UNIVERSITI PUTRA MALAYSIA

EFFECTS OF LIGHT INTENSITY AND AGRONOMICAL PRACTICES ON GROWTH, YIELD AND QUALITY OF SABAH SNAKE GRASS (*Clinacanthus nutans* (Burm.f.) Lindau)

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By

ALIREZA NASIRI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

MY FATHER AND MOTHER,

I Love You Forever

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of requirement for the Degree of Doctor of Philosophy

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Chairman : Associate Professor Izham Bin Ahmad, PhD
Faculty : Agriculture

Sabah snake grass (*Clinacanthus nutans* (Burm. f.) Lindau) is a medicinal herb that needs to be investigated since there are a number of claims that this plant is traditionally used as an anti-snake venom, treatment for dysentery, diabetes, fever, regulating menstrual function, relieving pain, anemia, jaundice and setting of fractured bones. In this study, the effect of some environmental factors (light intensity and plant density and nitrogen fertilizer) on physiological and biochemical changes of *C. nutans* were investigated. Information on the polyphenolic compounds (flavonoids and phenolic acids, saponin and tannin content) of *C. nutans* and its biological activities are still scarce and such data would be useful to provide information on herbs containing high levels of beneficial components. The first experiment was conducted to determine the effect of different light intensity (200, 395, 600 and 790 $\mu mol/m^2/s$) on yield and quality of *C. nutans*. Effect of different plant densities (30×30 cm, 30×40 cm, 40×40 cm and 40×50 cm) was examined in the second experiment. Third experiment was conducted to evaluate the response of *C. nutans* to nitrogen (N) fertilizer (0, 100, 150, 200 kg N/ha and chicken dung at 8 ton/ha). All three experiments were conducted based on randomized complete block design with four replications.. The result showed that as light intensity increased carbohydrate content also increased while the protein content decreased for all light intensities. Among all light intensities, the highest total flavonoids content (TF) and total phenolics (TP) was gained under a light intensity of 790 $\mu mol/m^2/s$ with 6.91 mg rutin equivalent /g dry weight and 10.81 mg gallic acid equivalent/g dry weight respectively. The order of TF and TP partitioning under different light levels was: 790 $\mu mol/m^2/s$ > 600 $\mu mol/m^2/s$ > 395 $\mu mol/m^2/s$ > 200 $\mu mol/m^2/s$. Tannin content also was the highest under 790 $\mu mol/m^2/s$, while saponin content was low in this light intensity. Antioxidant activities increased significantly with increasing of TF and TP concentration, and high antioxidant activity, was observed respectively, in *C. nutans* grown under 790 $\mu mol/m^2/s$. The results of HPLC analysis indicated that, except pyrogallol, synthesis and partitioning of orientin, isoorientin, vitexin, isovitexin and rutin were high in plants grown in the open field (790 $\mu mol/m^2/s$). In second experiment both TF and TP, reached to the maximum level in plants with 40×40 cm distance, however there was not a significant difference between 40×40 cm and 40×50 cm distance. The results showed that the highest content of carbohydrate and protein content and also photosynthesis rate was obtained in 40×40 cm distance. Based on the results obtained, orientin, isoorientin and pyrogallol amount were higher under the planting distance of 40×40 cm while the highest amount of vitexin and isovitexin was detected in planting distance of 30×40 cm. Activity of 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and Ferric Reducing Antioxidant Potential (FRAP) assay increased at the 40×40 cm planting distance, respectively. With increasing antioxidant power malondyaldehyde (MDA) level decreased for all four planting densities. In addition, *C. nutans* extract exhibited the highest anticancer activity in MCF–7 cancer cells with IC_{50} values of 102.19 and 106.21µg/ml for planting distance of 40×40 cm and 40×50 cm respectively. The results of the third experiment showed that, by using an adequate N rate, it was possible to significantly increase and optimize the bioactive compound levels. Highest photosynthesis rate, carbohydrate and protein production were recorded by supplying 150 kg N/ha. At 150 kg N application, the plant contained higher phenolics and flavonoids as well as higher antioxidant activity compared to other nitrogen levels. Application of chicken dung (after 150 kg N) also showed high antioxidant activity. The result also demonstrated that, except rutin, other flavonoids were detected in highest value in 150 kg N treated plants. Cancer cell line (MCF-7) exposed to the extracts showed cytotoxicity with a range of 58.12% for 150 kg N application. These results indicate that the yield and pharmaceutical quality of *C. nutans* can be enhanced by controlling the agronomic practices.

Abstrak tesis yang telah dikemukakan kepada Senate Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

KESAN KEAMATAN CAHAYA DAN AMALAN AGRONOMI YANG MEMPENGARUHI PERTUMBUHAN, HASIL DAN KUALITI DAUN BELALAI GAJAH (*Clinacanthus nutans*)

Oleh

ALIREZA NASIRI

Januari 2016

Pengerusi : Profesor Madya Izham Bin Ahmad, PhD
Fakulti : Pertanian

Belalai Gajah (*Clinacanthus Nutans*) adalah tanaman herba yang perlu dikaji kerana ramai yang mengatakan bahawa tanaman ini boleh digunakan secara tradisional untuk merawat racun ular, cirit-birit, kencing manis, demam, mengawal kitaran haid, melegakan kesakitan, anemia, penyakit kuning dan menetapkan tulang patah. Dalam kajian ini, kesan daripada beberapa faktor persekitaran (cahaya, kepadatan tanaman dan baja nitrogen) ke atas perubahan fisiologi dan biokimia *C. nutans* telah dikenal pasti. Maklumat mengenai polifenolik (flavonoid dan asid fenolik, saponin dan kandungan tanin) dan aktiviti biologi tanaman ini masih kurang didapati dan data tersebut adalah berguna bagi tanaman herba. Kajian pertama telah dijalankan bagi menentukan kesan kadar pencahayaan yang berbeza (200, 395, 600 dan 790 μmol/m^2/s) pada hasil dan kualiti C. nutans. Kepadatan tanaman yang berbeza (30 × 30 cm, 30 × 40 cm, 40 × 40 cm dan 40 × 50 cm) telah dijalankan untuk kajian kedua. Kajian ketiga telah dijalankan bagi mengenalpasti tindakbalas *C. nutans* kepada nitrogen baja (N) (0 , 100, 150, 200 kg N/ha dan baja tahi ayam pada 8 tan / ha). Hasil yang diperolehi menunjukkan bahawa kadar pencahayaan meningkatkan kandungan karbohidrat manakala kandungan protein menurun untuk semua kadar pencahayaan. Daripada semua kadar pencahayaan, peningkatan pada kandungan total flavonoids (TF) dan total phenolics (TP) telah dikenalpasti pada kadar pencahayaan 790 μmol/m^2/s dengan masing-moasing 6.91 mg rutin equivelent/g berat kering dan 10.81 mg gallic acid equivelent /g berat kering. Susunan TF dan pembahagian TP di bawah tahap pencahayaan yang berbeza adalah: 90 μmol/m^2/s > 600 μmol/m^2/s > 395 μmol/m^2/s > 200 μmol/m^2/s. Kandungan tanin juga tinggi pada 790μmol/m2/s, manakala kandungan saponin adalah rendah pada kadar pencahayaan ini. Aktiviti antioksidan meningkat dengan ketara selaras dengan peningkatan TF dan kepekatan TP telah dikenapasti pada *C. nutans* yang ditanam di bawah pencahayaan 790 μmol/m^2/s. Keputusan analisis HPLC menunjukkan bahawa penghasilan dan pembahagian orientin, isoorientin, vitexin, isovitexin dan rutin didapati tinggi apabila tanaman ditanam secara terbuka (790 μmol/m^2/s). Pada kajian kedua, kandungan TF dan TP telah mencapai ke tahap maksimum dalam tanaman yang ditanam pada jarak 40×40 cm. Walau bagaimanapun, ianya tidak memberi perbezaan yang ketara apabila dibandingkan dengan tanaman yang ditanam pada jarak 40×50 cm. Hasil kajian

menunjukkan bahawa kandungan yang tinggi pada karbohidrat dan kandungan protein serta kadar proses fotosintesis telah diperolehi pada jarak tanaman 40×40 cm. Berdasarkan keputusan yang diperolehi, orientin, isoorientin dan jumlah Pyrogallol adalah lebih tinggi pada jarak tanaman 40×40 cm manakala jumlah tertinggi vitexin dan isovitexin dikesan pada jarak tanaman 30×40 cm. Aktiviti 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) and Ferric Reducing Antioxidant Potential (FRAP) assay assay masing-masing meningkat pada jarak tanaman 40×40 cm. Dengan peningkatan malondyaldehyde kuasa antioksidan (MDA) menurun untuk keempat-empat jarak tanaman. Di samping itu, ekstrak *C. nutans* mempamerkan aktiviti anti kanser yang paling tinggi di MCF-7 sel-sel kanser dengan nilai-nilai IC_{50} daripada 102,19 dan 106.21μg / ml untuk jarak penanaman 40×40 cm dan 40×50 cm. Keputusan kajian ketiga pula menunjukkan bahawa dengan menggunakan kadar N yang mencukupi, ianya mungkin dapat meningkatkan dan mengoptimumkan kadar kandungan sebatian bioaktif. Kadar fotosintesis, karbohidrat dan pengeluaran protein yang paling tinggi telah direkodkan pada kadar pembajaan 150 kg N / ha. Pada kadar pembajaan 150 kg N, kandungan fenolik dan flavonoid serta aktiviti anti-oksidan didapati lebih tinggi berbanding dengan tahap nitrogen yang lain. Pembajaan tahi ayam (selepas 150 kg N) juga menunjukkan aktiviti antioksidan yang tinggi. Kajian juga menunjukkan bahawa nilai tertinggi telah dikenalpasti pada flavonoid yang lain, kecuali rutin pada kadar pembajaan 150 kg N. Sel kanser (MCF-7) yang telah didedahkan kepada ekstrak menunjukkan cytotoxicity dengan kadar 58,12% bagi 150 kg N pemberian baja. Keputusan ini menunjukkan bahawa hasil dan farmaseutikal kualiti *C. nutans* dapat ditingkatkan dengan mengawal amalan agronomi.

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LIST OF ABBREVIATIONS

°C	Degree centigrade
g	gram
g/kg	gram per kilogram
g/l	gram per liter
h	hour
IC_{50}	Values were calculated as the concentrations that show 50% inhibition of proliferation on any tested cell line.
l	liter
M	mole
m bar	millibar
mg	milligram
ml	milliliter
mM	milimole
mm	millimeter
mmol/m2/s	Milimol per meter square per second
nm	nanometer
ppm	parts per million
rpm	Round per minute
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
µg	microgram
µg/ l	microgram per litter
µg/g	microgram per gram
µl	microliter
µm	micromilimeter
µM	Micromol
µmol/m2/s	Micromol per meter square per second
µmol/mol	Micromol per mol

ANOVA	Analysis of Variance
MSTATC	Analytical Software
P	Probably
RCBD	Randomized Complete Block Design
SAS	Statistical Analysis Software
SD	Standard Deviation
SEM	Standard Error of Means

LIST OF ABBREVIATIONS OF CHEMICAL MATERIALS

$AlCl_3$	Aluminium chloride
BHT	Butylated Hydroxytoluene
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DMSO	Dimethyl Sulfoxide
FBS	Fetal Bovine Serum
$FeCl_2$	Iron(II) chloride
$FeCl_3$	Iron(III) chloride
FRAP	Ferric Reducing Antioxidant Potential
GAE	Gallic acid
H_2O_2	Hydrogenperoxide
HCl	Hydrochloric Acid
KCl	Potassium Chloride
MCF-7	Breast cancer cell line
MDA-MB-231	Breast cancer cell line
MeOH	Methanol
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na_2Co_3	Sodium carbonate
$NaNO_2$	Sodium nitrite
NaOH	Sodiume hydroxide
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute medium
SDS	Sodium Dodecyl Sulphate
TPTZ	2,4,6-tripyridyl-*s*-Triazine
Vitamin C	Ascorbic acid

CHAPTER 1

INTRODUCTION

Herbs and natural products are valuable sources of medicinal compounds and since ancient times, their benefits and healing properties are well known. According to ancient documents, herbs and medicinal plants have a long history in treating of human diseases and they still play an important role in curing diseases (Boyer and Liu, 2004).

Nowadays, evaluation of phytochemicals for their possible biological activities and benefits of physiological action in the human body is an important research area in the medical and food industry (Ao et al., 2008). Phytochemicals as important compounds in medicinal plants, are not only required for our normal bodily function, but they have an active and positive effect on the health or amelioration of many diseases. Many phytochemicals have been identified, although a great number are yet to be identified (Boyer and Liu, 2004). These compounds include a wide range of chemicals, some of which are secondary metabolites like vitamins, phenolics, terpenoids, and alkaloids (Grusak, 2002). Several biological functions of phytochemicals in different plants were reported by previous studies. For instance, secondary metabolites were reported to have an adaptive function in plants against biotic and abiotic stresses (Kliebenstein, 2004; Mithofer et al., 2004). Also, it is well known that oxidative stress, which is the main cause of tissue damage, is the hall-mark of several chronic disorders and cell death (Mates et al., 2002). Oxidative stress plays an important role in heart diseases, neurodegenerative diseases, cancer and it is also involved in the aging process (Zima et al., 2001; Astley, 2003). This theory is supported by evidences suggesting/indicating that oxidative damage is involved in the development of chronic, age-related degenerative diseases, and that dietary antioxidants oppose this and lower risk of disease (Atoui et al., 2005). Polyphenols are very important in the control and prevention of tissue damage done by activated oxygen species due to their antioxidative effects (Ibrahim and Jafaar, 2012). The therapeutic potentials of medicinal plants as natural antioxidants in reducing such free radical induced tissue damage and in the maintenance of health and protection from some age-related degenerative disorders such as cancer and coronary heart diseases is established (Atanassova et al., 2011). Several types of polyphenols (phenolic acid, hydrolyzable tannins and flavonoids) show antioxidant activity, anti-carcinogenic, anti-inflammatory and anti-mutagenic effect (Barbosa, 2007). Epidemiological studies have reported a correlation between ingesting phenolic compounds and improved health condition (Boker et al., 2002; Knekt et al., 2002). As a consequence of extensive research, a number of clinically useful and market approved drugs from plants are now available for use (Shoeb, 2006). One of the main disadvantages of synthetic antioxidants is the side effects when consumed *in vivo* (Chen et al., 1992). In recent years, there has been an increase in the usage of herbs as sources of natural antioxidants for scavenging of free radicals (Galati and Brien, 2004). As our scientific understanding of herbs grew in the modern days, many prefer the more natural source of supplements rather than chemically derived-products as a mean of keeping good health in the fast paced lifestyle. This, thus, has given rise to the blooming herbal industry in the modern days. Research on the utilization patterns of complementary medicine indicates that between a third and a half of the population of industrial countries may be using some form of complementary medicine. The increased demand for herbal medicines in both industrial and non-industrial countries is creating new patterns for medicinal plant harvesting. Reports indicate that these patterns are exceeding the capacity of supply (Brinckmann, 2009). Yet with appropriate policies for conservation, cultivation, processing and marketing, the World Bank has argued that medicinal plants are a possible bridge between sustainable economic development, affordable health care and conservation of vital biodiversity (Bodeker, 2000). Medicinal plants have a promising future because there are about half million plants around the world, and most of their medical activities have not been investigated yet, and their medical activities could be decisive in the treatment of present diseases for future.

Among the plants that have been known to provide antioxidant compounds is *Clinacanthus nutans (C. nutans)*, belonging to the family of Acanthaceae, which is a well-known medicinal plant (Okigbo et al., 2008). This plant is a small shrub that can be found throughout South East Asia, primarily indigenous to Thailand, Indonesia and Malaysia (Nesheim et al., 2006). It has been used traditionally as anti-venom, anti-inflammatory, analgesic, antidiabetic, anti-rheumatism, antiviral and antioxidant (Afiq, 2011; Arullappan et al., 2014). The English name of this plant is Sabah snake grass because this plant is well-known and can be found easily in East Malaysia, Sabah. However, in Thailand the plant is denoted as Saled pangpon tua mea, Phaya-Yor, Phak Man Kai, Phak Lin Khiat (Arullappan et al., 2014). In Thailand, scientists found that dysentery and fever can be treated by this plant. Due to its anti-cell lysis property, the plant has been used as anti-venom for snake and scorpion bites and also removes nettle rashes (Afiq, 2011). In China, this plant is used to treat inflammation such as hematoma, bruises on eye, anxieties, injuries and rheumatism (Senny, 2009). This plant's natural minerals help in adapting the normal menstrual function, relieving pain, anemia, repairing of fractured bones and jaundice. Malaysia and other Asian countries broadly use this plant to treat uric acid, gout, urinates neuropathies, liver cancer, kidney syndrome, nasal cavity cancer and uterine fibroid. This plant has been endorsed for treatment of herpes simplex, herpes zoster and skin psoriasis in the Primary Health Care Program (Wanikiat et al., 2008) Anti-inflammatory is another property of this plant which relieves major skin inflammation, skin rashes and insect bites (Siriporn and Vajrabhaya, 2002).

The absorption of total flavonoid and phenolic metabolites was found to be influenced by environmental conditions such as light intensity, carbon dioxide levels, temperature, fertilization, salinity, soil microorganisms and other biotic and abiotic factors which can alter the concentration of these compounds (Fine et al., 2006).

Irradiance is known to regulate not only plant growth and development, but also the biosynthesis of both primary and secondary metabolites. Phenolics biosynthesis requires irradiance or is enhanced by irradiance, and flavonoids formation is absolutely irradiance dependent where its biosynthetic rate is related to irradiance intensity and density. Different plants have different responses to irradiance intensity that result in differences in their production of secondary metabolites (Ibrahim and Jafaar, 2012).

The concentration of chemical compounds such as total flavonoids and phenolics is influenced by biotic and abiotic factors (Fine et al., 2006). Cultural practices such as planting density have been shown to have significant influence on herbs growth and productivity.

Fertilizer effects on antioxidant activity in medicinal plants have also been reported. Although fertilizer is a greatly needed input on plant growth, excess fertilizer use can result in serious damage to the soil and water contamination, and quality of the produce. The quantity of fertilizer used in nutrient management has important effects on the quality and sustainable production of herbs (Hassan et al., 2012).

Despite of the fact that the *C. nutans* have the physiological and biochemical benefits, the growth and development that are influenced by the biotic and abiotic factors are still inadequate. In order to hasten the development of medicinal aspects of *Clinacanthus nutans*, it is pertinent to establish the scientific knowledge of its medicinal properties under different agronomic practices, as little is known about this plant. Thus, this study was conducted to:

1) To investigate and determine changes in the accumulation and profiling of yield and antioxidant activity with changes in light intensity in *C. nutans*

2) To determine the best planting density for *C. nutans* to get the highest yield, antioxidant activity.

3) To evaluate the effect of Nitrogen fertilizer on yield, and accumulation of biochemical compounds of *C. nutans*

4) To investigate and determine the anticancer activity of *C. nutans.*

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LIST OF PUBLICATIONS

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