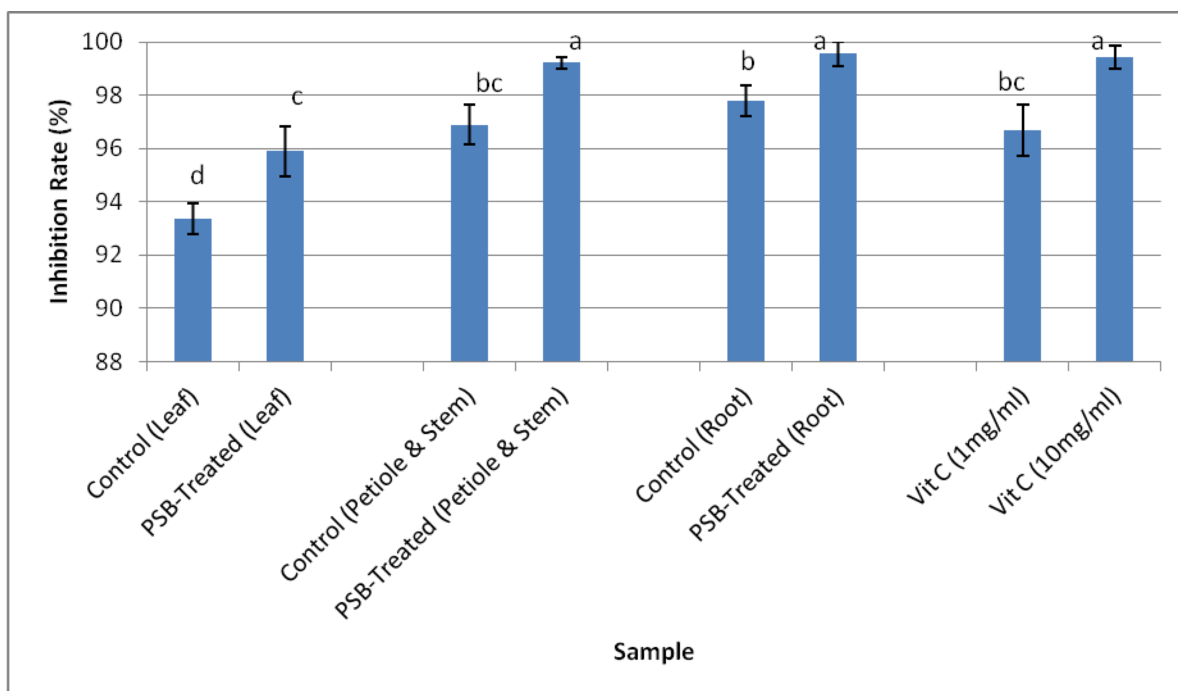


Figure 4.8. SOD activities of *C. asiatica* treated with the beneficial bacteria are presented as inhibition rate. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.3.3. Determination of Phenolic Compounds

4.3.3.1. Total Phenolic Content Determination

Table 4.8 shows that total phenolic content in all parts of treated *C. asiatica* was increased. Although this enhancement was not significant in petiole-stem of plant, the phenolics production was significantly increased in both leaf and root parts after treatment with the PSB. The total phenolic contents of leaf and root parts of *C. asiatica* were not significantly different before and after the treatment.

Table 4.8. Total phenolic content of *C. asiatica* treated with the beneficial bacteria and the control plants presented as gallic acid equivalent.

Sample	Gallic Acid Equivalents (g/100g)
Control (Leaf)	5.00 ± 0.157 [b]
PSB-Treated (Leaf)	7.76 ± 0.155 [a]
Control (Petiole-Stem)	4.02 ± 0.168 [c]
PSB-Treated (Petiole-Stem)	4.26 ± 0.229 [c]
Control (Root)	5.05 ± 0.14 [b]
PSB-Treated (Root)	7.8 ± 0.519 [a]

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.3.3.2. Detection of phenolic compounds using high performance liquid chromatography

The same phenolic standards namely gallic acid, catechin, p-coumaric acid, chloramphenical, phorozin and quercetin, which were used for HPLC study of the C. asiatica inoculated with pathogenic bacteria, were applied in this study (Table 4.5 shows the times of retention of the five selected phenolic while their HPLC chromatogram is shown in Appendix B-1).

The HPLC analysis result of this study is summarized in Table 4.9. Although the production of (+)-catechin in both leaf and root of treated plant was increased, the level of (+)-catechin was reduced in petiole-stem of treated *C. asiatica*. Although the amount of p-comaric acid was slightly reduced in root part of treated plant, there was no change in amount of this compound in other parts of treated plant. The content of phlorizin in plant leaves was sharply reduced after treatment while the amount of the same compound was raised in both petiole-stem and root parts of treated *C. asiatica*. Although quercetin content was reduced in treated plant root, the production of quercetin was enhanced by treatment

of the plant with the used beneficial bacteria. The HPLC chromatograms of this study are shown in Appendix B-11 to B-16.

Table 4.9. Number of detected phenolic compounds and the amount of the detected phenolic compounds based on standard phenolic compounds in different parts of *C. asiatica* treated with the beneficial bacteria and non-treated *C. asiatica* (Control).

Sample	Content (mg/10g of dry weight)					Number of Total Detected Phenolic Compounds
	gallic acid	(+)-catechin	p-coumaric acid	phlorizin	quercetin	
Control (Leaf)	ND	4 ± 1	1 ± 0.0	10 ± 1	1 ± 0.0	33
PSB-Treated (Leaf)	ND	17 ± 2	1 ± 0.0	3 ± 1	15 ± 1	44
Control (Petiole & Stem)	ND	6 ± 1	< 1	< 1	1 ± 0.0	28
PSB-Treated (Petiole & Stem)	ND	3 ± 1	< 1	1 ± 0.0	2 ± 0.0	29
Control (Root)	ND	3 ± 1	1 ± 0.0	< 1	4 ± 0.0	37
PSB-Treated (Root)	ND	15 ± 1	< 1	3 ± 1	2 ± 0.0	46
Blank (Methanol)	ND	ND	ND	ND	ND	0

ND: Not Detected

4.4. *Centella asiatica* Associated Endophytic Bacteria

4.4.1. Isolation and Identification of *Centella asiatica* Associated Endophytic Bacteria

Table 4.10 shows the isolated and identified bacteria from different surface sterilized parts of *C. asiatica*. There were 6 strains of endophytic bacteria isolated from *C. asiatica* petiole-stem part as well as 6 strains from leaf blades while three strains are common to both parts. The accession numbers of the strains deposited in NCBI data bank are mentioned in the table (The 16S rRNA gene partial sequences of the identified endophytic bacteria which are submitted to the NCBI data bank are shown in Appendix C). The only Gram-positive bacteria isolated were *Bacillus gibsonii* from the petiole-stem part of *C. asiatica*.

Table 4.10. Isolated *Centella asiatica* endophytic bacteria

Bacteria Name	Bacteria Strain	Accession No. (NCBI)	Source	Gram-
Bacillus gibsonii	AR_PBSTSB	HM582875	P-S	Positive
Methylobacterium radiotolerans	AR_PSLBHI	HM582876	L & P-S	Negative
Pantoea agglomerans	AR_PSBH2	HM582877	L & P-S	Negative
Pantoea agglomerans	AR_PINLBH4	HM582878	L & P-S	Negative
Erwinia tasmaniensis	AR_PINLTS5	HM582879	L	Negative
Erwinia soli	AR_PINLTS1	HM582880	L	Negative
Providencia vermicola	AR_PSBH1	HM582881	P-S	Negative
Pseudomonas fulva	AR_PSTS1	HM582882	P-S	Negative
Xanthomonas axonopodis	AR_PINLBH3	HM582883	L	Negative

P-S: Petiole-Stem; L: Leaf

4.4.2. Antibacterial Activity Evaluation of the Endophytic Bacteria isolated from *C. asiatica*

The result of antibacterial activity of isolated endophytic bacteria is shown in Table 4.11. Based on this result, only *B. gibsonii* had no antibacterial activity against the used pathogenic bacteria while all other isolated endophytic bacteria suspension inhibited the growth of *P. aeruginosa*. *P. vermicola* showed the highest antibacterial activity against *P. aeruginosa* which was significantly higher than other isolated endophytic bacteria but it could not be as high as applied positive control (30 μg of tetracycline) in this study. The observed antibacterial activity of isolated endophytic bacteria against *P. aeruginosa*, except *P. vermicola,* was not significantly different compared to each other. No antibacterial activity was observed from the isolated endophytic bacteria against *E. coli*, *B. cereus* and *S. aureus*.

Table 4.11. Antibacterial activity of a suspension of endophytic bacteria against the bacterial species tested by disc diffusion assay.

Sample	Inhibition Zone (mm)			
	Escherichia coli	*Pseudomonas aeruginosa*	*Bacillus cereus*	*Staphylococcus aureus*
B. gibsonii (HM582875)	NI^{b}	NI^{d}	NI^{b}	NI^{b}
M. radiotolerans (HM582876)	NI^{b}	$9^{c} \pm 0.77$	NI^{b}	NI^{b}
P. agglomerans (HM582877)	NI^{b}	$9^{c} \pm 0.89$	NI^{b}	NI^{b}
P. agglomerans (HM582878)	NI^{b}	$9^{c} \pm 0.77$	NI^{b}	NI^{b}
E. tasmaniensis (HM582879)	NI^{b}	$9^{c} \pm 0.89$	NI^{b}	NI^{b}
E. soli (HM582880)	NI^{b}	$9^{c} \pm 1.18$	NI^{b}	NI^{b}
P. vermicola (HM582881)	NI^{b}	$11^{b} \pm 0.44$	NI^{b}	NI^{b}
P. fulva (HM582882)	NI^{b}	$8^{c} \pm 0.89$	NI^{b}	NI^{b}
X. axonopodis (HM582883)	NI^{b}	$8^{c} \pm 0.77$	NI^{b}	NI^{b}
Tetracycline (30 μg)	$26^{a} \pm 0.89$	$18^{a} \pm 1.18$	$22^{a} \pm 0.89$	$31^{a} \pm 0.77$
Sterilized dH_2O	NI^{b}	NI^{d}	NI^{b}	NI^{b}

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$; NI: No Inhibition

4.4.3. Antioxidant Activity Evaluation of the Endophytic Bacteria isolated from *C. asiatica*

4.4.3.1. Erythrocytes hemolysis assay

The result shown in Figure 4.9 shows that the highest prevention against hemolysis was obtained from *P. agglomerans* (HM582877) followed by *E. tasmaniensis* (HM582879). All the examined bacteria showed significantly higher hemolysis percentages compared to positive control (vitamin C at 1 mg/ml).

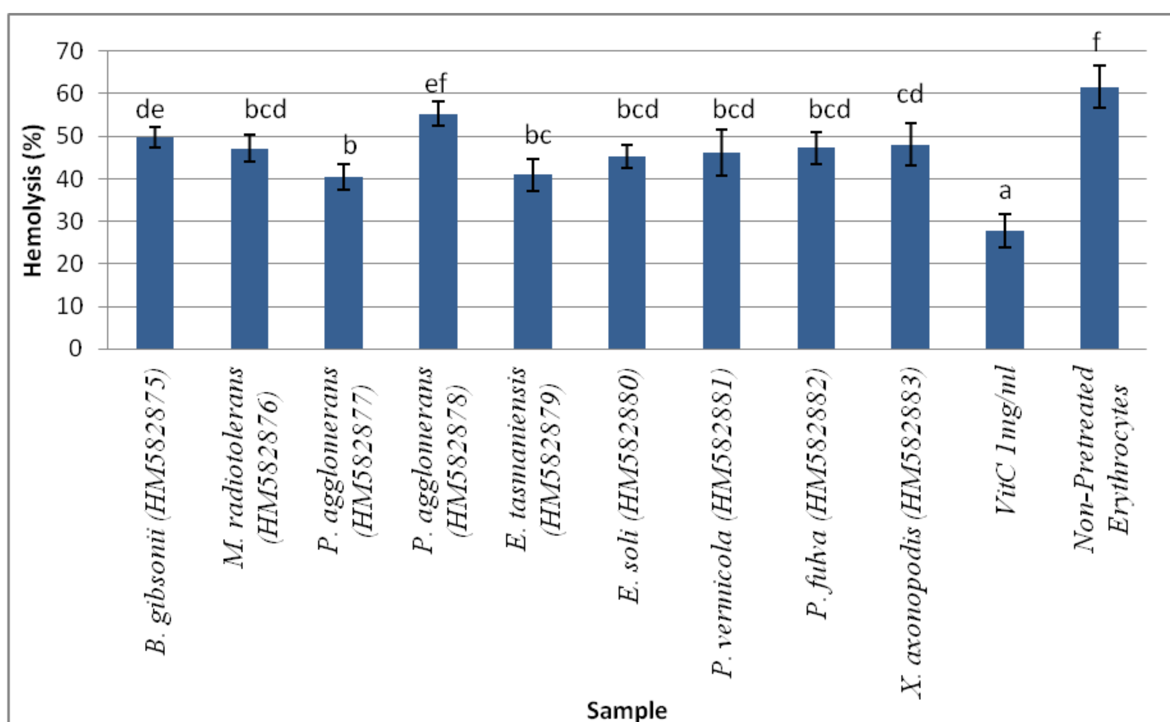


Figure 4.9. Hemolysis of rabbit erythrocytes is expressed as percentage values for *C. asiatica* associated endophytic bacteria samples. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.4.3.2. Free radical scavenging activity

The result of this study (Figure 4.10) shows that the highest radical scavenging activity obtained by *P. agglomerans* (HM582877) and followed by *P. vermicola*. The DPPH free

radical scavenging activity of all examined bacteria was significantly lower than positive control (vitamin C at 1 mg/ml).

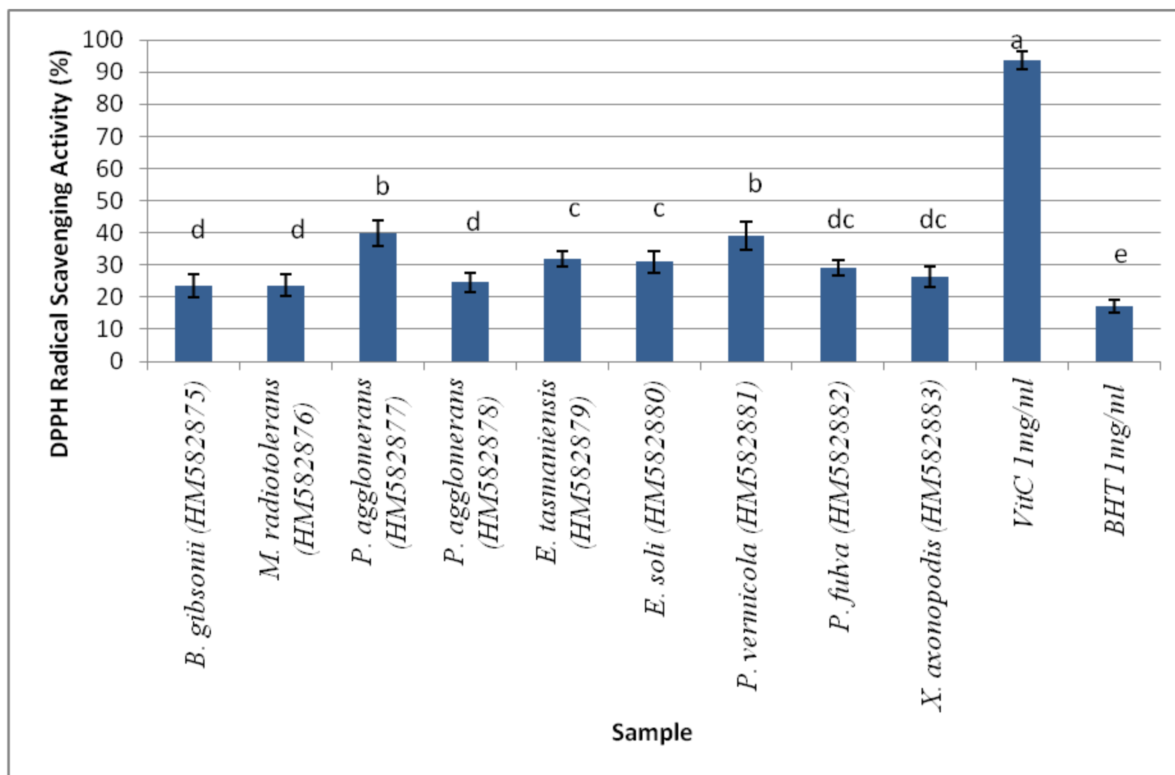


Figure 4.10. DPPH assay of endophytic bacteria associated with *C. asiatica*. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.4.3.3. Superoxide dismutase (SOD) activity assay

The result in Figure 4.11 shows while the highest inhibition rate among the examined bacteria was obtained with *Erwinia soli,* all of the bacteria showed significantly lower SOD activity compared to the positive control (vitamin C at 1 mg/ml).

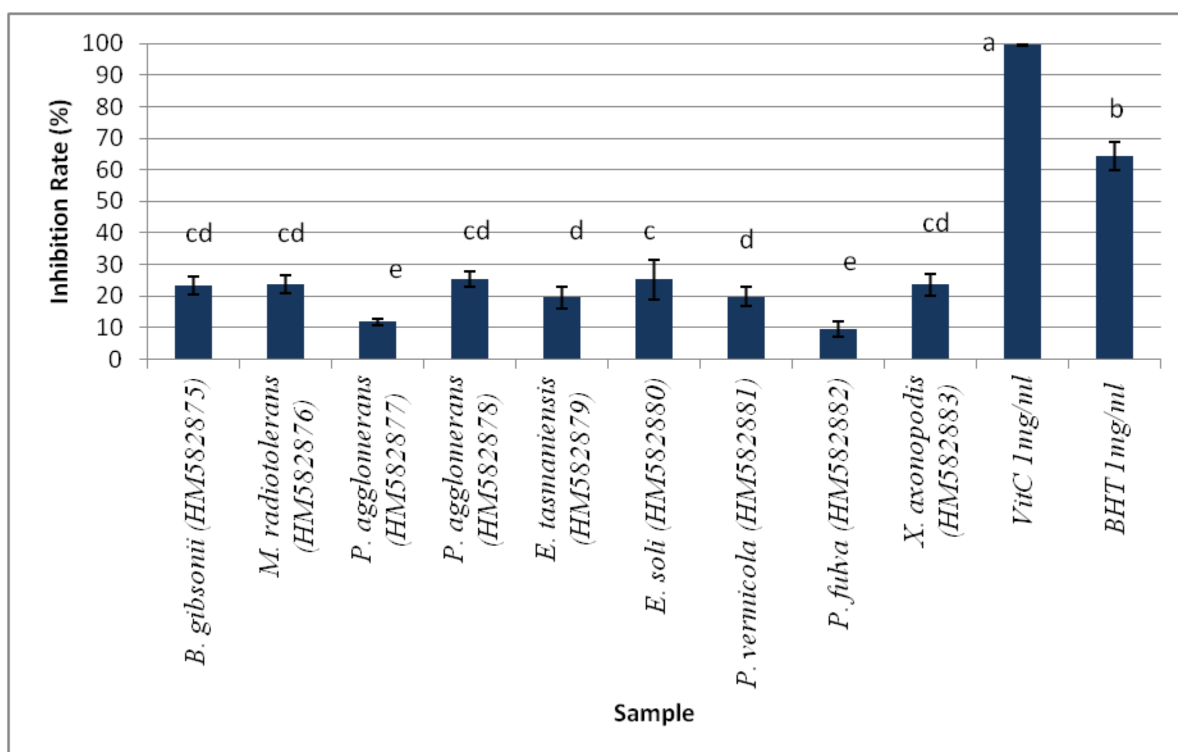


Figure 4.11. SOD activities of the endophytic bacteria associated with *C. asiatica* are presented as inhibition rate. Means followed by the same letter (s) are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.5. Production of Bacteria-Free *Centella asiatica* Callus via Tissue Culture

4.5.1. Callus Induction from Leaf Explant

Based on the result of this study (Table 4.12), the highest percentage of callus formation (100%) as well as the highest average of callus fresh matter (88.25 mg) were obtained from the treatment containing 3mg/L NAA and 3mg/L BAP that was significantly higher than other treatments.

Table 4.12. Callus formation and average of callus fresh matter after 6 weeks of culture on MS medium with different NAA and BAP concentrations.

Growth Regulator	Callus Formation (%)	Average of callus fresh matter (mg)
0 mg/L NAA + 0 mg/L BAP	0 [b]	0.00 [d]
1 mg/L NAA + 1 mg/L BAP	25 [b]	36.11 [c]
3 mg/L NAA + 3 mg/L BAP	100 [a]	88.25 [a]
5 mg/L NAA + 5 mg/L BAP	75 [a]	59.37 [b]

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's multiple range test at $p = 0.05$.

4.5.2. Bacteria-Free *C. asiatica* Callus Tissue Production

Callus tissues produced on 3 mg/L of NAA + 3 mg/L BAP were subcultured on the MS medium containing the same plant growth regulators three times. Each subculture period took 4 weeks. The calli were produced on subcultured MS medium containing 3 mg/L of NAA + 3 mg/L BAP for three subculture periods of 4 weeks each. After a total subculture of 12 weeks the calli were subcultured on MS medium containing the same plant growth regulators as well as 5 g/L peptone to screen the contamination. The calli were screened 4 weeks after culturing on the MS medium containing peptone. The applied technique resulted in 75% contaminant-free calli while 25% of calli were contaminated.

DISCUSSION

5.1. Evaluation of Antibacterial and Antioxidant Activities as well as Total Phenolic Contents of Two Different Subspecies of *Centella asiatica*

5.1.1. Plant Subspecies

Based on several reports, different sub-species, cultivars or varieties of the same plant might show different levels of bioactivities. For example, the different antioxidant properties of eleven varieties of grape have been shown by Kedage et al. (2007) while Henríquez et al. (2009) reported the different antioxidant capacity of five Chile apple cultivars. A variation in the antibacterial activities of different varieties of *Curcuma longa* (Naz et al. 2010) is another example which confirms the probability of different bioactivity levels in different varieties of the same plant.

In the present study, two different subspecies of indigenous *Centella asiatica* have been examined to evaluate their antibacterial and antioxidant activities as well as their total phenolic contents. The subspecies which showed better bioactivities and a higher content of phenolic compounds could be the ideal sample to show the changes in target parameters in our next studies. Therefore the more potent subspecies in terms of bioactivities and total phenolic contents was used in this study.

5.1.2. Plant Morphological Parts

Based on the plant tissue-specific promoters of genes which express the gene in different parts of a plant variably, the production of compounds in different parts of a plant differs (Seigler 2002). For example, Jang et al. (2010) reported that the different parts of

persimmon fruit produce variable amounts of phenolic compounds and show different antioxidant activities. Hence, *C. asiatica* plant parts were separated into three groups namely two from the aerial parts of the plant and one comprising the plant root. The results obtained in this study were also in agreement with the above reports and showed that bioactivities and production of biocompounds vary with type of tissue.

5.1.3. Plant Extraction

Plant extract contains a variety of bioactive compounds which are dependent on the solvent employed for plant extraction. Sultana et al. (2009) has explained how extraction solvents and extraction techniques can affect amount of phytocompounds present in a plant extract. An extraction technique was developed based on the available reports in this study. Since our focus in this study was on the antibacterial and antioxidant phenolic compounds, ethanol was selected based on its wide use. For example, Koffi et al. (2010) showed that ethanol is the best solvent to extract the phenolic compounds from twenty three Ivorian plants compared to acetone, water and methanol. Núñez et al. (2003) reported the same ability of ethanol in different plants such as grape and apple pomaces, pine sawdust, and almond hulls and proposed that ethanol should be used as a solvent for extraction since it offered the best yield compared with methanol and water. Although antioxidant (Chauhan et al., 2010) and antimicrobial (Mamtha et al., 2004; Jagtap et al., 2009) activities of ethanolic extract of *C. asiatica* has been previously reported, extraction of phenolic compounds from this plant has been mostly performed using methanol (Mohd Zainol et al., 2003; Huda-Faujan et al., 2009) and water (Pittella et al., 2009). Nonetheless, Chauhan et al. (2010) reported that ethanol is a better choice compared to methanol for extraction of the phenolic antioxidants from *C. asiatica* leaves.

The effect of temperature of extraction on chemical composition and therefore the bioactivities of plant extracts have been reported (Cordenunsi et al., 2005). Palma et al. (2001) showed that the recovery of phenolic compounds in plant extracts was lower when high temperatures (65 - 150 °C) were applied. Further, Türkben et al. (2010) showed freezing can significantly decrease the content of some of the important phenolic compounds in raspberry and blackberry cultivars. Hence the extraction techniques which requires freezing and boiling were deemed not suitable for such studies. In the present study, the temperature was not allowed to exceed 40 °C in both plant drying and solvent evaporation procedures. Several steps of our method of extraction such as drying the plant parts and soaking in the solvent were conducted in the dark as previous studies showed that light can induce the oxidation of phenolic compounds (Shukla et al., 2010).

5.1.4. Antibacterial Activity of *C. asiatica* Subspecies

The standard disc diffusion method was selected as the method to evaluate the antibacterial potential of samples in this study because it is a convenient, cheap and very rapid technique (Kumar et al., 2010b). This method was also reported to be accurate and reliable (Serrano et al., 2004). Hence, disc diffusion has been selected by many researchers in their antimicrobial activity tests. The antimicrobial activity of *C. asiatica* was also evaluated using the same technique by several researchers. For example, Panthi and Chaudhary, (2006), Wei et al. (2008), Taemchuay et al. (2009) and Ullah et al. (2009), has employed a disc diffusion method to investigate the antimicrobial activity of *C. asiatica* extract against pathogenic microorganisms. Meanwhile, agar well diffusion technique, which is another widely used method for *in vitro* antimicrobial activity study, has also been employed to measure the antimicrobial capacity of *C. asiatica* (Mamtha et al., 2004; Jagtap et al., 2009)

but to our knowledge there is no comparative study to show which method is more suitable for the evaluation of antimicrobial activity of *C. asiatica*.

Selection of ethanol as the solvent to extract the antibacterial compounds from *C. asiatica* in this study has been also supported by Jagtap et al. (2009) as they have reported that the ethanolic extract of *C. asiatica* has higher antimicrobial potential compared to either petroleum ether or water extracts.

The present study is unique in the aspect of evaluating the antibacterial activity of different parts of the *C. asiatica* plant. The result of our study shows that the extracts of both subspecies (100 mg/ml) did not inhibit *Escherichia coli* and *Staphylococcus aureus*. This result is supported by the studies of Taemchuay et al. (2009) where they have reported no antibacterial activity from 100 mg/ml ethanol extract of *C. asiatica* leaf against *S. aureus*. Wei et al. (2008) have also reported that 250 mg/ml of methanol and aqueous extracts of whole *C. asiatica* plant did not inhibit the growth of *E. coli*. Panthi and Chaudhary (2006) also did not observe any antibacterial activity against *E.coli* from methanol extract (1000 mg/ml) of the entire *C. asiatica* plant while it could inhibit the growth of *S. aureus*. Meanwhile, Jagtap et al. (2009) interestingly reported that petroleum ether, ethanol and water extracts of *C. asiatica* inhibited the growth of *E. coli* and *S. aureus* at a concentration as low as 1 mg/ml which is not in agreement with other reports as well as the results from this study.

The result of this study has also confirmed the previous reports which had shown variable antibacterial activity among the varieties, cultivars or subspecies of same plant (Kedage et

al., 2007; Henríquez et al., 2009). The root of subspecies A inhibited the growth of *Bacillus cereus* while the root of subspecies B had no antibacterial activity against the same bacteria. The antibacterial potential of root extract of subspecies A against *Pseudomonas aeruginosa* was also significantly higher than root extract of subspecies B.

5.1.5. Antioxidant Activity of *C. asiatica* Subspecies

Three different methods namely erythrocytes hemolysis, DPPH free radical scavenging and SOD assays were used to evaluate the antioxidant capacity of samples in this study because more reliable result can be achieved when more than one antioxidant assays are applied (Nielsen 2010). To our knowledge, a good combination of enzymatic and non-enzymatic *in vitro* methods as is used in this study to evaluate the antioxidant activity of *C. asiatica* has not been reported previously for this plant. For example, there is no report on application of erythrocyte hemolysis technique to evaluate the antioxidant potential of *C. asiatica*. In some studies, only one method was employed to measure the antioxidant activity of *C. asiatica*, as in Ullah et al. (2009) that only used DPPH free radical scavenging assay or Ashawat et al. (2007) that only applied $FeCl_3$ reducing power method. Ethanol which was reported as a suitable solvent to extract the antioxidant compounds from *C. asiatica* compared to other solvents like methanol (Chauhan et al., 2010) was selected as the extraction solvent in this study.

This study confirmed the previous studies which reported the high antioxidant potential of *C. asiatica.* For example, Ullah et al., (2009) reported the high DPPH free radical scavenging activity of concentrated methanol extract of whole plant of *C. asiatica* which is completely in agreement with the current study result (Section 4.1.2) on DPPH scavenging

assay for ethanol extract using different parts of the plant or the high SOD activity of ethanol extract of *C. asiatica* leaf as reported by Chauhan et al. (2010).

Comparison between antioxidant potential of different parts of *C. asiatica* showed that in subspecies A, all three assays showed similar results with the root part showing the highest antioxidant activity compared to other parts of the plant. Although erythrocytes hemolysis assay results showed leaf extract of subspecies A had the highest antioxidant activity followed by the root extract, both DPPH radical scavenging activity and SOD assays showed that the petiole-stem extracts was the second most bioactive part of this subspecies A. In subspecies B, root extract also had the highest antioxidant activity based on all three assays. While both erythrocytes hemolysis and SOD assay results showed that leaf extract of subspecies B was highest for antioxidant activity followed by root extract, DPPH radical scavenging activity assay showed the antioxidant potential of petiole-stem extracts was higher than leaf extract in this subspecies B.

The leaf extracts of subspecies A showed a higher antioxidant potential compared to subspecies B in all three assays but with only erythrocyte hemolysis assay showing statistical significance. Again, all three methods of antioxidant measurement were in agreement for the petiole-stem extract of subspecies A showing a higher antioxidant activity compared to subspecies B and this difference was statistically significant in both DPPH radical scavenging and SOD assays. In root extract, while both erythrocytes hemolysis and SOD assays showed a significantly higher antioxidant activity of subspecies A, DPPH radical scavenging method did not show any difference between both subspecies.

Hence, subspecies A showed a totally higher antioxidant potential in the different examined morphological parts compared to subspecies B of *C. asiatica*.

5.1.6. Estimation of Total Phenolics Content

Although total phenolics content of *C. asiatica* was previously reported (Mohd Zainol et al., 2003; Chauhan et al., 2010), the results are not comparable as the extraction methods as well as subspecies were not be same. Mohd Zainol et al. (2003), using a freeze-drying method of extraction and employing methanol as the extraction solvent, reported that leaf parts (3.23 g/ 100g dry sample) followed by root and petiole parts contains the highest amount of phenolic compounds. However, Chauhan et al. (2010) reported lower values of 2.74 g/100 g and 2.71 g/100 g total phenolic content from ethanolic and methanolic extract of *C. asiatica* leaf respectively.

In this study, a widely used method namely Folin-Ciocalteau used to estimate the total phenolic content of different morphological parts of *C. asiatica*. The same method had been reported for the same plant previously by a few researchers such as Mohd Zainol et al. (2003) and Chauhan et al. (2010). Based on the results, root extract contained the highest amount of phenolic compounds followed by leaf and petiole-stem extracts in subspecies A in which the difference between the amount of phenolics in root and leaf was not significant. In subspecies B while the highest amount of phenolic compounds was obtained from the leaf extract, it was significantly higher than root but not as high in petiole-stem extract. These results clearly showed that the amount of phenolic compounds in *C. asiatica* varies among the subspecies and the production level of phenolic compounds in different parts of the plant is variable.

Comparing the two subspecies, although leaves of subspecies A contained higher amount of phenolic compounds than that of subspecies B, it was not significantly different. Meanwhile subspecies B petiole-stem extract showed significantly higher amount of phenolic content while total phenolic content of subspecies A root extract was significantly higher than root extract of subspecies B. Therefore it is not easy to point out which one produces higher amount of phenolic compounds compared to the other.

5.2. Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents of *Centella asiatica* after Inoculation with Pathogenic Bacteria

This study was carried out to determine how plant defense system is induced by stress and pathogens thereby enhancing the production of certain secondary metabolites (Hammond-Kosack & Jones, 1997; Kliebenstein et al., 2005; Poiatti et al., 2009). Bioactivities of many of these secondary metabolites which are produced to defend plants against stress and pathogens have been reported. For example, phenolics are from a group of compounds whose production is increased by pathogen attacks (Ortega et al., 2005; Petkovšek et al. 2008; Poiatti et al., 2009). Several bioactivities of these phenolic phytochemicals such as antimicrobial and antioxidant activities are reported. The defense strategies of different plants might be changed at different stages of infection by pathogens (Wojtaszek 1997) as they have variable responses such as cell wall reinforcement, production of phytoalexin and accumulation of antimicrobial compounds (Kombrink 2000). Hence, production of bioactive compounds in *C. asiatica* in early and late stages of pathogenic bacteria attack was measured and compared to the normal plants (control) to understand if pathogenic bacteria can enhance the production of pharmaceutically valuable compounds in this medicinal plant. It is understood that apart from the different stages of infection, the

different morphological parts of the plant were also be investigated based on the tissue-specific defence system in some plants (Thomma & Broekaert, 1998; Hoegen et al., 2002).

5.2.1. Antibacterial Activity of Inoculated Plant

The result of this study proved the hypothesis that the antibacterial activity of the plant under study was enhanced with bacterial infection. Antibacterial activity in all three morphological parts of the plant namely leaf, petiole-stem and root was increased against *Pseudomonas aeruginosa*. Although this enhancement was not significant in the root part, the antibacterial potential was significantly increased in both leaf and petiole-stem parts. Interestingly, the leaf part of the plant which did not show any antibacterial activity in normal situation (control), inhibited the growth of *P. aeruginosa* after inoculation with *Enterobacter sp.*. The same effect was repeated with petiole-stem part of control plant which did not show antibacterial activity against *Bacillus cereus* while the same morphological part of the plant after inoculation with pathogenic bacteria inhibited the growth of *B. cereus.* The result also showed that the roots of normal *C. asiatica* could not inhibit the growth of *Escherichia coli* but the root of inoculated plant could bring inhibition. No inhibition against the growth of *Staphylococcus aureus* was reported before and after inoculation. Based on the enhancement of antibacterial activity in inoculated *C. asiatica*, deduction can be made that the production of antibacterial compounds were supposedly increased with the enhanced defense system of the plant in all examined parts of the plant. However, the produced antibacterial phytochemicals could still not inhibit the growth of some of the test bacteria in this study. For example, the antimicrobial compounds produced in leaves of inoculated plant only inhibited the growth of *P.*

aeruginosa and did not show any antibacterial activity against *E. coli*, *B. cereus,* and *S. aureus* (Table 4.3).

The results of the current study also showed that production of antimicrobial compounds based on the enhanced defense system is tissue-specific. For example, antimicrobial compounds produced in inoculated plant root could inhibit the growth of *E. coli* while the same antimicrobial compounds were not produced in other parts of the plant. It is also possible that the level of production was not adequately high as in the roots to develop antibacterial activity against the same test microorganism. From plant pathological point of view this finding is also very important as it shows the defense strategy of the plant against a systematic bacterial disease.

Comparing the results of early and late stages of infection, there is no significant difference between the antibacterial activities of the extracts. For example, although the antibacterial activity of petiole-stem part of plant was increased (Table 4.3) in late stage of infection (seven days after inoculation) compared to the early stage (three days after inoculation). However, this difference was not significant (Table 4.3). Based on this investigation, it is deduced that *C. asiatica* started to produce the antibacterial compounds as one of the early defence systems of the plant.

The modified method of inoculation used in this study is confirmed to be a suitable and practical method for *C. asiatica* as the symptoms of disease could be seen in different parts of the test plant although only the leaves were inoculated. The enhanced defense system of

plant in other parts of plant like roots also showed the inoculation system can be used in similar studies in the future.

Overall, the designed study showed that enhancing the defense system of *C. asiatica* plant using a plant pathogenic bacterium, thereby inducing a systematic disease, is a practical method to increase the production of antibacterial compounds and/or to produce new antimicrobial compounds in the plant tissues. This needs further investigation as similar studies have not been reported in the past based on the review of this research.

5.2.2. Antioxidant Activity of Inoculated Plant

Enhancement of antioxidant activity in the early stage of infection in all examined parts of inoculated *C. asiatica* was confirmed by both DPPH and SOD assays. Antioxidant activity in some parts of the plant was increased as high as 10 mg/ml ascorbic acid which is a strong antioxidant three days after inoculation. The results of both assays (Figure 4.4 and 4.5) were in agreement in that the antioxidant activity dropped in all parts of the plant in late stage of infection (seven days after inoculation). The antioxidant capacities of some parts of the plant in early and late stages of infection were significantly different. For example, antioxidant potential of leaf and petiole-stem parts of *C. asiatica* seven days after inoculation was significantly reduced compared to the antioxidant activity of the same parts three days after inoculation. In some cases the antioxidant capacity in later stages of infection became lower than the control plants but the difference was not significant. For example, SOD activity of leaf and petiole-stem parts was reduced in late stage of infection. These increase and reduction of antioxidant activities in different stages of infection are in relationship with the amount of phenolic compounds as the main group of antioxidant

compounds in plants as mentioned in the literature. For example, Yi et al. (2010) showed that phenolic compounds production increased in potato inoculated with *Peronophythora litchi* as a pathogen. Kużniak and Sklodowska (2005) also showed various antioxidant activities at initial and advanced stages of infection in leaf of tomato plants. They reported significant changes in the peroxisomal antioxidant system at advanced stage of infection led to failure of the protective system in the infected plants. The reduction in antioxidant activity of infected *C. asiatica* in late stage of infection might have the same reason reported by Kużniak and Sklodowska (2005).

The designed method seems to be an ideal method to enhance the production of antioxidant compounds in whole parts of *C. asiatica* but the plant should be harvested at early infection stages for extraction of the antioxidant phytochemicals. The modified technique of inoculation was also a practical method which was able to influence the defense system throughout the plant.

5.2.3. Total Phenolic Content of Inoculated Plant

Production of phenolic compounds was increased by infection of *C. asiatica* in early stages of infection and this increasment was only significant in root part of the plant. The high amount of total phenolic content in *C. asiatica* plants three days after inoculation was significantly dropped in late stage of infection (seven days after inoculation) in all examined parts of plant. Therefore, the amount of phenolic compounds extracted (Table 4.4) from the plant seven days after inoculation was significantly lower than the amount of phenolic compounds extracted from the control plants (uninfected plants). This result

clearly shows that production of phenolic compounds is a method of defense by plant in early stages of infection.

The result of this study showed that phenolic compounds are essential phytochemicals for the defense system of *C. asiatica* in pathogenic attacks with similar results reported in other plants such as *Capsicum annuum* (Gayoso et al., 2004) and *Arabidopsis thaliana* (González et al., 2006). In other cases such as environmental stress the production of phenolic compounds are also reported to be enhanced as a hemoestatic mechanism. For example, during heavy metal stress, high amount of phenolic compounds are produced not only to act as metal chelators but also to scavenge the generated harmful active oxygen species directly (Michalak 2006). Based on the result of this study we can deduce that the production of phenolic compounds are reduced and/or the produced phenolic compounds were degraded when the plant is completely infected (late stages of infection).

Based on the antimicrobial activity of most of the phenolic compounds which are expressed as a response to infection, injury, and stress (Michalak 2006), the present study can confirm that phenolics produced in infected *C. asiatica* were at least one of the major groups of antibacterial compounds which increased the antibacterial activity of the plant tissues. In addition, the antioxidant activity of phenolic compounds is also apparent. Therefore, the antioxidant activity of different plants were enhanced by inducing the production of phenolic compounds from environmental stress such as heavy metal stress (Winkel-Shirley 2002; Michalak 2006) or salt stress (Ashraf et al., 2010). The present result (Table 4.4) also showed an enhancement in the amount of phenolic compounds after inoculation of *C. asiatica* with pathogenic bacteria thereby increasing the antibacterial

potential of plants. Hence, this study can be a suitable method to increase the production of phenolic compounds in *C. asiatica* but the infected plants should be harvested for extraction in the early stages of infection before the level of phenolic compounds is reduced.

5.2.4. Phenolic Compounds Determination Using High Performance Liquid Chromatography

Evaluation of total phenolic contents of different parts of the inoculated *C. asiatica* with pathogenic bacteria clarified the enhancement in production of phenolic compounds in early stage of infection and the reduction in the amount of phenolics in late stage of infection. Analyzing the phenolic compounds using high performance liquid chromatography (HPLC) not only confirmed the previous obtained data from evaluation of total phenolic content study but also provided a detailed profile of phenolic compounds and their changes during the bacterial infection. Identification of all detected phenolic compounds using different methods such as electrophoresis coupled with electrospray ionization time-of-flight-mass spectrometry (Arráez-Roman et al., 2007) or LC/TOF-MS and LC/MS/MS (Hokkanen et al., 2009) is costly and needs long time. Hence some valuable phenolic compounds are usually used as standard to identify at least a few important phenolics based on the detection of these standards. For example, while gallic acid, (+)-catechin, (-)-epicatechin, (-)-epigallocatechin gallate (EGCG), quercetin and rutin were selected as the standards for determination of phenolic compounds in broccoli seeds (Chuanphongpanich & Phanichphant, 2006), catechin, chlorogenic acid, *p*-coumaric acid, syringic acid, ferulic acid, hesperidin, *t*-cinnamic acid, and kampferol were used as standard phenolics to analyze phenolic compounds in rice grain (Chi et al., 2007).

In this study the standard phenolic compounds were selected based on reports which had been confirmed for their antioxidant and antimicrobial activities. Antimicrobial and antioxidant potentials of gallic acid (Chanwitheesuk et al., 2007; Li et al., 2005), catechin (Friedman et al., 2006; Huang & Frankel, 1997), p-coumaric acid (Lawrence et al., 2009; Zhang et al., 2007), phlorizin dihydrate (Kutner et al., 1987; Xu et al., 2010) and quercetin (Ramos et al., 2006) have been reported.

Result of the total phenolic production changes after inoculation in this study showed that *C. asiatica* in early stage of infection tend to increase the production of phenolic compounds in all parts of the plant which was confirmed by the number of detected phenolic compounds in each extract (Table 4.6 and Appendix B-3 to B-10). For example, while the total phenolic content of root part was increased from 5.08 to 5.74 (g/100g) in early stage of infection, the HPLC results also showed that the number of detected phenolic compounds have been increased from 34 to 39 compounds in the same sample (Table 4.6, Appendix B-8 and B-9). Although analyzing the total detected phenolic compounds in different stages is simple, the specific compound changes analyzing seems to be complicated. For example, while catechin production in plant leaf and root parts was increased three days after inoculation, the amount of this compound dropped in petiole-stem parts at the same time. The amount of phlorizin sharply reduced in both leaf and root of *C. asiatica* three days after inoculation while its amount increased in petiole-stem part of plant at the same time (Table 4.6, Appendix B-5 and B-6). The reason for this kind of uncoordinated changes is not known and might show a tissue-specific production strategy for each phenolic compound involved in the defense mechanism of *C. asitica* in response to the pathogenic bacteria attack. Further study to clarify this aspect is recommended based

on the current investigation. Among these erratic changes of the phenolic compounds in different parts of the inoculated plant, the change in level of quercetin was similar in the late stage of infection and the compound could not be detected throughout the plant.

Based on the results obtained in this study, the designed inoculation technique seems to be a practical method to enhance the production of some particular phenolic compounds in some specific parts of *C. asiatica*. For example, infection acquired seven days after inoculation using pathogenic bacteria could increase the production of (+)- catechin, p-coumaric acid, and phlorizin in petiole-stem part of *C. asiatica* (Table 4.6 and Appendix B-7). These phenolic compounds play critical roles in defense system of plants against bacterial pathogens. For example, Li et al. (2009) showed that p-coumaric acid production in infected plant with plant bacterial pathogen *Dickeya dadantii* interfering the expression of T3SS genes of the pathogens.

5.3. Evaluation of Antibacterial and Antioxidant Activities as Well as Total Phenolic Contents of *Centella asiatica* after Inoculation with Beneficial Bacteria

C. asiatica was treated with a strain of phosphate solubilizing bacterium (PSB) namely *Pseudomonas sp*. to study if this method can induce the production of valuable bioactive compounds in the plant tissues. Application of beneficial soil microbes is mostly studied to evaluate the growth promotion and enhancement of crop yields (Balandreau 2002; Elmerich & Newton, 2007).

5.3.1. Antibacterial Activity of Treated Plants

Although treating *C. asiatica* with PSB could not enhance the antibacterial activity in leaves of the treated plants, it significantly increased the antibacterial activity against *Pseudomonas aeruginosa* in both petiole-stem and root parts as well as antibacterial activity against *Bacillus cereus* in only roots of plants. This result confirmed the enhanced production of antimicrobial compounds in at least two examined parts of the plant namely petiole-stem and root. The extracts of treated plant parts did not show inhibiting the growth of any of those test bacteria similar to the control treatment wherein the normal plant extract did not inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* (Table 4.7). Hence, it seems that no new antibacterial compound was produced after treating *C. asiatica* with beneficial bacteria in this study.

Based on past research, rarely are beneficial bacteria used to enhance the antibacterial activity of plants. One example is the production of antimicrobial compounds in sugar beet increased by treating its seeds with a strain of *Pseudomonas fluorescens* as a beneficial bacterium (Thrane et al., 2000).

The method of inoculation and treatment used in this study was similar to what is usually applied for treating the crops in a field applying bio-fertilizers through irrigation procedure. This method is easier and faster to be carried out compared to ones which required treating the plantlets (Han et al., 2006) or seeds (Verma et al., 2010) before planting or treating the soil separately (Son et al. 2006). The nutrient media used as the bacterial growth media were removed from the suspension before treating the plants because the rich bacterial growth media might affect the plants itself and it could also let

other microorganisms to grow rapidly in the soil and then interrupt the experiment undertaken in this study.

5.3.2. Antioxidant Activity of Treated Plants

The results of both DPPH free radical scavenging and SOD assays confirmed that antioxidant activity of all parts of the plant was increased after treatment with PSB. Although this enhancement was significant for all examined parts of treated plant in SOD activity test, the enhancement in DPPH radical scavenging activities of those parts were not significant. The radical scavenging capacity and SOD activity of petiole-stem and root parts of treated *C. asiatica* was significantly high at 10 mg/ml BHT and 10 mg/ml ascorbic acid (two strongest positive controls used in these two assays) respectively. Hence, it showed that the designed method has the potential to enhance the production of antioxidant compounds in *C. asiatica* to make them compatible to well-known commercially available antioxidants.

The result of this study showed that the treatment technique used for treating the plants was a suitable method as the production of antioxidants in all parts of the plant was increased. To our knowledge, there are not many studies carried out to investigate the effect of beneficial microbes on antioxidant activity of plants. One example provided by Ordookhani et al. (2010) which showed the enhancement of antioxidant capacity of tomato plants treated with a combination of different beneficial bacteria and fungi.

Based on this study, application of beneficial microbes as biofertilizers might not only increase the yield of crops but also enhance the production of valuable phytochemicals in them and thereby increase their bioactivities.

5.3.3. Total Phenolic Contents of Treated Plants

The total phenolic content of *C. asiatica* plants treated with the beneficial bacteria (PSB) was evaluated in different parts of plant and based on the results obtained from this study the bacteria were able to enhance the production of phenolic compounds in whole plant parts. Although there was a sharp and significant increase in total phenolic contents of both leaf and root parts of treated plant, the difference between total phenolic contents of petiole-stem parts of treated plants with control plants was not significant. It might show a tissue-specific activity of applied bacteria in *C. asiatica*.

The current study showed to be a practical method to enhance the production of phenolic compounds in *C. asiatica*. The application of beneficial microbes to enhance the production of valuable phenolic compounds is not only economic, but it is a handy and ecologically safe and acceptable way. Based on differences in the activity of beneficial bacteria between the species and strains, a specific study can be designed to evaluate the effect of different bacteria on bioactivities of selected plants. Fuentes-Ramirez and Caballero-Mellado (2006) mentioned that the result of application of beneficial bacteria might be varied in different crops and cultivars. Hence, the established protocol in this study can be practiced for other plants to investigate the production of phenolic compounds.

5.3.4. Phenolic Compounds Determination Using High Performance Liquid Chromatography

Based on the number of total detected phenolic compounds in this study, treatment of *C. asiatica* with the beneficial bacteria (PSB) increased the amount of phenolic compounds in the whole plant. Although, this increase was very slight in the petiole-stem part of the treated plant but the number of detected phenolic compounds sharply increased in leaf and root parts. Therefore, the obtained result of this study confirmed the result obtained from the study for total phenolic content evaluation.

The only unknown issue observed from this result is the unrelated changes of different phenolic compounds within and among the plant parts. For example, while the amount of (+)-catechin and quercetin was enhanced in leaf part after treating the plant with beneficial bacteria, the amount of phlorizin dropped in the same morphological part (leaf) of the treated plant. The changes were also variable among the plant parts. For example, treating of plant with beneficial bacteria increased the production of (+)-catechin in leaf but decreased in petiole-stem. These unrelated changes might show that the effect of beneficial bacteria on production of different compounds even from the same chemical groups showed variation in a specific plant tissue and also affected production of a particular compound at variable levels among the plant tissues.

The result of this study clearly showed that the designed protocol is practical for enhancement of selected bioactive compounds in specific parts of plant. This is more relevant to some compounds such as (+)-catechin and quercetin in leaves of *C. asiatica* in which their production was increased several times compared to the control.

5.4. Endophytic Bacteria Associated with *C. asiatica*

5.4.1. Isolation of Endophytic Bacteria

While Hallmann et al. (1997) showed that bacterial endophytes can be isolated from both surface disinfected plant tissue and internal plant tissue extracts, several methods for the isolation of endophytic bacteria have been reported recently. Qin et al. (2009) applied three different methods of isolation of endophytic actinobacteria from the dried surface sterilized plant samples after a 15 minute heat surface treatment at 100 ºC. Although Qin et al. (2009) stated that using various methods of isolation increase the possibility of obtaining many endophytic bacteria of interest compared to the usage of a single isolation procedure, the single method used in this study showed to be able to isolate a variety of endophytic bacteria from different families from different parts of plant. The result confirmed the efficiency of the methods which employed macerating the plant tissue and then plating them to increase the chance of isolation (Reed et al. 1995; Dias et al 2009; Tiwari et al. 2010).

The endophytic bacteria in this study were only isolated from aerial parts of plant namely leaf and petiole-stem parts to avoid isolation of soil environmental bacteria from root part. Roots of plants are highly contaminated with soil microflora and usually surface sterilization methods cannot perfectly remove all microorganisms on the surface of roots (Habiba et al, 2002). Most of reports on endophytic bacteria from plant roots indicated that the diversity of bacteria is mostly the same as the diversity of rhizosphere bacteria (Germida et al., 1998). It can be accepted that the source of some endophytic bacteria associated with plant is the rhizosphere but high number of similar bacteria in both soil and root and reports on difficulty of root surface sterilization raise doubts if all the isolated

bacteria from roots are the endophytic bacteria from the plant. For example, Marquez-Santacruz et al. (2010) showed endophytic bacterial diversity associated with Mexican husk tomato is a subset of the rhizosphere bacterial population.

5.4.2. Identification of Isolated Endophytic Bacteria

The isolated bacteria were identified using a 16S rRNA gene sequencing method after an initial Gram-staining study. The selected methods of identification were able to identify the isolated bacteria to species level. The accuracy and reliability of 16S rRNA gene sequencing as a method of bacterial identification compared to other methods of identification have been reported many times (Petti et al., 2005; Siqueira & Rôças, 2005; Mignard & Flandrois, 2006; Philip et al., 2008). This method has been reported as a single method of identification to identify many endophytic bacteria from different plants. For example, Thomas et al. (2008) and Sun et al. (2008) used only the 16S rDNA sequencing technique to identify the endophytic bacteria from banana and rice respectively.

Bacillus gibsonii which is the only Gram-positive bacteria isolated from *C. asiatica* is mostly reported as a soil bacterium. For example, Sturz et al., (2001) isolated a strain of *B. gibsonii* from potato growing soils while Li et al. (2005) isolated another strain of *B. gibsonii* from alkaline soil. Therefore, the source of this endophytic bacterium in *C. asiatica* could be the rhizobacteria which could be successfully introduced into the plant tissue. *Methylobacterium radiotolerans* which was isolated from both leaf and petiole-stem parts of *C. asiatica* was previously reported as the endophytic bacteria isolated from and associated with other plants such as *Xylella fastidiosa* subsp. *pauca* (Lacava et al., 2008) and *Oryza sativa* (Kaga et al., 2009). Two different strains of *Pantoea agglomerans* were

collected from both leaf and petiole-stem parts. Both strains were present in both morphological parts of *C. asiatica*. *Pantoea agglomerans* was also previously reported as endophytic bacteria associated with different plants such as coffee (Vega et al., 2005) and rice (Feng et al., 2006). *Erwinia tasmaniensis* which was isolated from the leaf of *C. asiatica* has been reported as a non-pathogenic *Erwinia* species and previously was isolated from different plants such as apple and pear trees (Geider et al., 2006). *Erwinia soli* was another species belonging to the genus *Erwinia* which was isolated from leaves of *C. asiatica*. This bacterium was also isolated from different plant sources. For example, Papalexandratou et al. (2011) isolated this bacterium from coca bean. To our knowledge, it is the first report on isolation of *Providencia vermicola* as endophytic bacteria while no other reports was found to mention the isolation of this species of bacteria from any other plant sources. In this study *Pseudomonas fulva* was isolated from petiole-stem part of C. asiatica. *P. fulva* was also reported as endophytic bacteria previously. For example, Pokojska-Burdziej et al. (2004) isolated a strain of *P. fulva* from pine tree. *Xanthomonas axonopodis* isolated from leaf of *C. asiatica* is known as one of the citrus pathogens in Malaysia (Derso et al., 2007).

5.4.3. Antibacterial Activity of Isolated Endophytic Bacteria

Antimicrobial activity of endophytic microbes has been studied more than any other bioactivity properties. Some of the endophytic fungi isolated from different plants showed potent antimicrobial activity. Ramos et al. (2010) reported the antimicrobial properties of some endophytic fungi isolated from *Smallanthus sonchifolius* roots. Other report presented by Maria et al. (2005) showed that the antimicrobial potential of some mangrove endophytic fungi from the southwest coast of India while Sette et al. (2006) showed similar

bioactivity in coffee plant. Several of the endophytic bacteria species isolated from Thai medicinal plants also showed promising antibacterial activity against various human pathogenic bacteria (Laorpaksa et al. 2008). Roy and Banerjee (2010) showed that endophytic bacteria associated with *Vinca rosea* plant have the potential as a new source of antimicrobial compounds. The activity of endophytic actinomycetes isolated from tomato plants against *Ralstonia solanacearum* (Tan et al. 2006) is another example to clarify the importance of endophytic bacteria as the bioactive phytochemicals producer. All isolated endophytic bacteria in this study, except *B. gibsonii* (HM582875), showed good inhibition activity against *P. aeruginosa* but no inhibition against the other test bacteria. The reason for such selectivity is unclear and needs further investigation although there are other reports which showed similar selectivity among other endophytic bacteria. For example, a few endophytic bacteria isolated from *Vinca rosea*, called Vrl42, Vrb44, and Vrb45 could inhibit the growth of *E. coli* while these had no antibacterial activity against *B. cereus* (Roy & Banerjee, 2010). Results from this study also suggested that the secondary metabolites produced by *C. asiatica*-associated endophytic bacteria can be examined in terms of antimicrobial potential in future studies.

5.4.4. Antioxidant Activity of Isolated Endophytic Bacteria

Although endophytic microorganisms have been shown to be a profuse source of bioactive compounds, they are seldom screened and evaluated for their properties (Strobel 2007). Evaluation of antioxidant activity of endophytic *Pestalotiposis microspora* fungus showed high antioxidant capacity of pestacin (Harper et al. 2003) and isopestacin (Strobel et al. 2002) produced by this fungus. Although there are a few reports on antioxidant activity of endophytic fungi, to our knowledge there is no specific report on antioxidant potential of

endophytic bacteria and the current study may be the first report of its kind. Application of various antioxidant evaluation methods to achieve a more reliable result is recommended (Nielsen 2010). Therefore, in the present study a combination of enzymatic and non-enzymatic assays to measure the antioxidant properties of endophytic bacteria metabolites was applied. The study clearly showed that the result of each antioxidant assay may or may not be in agreement with the other assays. For example, *P. agglomerans* (HM582877) showed the highest antioxidant activity based on erythrocytes hemolysis and free radical scavenging assays while it showed one of the lowest SOD activities among all isolated endophytic bacteria. Hence, using different antioxidant activity techniques is necessary to gain complete assessment.

In comparison to positive controls used in this study, none of the bacterial sample showed antioxidant activity as high as 1 mg/ml concentration of ascorbic acid in all three assays showing a low or fair antioxidant capacity for endophytic bacteria isolated from *C. asiatica*.

5.5. Production of Endophytes-Free Callus

Although endophytic bacteria associated with *C. asiatica* producing antibacterial and antioxidant compounds which might affect the total antibacterial and antioxidant capacity of plant extract, they might also induce the production of bioactive compounds by plant cells. In other words, the bioactivities of the plant cells associated with endophytic bacteria might be different with the plant cells without association with those bacteria. Therefore, the bioactivities of endophytes associated with plant should not be the only study and their direct effects of these bacteria on the plant cells should also be investigated. Most of the

reports in this area have been only focused on bioactivity of the endophytices and the biocompounds synthesized by them (Owen & Hundley, 2004) while there is not many studies on effect of endophytes on the production of biocompounds from the plant cells. For example, Hsieh et al. (2009) focused only on bioactivities of endophytes isolated from Taiwanese herbal plants, but not the effects of them on bioactivities of the plants. Similarly, Arunachalam and Gayathri (2010) studied only the bioactivities of isolated endophytic bacteria from *Andrographis paniculata* while they have not evaluated the effects of those bacteria on *A. paniculata* bioactivities.

To investigate the effect of endophytic bacteria on bioactivities of a plant, two main models of study can be performed. The first type of study is to evaluate the bioactivity of a plant treated with its own endophytic bacteria to clarify if they can induce the plant bioactivities after inoculation. The second model is to produce an endophytes-free plant and comparing its bioactivities with the control plant, which is the normal plant associated with its endophytes. The first type of study has the main disadvantage that it can only show the effect of endophytes on bioactivity of plant and it is not able to clarify the effect of present diversity of endophytes in their normal population on plant activities. For example, fungal endophytes isolated from *Theobroma cacao* were re-introduced to the plant to measure the enhancement of plant disease tolerance of inoculated *T. cacao* plants (Mejía et al., 2008). Although they showed the positive effect of endophytic fungi of the plant on the plant defense system, level of the effect by normal population of endophytic fungi presence in *T. cacao* was not measured.

To our knowledge there is no specific report to compare the bioactivities of a plant without endophytes to the same plant associated with its endophytes. To perform such a study, production of endophyte-free plant is a prerequisite. There are some reports on production of pathogen-, bacteria-, fungi- and virus-free plants using different *in vitro* techniques (Pierik 1987; Pushkar 2009). In some cases, other techniques such as chemotherapy (Taji et al., 2002) and thermotherapy (Agrios 2005) have been combined with *in vitro* methods to achieve axenic plant culture. Production of endophyte-free plant might take a long time because several tissue culture protocols should be optimized and in case the method fails, other techniques should be practiced.

In this study, the procedure of production of endophytes-free *C. asiatica* tissue has been set up. In order to produce a complete endophyte-free plant from the endophyte-free tissue, a separate research project should be designed. Shoot regeneration and root development protocols as well as acclimatization protocol for transferring the plant into sterile soil in sterile condition must be optimized. This was not achieved due to limited time available for this study. One of the common tissue culture techniques namely multiple callus culture, which had been previously used in some studies (Segare et al., 2000; Ayadi & Guiller, 2003; Kumar et al., 2010a), was used to produce endophytes-free callus tissue of *C. asiatica* in this study.

5.5.1. Callus Induction

Optimization of callus induction from leaf explants of *C. asiatica* were carried out using different concentration of BAP and NAA plant regulators. BAP, which is a widely used synthetic cytokinin, and NAA, which is commonly used auxin plant regulator have been

used several times to produce callus tissue in different plants such as *Podophyllum hexandrum* (Ahmad et al., 2007) and *Camellia sinensis* (Seran et al., 2007).

The callus induction of *C. asiatica* has been reported using various types of explants. A precise combination of auxins and cytokinins is mostly essential to fulfill the requirements for callus induction (Pierik 1987). Patra et al. (1998) established an effective protocol for the regeneration of callus cultures of *C. asiatica* from stem and leaf explants. The stem and leaf explants were cultured on MS media supplemented with 2.0 mg/L Kinitin and 4.0 mg/L of NAA. Results of their study showed that when calli were cultured in medium supplemented with BA (4.0 mg/L), Kinitin (2 mg/L), NAA (0.25 mg/L) and adenine sulphate (20 mg/L), green nodular structures which developed into dark green shoots were observed after 4 weeks and 6 weeks of culture. The regeneration study of Patra et al. (1998) showed that the calli produced from leaves could be easier regenerated compared to stem-based calli. Rao et al. (1999) claimed that stem explants are better choice for induction of callus compared to leaf explants. It was suggested that the best combinations of growth regulators for optimum callus induction was 2 mg/L NAA in combination with 0.5 mg/L Kinitin. The derooted nodal segments of *C. asiatica* cultured on MS medium supplemented with BAP (10 ppm) and NAA (2 ppm) produced both shoot and callus (Indumathy et al., 2004). Another successful procedure was developed for callus induction and plantlet regeneration from nodal explants of *C. asiatica* by Naidu et al. (2010). In their study, 92% callus induction was observed when explants were grown in MS medium supplemented with 4 mg/L of NAA in combination with 2 mg/L of 2,4-dichlorophenoxyacetic acid (2, 4-D). The formation of calli was obvious by 8th day on the callus induction medium supplemented with auxin. Callus proliferated within 12-16 days

from all nodal explants in all auxin concentrations and exposure to both light and dark conditions. Deshpande et al. (2010) also showed that combination of auxin and cytokinin is the ideal factor to produce callus tissue in *C. asiatica*. They optimized a high frequency callus induction protocol using NBP and NAA plant growth regulators and found that 6 μM BAP in combination with 6 μM NAA is the best treatment among others. Based on available reports, application of NAA and BAP was chosen for this study and leaf was selected as the explants. The result confirmed that leaf is a suitable explant for producing callus tissue and also showed that a combination of NAA and BAP as auxin and cytokinin is suitable for this study.

5.5.2. Callus Multi-Subculture Method to Obtain Bacteria-Free *C. asiatica* Callus and Screening

The protocol optimized for callus tissue induction was used to produce callus from leaf. The leaf-derived calli were subcultured again on the same medium containing 3 mg/L BAP and NAA three times. The results showed that multiple callus culture method which had been used previously for other medicinal plants such as *Corydalis yanhusuo* (Sagare et al., 2000) and *Gymnema sylvestre* (Kumar et al., 2010a), is also a practical method for *C. asiatica*. There are several reports that show the endophytes associated with plant tissue can become apparent a few weeks after culturing in an *in vitro* condition (Odutayo et al., 2007). Therefore, several subculturing, which was carried out in our study, could produce long-term micropropagated tissues to reduce the risk of endophytes hiding. In addition, a rich media was selected to screen the endophytes-free plants and evaluate the efficiency of the employed protocol. Thomas (2004) previously showed that a bacteriological media was a suitable media to detect the covert and endophytic bacteria in plant tissue cultures.

Bacteriological peptone, which is a high peptide and amino acid content, was used to enrich the plant growth medium and prepare a suitable media for growth of endophytic bacteria. The result of this study proved that the used media is a suitable screening media which could induce the growth of endophytic bacteria which had been covert within plant tissue. Since majority of obtained calli in this study were endophytes-free tissues, the applied method of producing endophytic bacteria-free callus is proven as a competent method.

Further future study is needed to produce endophyte-free complete plant from the endophytes-free calli in an *in vitro* condition. Then, comparison between bioactivity of endophytes-free *C. asiatica* plant and control (*C. asiatica* associated with endophytes) can show the total effect of endophytes on bioactive compounds production in *C. asiatica*.

CONCLUSION

This study was initiated as a scientific investigation to show some bioactivities of *Centella asiatica* that made it significant in traditional medicine for a long time. However, an investigation of this scale attempted to look at more evidence based data to reach a conclusive finality. Both examined indigenous subspecies of *C. asiatica* showed potent antibacterial and antioxidant activities in this study. They also contained high amounts of phenolic compounds. The bioactivities and total phenolic contents showed a variable range between different morphological parts of *C. asiatica* in both subspecies. It is important for pharmaceutical industries engaged in extraction procedures to determine which morphological part of the plant contains the highest amount of the compound of interest.

Bioactivities and production of phytochemicals in *C. asiatica* also differed between the subspecies and one of the subspecies (KLU047364) showed higher bioactivities than the other (KLU047552). This finding shows bioactivities of one subspecies or variety of a plant species cannot be representative for all subspecies or varieties of that species.

This study also confirmed the effect of bacteria on plant metabolism by using both plant pathogenic and beneficial bacteria. The hypothesis that pathogenic bacteria can enhance the production of valuable compounds and increase the bioactivity of plants by influencing the plants' defense system was confirmed in this study. Although the antioxidant activity of *C. asiatica* inoculated with the selected pathogenic bacteria (*Enterobacter sp.*) were only enhanced in the early stage of infection while the antibacterial activity of all examined parts of the plant was increased in both early and late stages of infection. The increase in

the level of phenolic compounds in the initial stage of infection (three days after inoculation) and the reduction of the phenolic contents in the late stage of infection (seven days after inoculation) were shown in this study for both total phenolic contents based on the Folin-Ciocalteu and HPLC assays.

On the other hand, the antibacterial activity of *C. asiatica* was not significantly changed after treating the plant with the selected beneficial bacteria (*Pseudomonas sp*.) in this study. Nevertheless, the antioxidant activity of the plant was significantly enhanced by the beneficial bacteria and the total phenolic contents in different morphological parts of the treated plants were increased. This increase of total phenolic contents was also confirmed by HPLC examination.

Some valuable phenolic compounds such as catechin, phlorizin, and quercetin on which their antibacterial and antioxidant activities had been previously reported in literature for this plant were detected in all examined morphological parts of *C. asiatica* using HPLC investigation in this study. Amounts of some of these important bioactive compounds were enhanced and reduced in specific morphological parts of *C. asiatica* after treating the plant with *Enterobacter sp.* and *Pseudomonas sp*. bacteria in this study. The reason for this irregular production of phenolic compounds is not clear and it needs to be investigated further. Meanwhile, levels of some compounds such as catechin and quercetin were sharply increased in leaves of the treated *C. asiatica* with *Pseudomonas sp*.

A wide range of endophytic bacteria belonged to different families were isolated from leaf and petiole-stem of *C. asiatica*. There were similar strains such as *Methylobacterium*

radiotolerans AR_PSLBHI and *Pantoea agglomerans* AR_PSBH2 which were found in both examined morphological parts of plant. This finding can confirm that some of the endophytic bacteria migrated between different plant parts. *Bacillus gibsonni* was the only Gram-positive endophytic bacteria isolated. All isolated endophytic bacteria except *B. gibsonii* could inhibit the growth of *Pseudomonas aeruginosa* as the pathogenic test bacteria. This confirmed that endophytic bacteria associated with *C. asiatica* are a novel source for antimicrobial compounds. Although the isolated endophytic bacteria cell-free cultures showed fair antioxidant activities compared to the applied positive controls, DPPH radical scavenging activity of these bacteria free cultures were significantly higher than one of the positive controls (BHT at concentration of 1 mg/ml).

In this study, a callus induction protocol for *C. asiatica* leaf explants using different concentrations of BAP and NAA as the plant growth regulators was optimized. This was followed by developing an *in vitro* protocol to produce endophytic bacteria-free *C. asiatica* callus tissue using the optimized callus induction protocol. The study showed that a multiple callus-subculturing method is a practical method to obtain endophyte-free tissue from *C. asiatica*.

Based on the findings in this study, some future prospects remain to be researched such as:

- Identification of detected phenolic compounds in HPLC study
- Investigation into the reason for irregular changes in the amounts of specific phenolic compounds after inoculation of the plant with bacteria
- Identification of the antimicrobial compounds produced by the isolated endophytic bacteria

- Production of endophytes-free *C. asiatica* plant from the endophytes-free calli to evaluate the total effect of endophytes on the bioactivities of *C. asiatica* by comparing the endophytes-free plant with the plant associated endophytic bacteria are suggested to be carried out.