

EFFECT OF SALT STRESS ON THE PHYSIOLOGICAL
RESPONSES, PHYTOCHEMICALS AND ANTIOXIDANT
ACTIVITIES OF *CLITORIA TERNATEA* VAR. *PLENIFLORA*

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DEDICATION

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ABSTRACT

Soil salinity is a process of increasing salt in soils. It has become a problem since it keeps spreading geographically from coastal areas to other fertile lands. Therefore, many plants have developed some series of adaptations to mitigate against salinity. Salinity limits the vegetative productions and changes the ecological health of estuaries due to unproductive lands. It lowers the crop yields for daily purposes as well as a threat for biodiversity in which living things loses habitat for both land and in water. Thus, the purpose of this study was to evaluate the growth traits of *Clitoria ternatea* var. *pleniflora* exposed to different concentrations of sodium chloride, NaCl. Accordingly, it also brings insights into the effect of various concentrations of salt stress on the qualitative phytochemicals content of tannins, flavonoids, glycosides, steroids, terpenoids, alkaloids, phenols and saponins. Moreover, the total phenolic and flavonoid contents were determined quantitatively. Ferric reducing antioxidant power (FRAP), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were conducted to evaluate the antioxidant activities of the *Clitoria ternatea* var. *pleniflora*. Thus, the results from this study revealed that all of the test growth traits—the number of branches and leaves, height of plants, fresh weight of leaves and dry weight of stem, leaves and roots—were the highest under 200 mM of NaCl treatment. Apart from that, under 200 mM of NaCl, *Clitoria ternatea* var. *pleniflora* was shown to contain the highest total of phenolic and flavonoid contents among of all the salt stress treatments. Thereupon, the antioxidant activities of FRAP, DPPH and ABTS were also showed to reach at their highest activities under 200 mM of NaCl. In conclusion, 200 mM of NaCl is the optimum concentration for *Clitoria ternatea* var. *pleniflora* to improve the growth traits, phytochemicals and antioxidants of *Clitoria ternatea* var. *pleniflora*.

ABSTRAK

Tanah masin adalah satu masalah kerana perkara ini sentiasa berkembang dari kawasan berdekatan laut ke tanah subur yang lain. Banyak tumbuh-tumbuhan juga telah mempunyai siri penyesuaian untuk mengatasi kemasinan tanah. Tanah yang masin menjadi tanah yang tidak produktif dan menjadi batas kepada pengeluaran tumbuhan. Ini akan memberi kesan kepada pengurangan bekalan makanan untuk keberlangsungan kehidupan manusia. Oleh itu, tujuan kajian ini adalah untuk menilai ciri-ciri pertumbuhan *Clitoria ternatea* var. *pleniflora* semasa pendedahan sodium klorida NaCl. Ini juga memberi asas penting kepada kesan terhadap fitokimia kualitatif yang ditentukan oleh kehadiran tanin, flavonoid, glikosida, steroid, terpenoid, alkaloid, fenol dan saponin. Selain itu, fitokimia kuantitatif juga dinilai berdasarkan kandungan total fenolik dan flavonoid. Ujian Ferric reducing antioxidant power (FRAP), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) dilakukan untuk menilai aktiviti antioksidan *Clitoria ternatea* var. *pleniflora*. Oleh itu, hasil kajian ini telah menunjukkan bahawa semua sifat pertumbuhan: jumlah dahan dan daun, tinggi tanaman, berat daun yang segar dan berat batang, daun dan akar yang kering, adalah tertinggi di bawah tekanan 200 mM sodium klorida. Selain itu, di bawah 200 mM NaCl, *Clitoria ternatea* var. *pleniflora* memperoleh jumlah kandungan fenolik dan flavonoid tertinggi. Aktiviti antioksidan FRAP, DPPH dan ABTS juga mencapai aktiviti tertinggi di 200 mM NaCl. Kesimpulannya, 200 mM NaCl adalah tekanan garam yang sesuai kepada *Clitoria ternatea* var. *pleniflora* untuk memperbaiki tumbesaran, fitokimia dan antioksidan *Clitoria ternatea* var. *pleniflora*.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	-	Degree celsius
%	-	Percentage
1/4	-	Quarter
cm	-	Centimetre
dSm ⁻¹	-	Decisiemens per metre
g	-	Gram
h	-	Hour
m	-	Metre
µg/mL	-	Microgram per millilitre
mg/g	-	Milligram per gram
mg/kg	-	Milligram per kilogram
mg/mL	-	Milligram per millilitre
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
mM/g	-	Millimolar per gram
nm	-	Nanometre

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CHAPTER 1

INTRODUCTION

1.1 Background and rationale of study

Nowadays, most countries around the world face soil salinity, which it is affecting plant growth. Salinity is a problem because it became one of the limiting factors towards the environment and life. It limits the production of plants and decreases the agricultural land areas. This problem is more challenging and threatening since 23% of 1.5×10^9 hectares of the world's cultivated land is saline (Shereen *et al.*, 2005). Earth is losing an average of 2000 hectares of soil into salt-affected lands continuously every day (Hamilton, 2014). In many countries, soil salinity is a threatening problem and is affecting human health and environmental resources. Currently, it is affecting around 1 billion hectares globally and is having significant impacts on the ecological balance of the soil physicochemical properties (Shrivastava & Kumar, 2015). Additionally, most researchers found that soil salinity is irreversible and it causes damages continuously and diminishes the capacity of the ecosystem services (Li & Li, 2017; Sangadji *et al.*, 2019). Furthermore, soil salinity leads to less food production and sustainability, damaging the ecological area and affecting plants growth (Hasanuzzaman *et al.*, 2013).

Moreover, the productivity and growth of plants are reduced since most of them have low tolerance towards soil salinity (Machado & Serralheiro, 2017). The majority of plants species are not well-adapted towards salt-affected soil, and only 0.14% can adapt towards salinity. These species suggest that salt tolerance adaptation is not a fundamental trait within them, but the ability appeared and occurred during their evolution (Flowers & Colmer, 2015) as a series of defences against damages.

Moreover, the plants undertake the salt tolerance evolution by two main mechanisms, either by limiting the salt's entrance through roots or controlling salts concentration and distribution (Hanin *et al.*, 2016) which is more relevant to improve their tolerance and adaptations. Currently, the developing countries are making few alternatives and strategies to understand the functional studies of the gene where it involves the engineered crop. It is to enhance the traits of salt stress-tolerance plants to overcome the salinity problem.

Many studies bring up the significant role of plants in overcoming soil salinity. For instance, the impacts of salt stress have been faced by species of *Clitoria ternatea* in semi-arid and arid regions, where they are considered as low to medium salt-tolerant (Talukdar, 2011). *Clitoria ternatea* has 6.4 dS m^{-1} of tropical legume tolerance rate based on the electrical conductivities, (EC) towards salinity (Keating *et al.*, 2015). This shows that *Clitoria ternatea* is more tolerant and can mitigate salt stress than other comparative legumes (Talukdar *et al.*, 2011; Nasim & Pa'ee, 2021).

Besides of its tolerance towards salinity, these attractive perennial climbers produce various beneficial phytochemicals in which contribute to the medicinal properties. Some *Clitoria ternatea*'s species extracts have medicinal values on its wide range of phytochemical constituents. They are beneficial for antipyretic, analgesic, diuretic, local anaesthetic, antidiabetic, insecticidal properties of vascular smooth muscle relaxing (Kumar *et al.*, 2017) and antimicrobial properties (Durga *et al.*, 2015; Jamil *et al.*, 2018). In addition, viable cells and cancer cells have been shown to decrease with the exposure of their leaves' methanol extract (Al-Snafi, 2016). The growth and germination of *Clitoria ternatea* may be influenced by the change of natural environment such as abiotic stress, including salinity. The changes of physiological, phytochemicals and antioxidants may be interrupted by the salinity during plant growth and development. Salt stress affects the growth of plants by inducing the osmotic stress and lead to sodium, Na^+ and Cl^- toxicities. Hence, it will cause the closure of stomata and reduce the rate of photosynthesis (Sarker *et al.*, 2018). Furthermore, many plants may be affected by pathogens interference, thus decreasing the crop yield of many plants during the stress. However, there are fewer studies on the effect of salt stress on the physiological responses and the phytochemical productions of *Clitoria ternatea* var. *pleniflora* (Nasim & Pa'ee, 2021). Besides, *Clitoria ternatea* var. *pleniflora* was chosen since, our literature review suggests there are fewer studies and discoveries on the scientific values of this variety (Nasim &

Pa'ee, 2021). Hence, this study was conducted to explore the new potential of *C. ternatea* var. *pleniflora* as salt-tolerant plant. Besides, this study will provide insights on the relation of the physiological, phytochemical and antioxidant activities responses of *C. ternatea* var. *pleniflora* towards the abiotic stress, soil salinity.

1.2 Problem statement

Natural process such as sea-level rise has induced seepage into areas that are below sea level. Furthermore, evapotranspiration and lack of rainfall to flush the soils also lead to soil salinity (Wada *et al.*, 2017; Corwin, 2020). These natural processes are unavoidable and will cause an increment of saline soils. In the early stages, salinity reduces soil productivity by affecting the metabolism of soil organisms, and it is becoming more serious when the vegetation and organisms in the soils are destroyed. Consequently, it transforms the productive and fertile land into barren land (Shrivastava & Kumar, 2015; Prasad *et al.*, 2016; Machado & Serralheiro, 2017). Presently, plants are the main sources for humans to sustain food availability, survival, and as a source of medications. Thus, the increment of soil salinization around the world will give impacts on the ecology and future survival of living things. Subsequently, the variety of *Clitoria ternatea* var. *pleniflora* is an underutilised plant (Prasad *et al.*, 2016; Tomaz *et al.*, 2020). Some of the varieties in the genus *Clitoria* were recorded to be adaptable to natural environmental changes and abiotic stresses—such as light and drought and able to produce different phytochemicals in their extract. However, there are fewer studies on the varieties of *Clitoria ternatea* var. *pleniflora* in physiological responses when treated with salt stress (Jamil *et al.*, 2018; Marget *et al.*, 2019).

Thus, an approach of a study using *Clitoria ternatea* var. *pleniflora* is important. This is because most of the practitioners are from different backgrounds (Quazi & Yogekar, 2020). Besides, their new potential for producing new and valuable phytoconstituents treated with salt stress was less scrutinized. In addition, the production of antioxidants of this variety is still uncovered wholly scientifically.

1.3 Significance of study

Saline soils are increasing day by day and many researchers are finding alternatives to overcome this arising problem. Saline soils can be controlled and mitigated by the introduction of salt-tolerant plants. Meanwhile, species of *Clitoria ternatea* was known to tolerate the saline soils. Therefore, this study will provide new insights into *Clitoria ternatea* var. *pleniflora*'s significance to tolerate on saline soils. On the other hand, *Clitoria ternatea* var. *pleniflora* was chosen because it can grow well under a certain concentration of salt stress.

This variety of *Clitoria ternatea* var. *pleniflora* is an alternative to most of the industrial sectors since it is easily grown in Malaysia. Although they are abundantly available in Malaysia, not many people are aware of the existence and importance of *Clitoria ternatea* var. *pleniflora*. In addition, *Clitoria ternatea* var. *pleniflora* was chosen because the plants will respond to abiotic stresses, leading to changes in their phytochemical constituents and antioxidants activities. Hence, this study provides an important opportunity for new discovery that may generate fresh insights and a useful option for future needs.

1.4 Study limitations

Due to the nature of research environment, there were few limitations occurred in this study. Firstly, the limitation was time constraint where, the longest period of one year and half was taken on the plant material to grow the samples. In addition, another 3 months were taken to treat the samples since they had diseases such as rust and verticillium wilt. Due to these constraints, they were also affecting the remaining time for the other analysis such as phytochemical and antioxidant analysis.

Besides, there was a limitation in data collections and analysis of soil moisture and air humidity during the experimental period. These analysis cannot be done because, some equipments such as air humidity meter and soil moisture meter were broken and not available in the workplace. Hence, these analysis cannot be done for the data collections.

Then, the other limitation was financial resources. Better financial resources was needed since, necessary equipment such as glassware to keep the stock of samples

was needed and they come in large quantities. Better financial resources also needed to grow the samples. The quantity of the samples was in hundreds, thus enough money was needed to purchase the pots, soils and seeds for growing the samples.

Besides, other limitation was the payment service for plant identification. Enough financial resources was important to pay the professional taxonomist to identify the species of *Clitoria ternatea* and to obtain the confirmation identification letter of the sample. Besides, there was a limitation in the availability of equipments. Some of the equipment such as UV-VIS spectrophotometer and oven dryer in a laboratory were always broken and required a long period of time to undergo maintenance. Thus, these limitations might be reflected in the results of the study.

1.5 Research objectives

The objectives of this study were:

- i. To measure the growth rate of *Clitoria ternatea* var. *pleniflora* exposed to different concentrations of sodium chloride, NaCl.
- ii. To determine the phytochemical content of *Clitoria ternatea* var. *pleniflora* with the effects of salinity treatment.
- iii. To evaluate the antioxidant activities of *Clitoria ternatea* var. *pleniflora* exposed to different concentrations of NaCl.

1.6 Research scopes

The scopes of this study were:

- i. The study was carried out in six different salt concentrations (0, 100, 150, 200, 250, 300) mM on *Clitoria ternatea* var. *pleniflora* in 8 weeks of propagation stage. This is to evaluate the physiological adaptation of *Clitoria ternatea* var. *pleniflora* based on their growth: height, increment number of leaves, braches and fresh and dry weight of each plants' part.
- ii. The study was designed to investigate the resulting potential secondary metabolites for qualitatively (tannins, saponins, steroids, alkaloids, glycosides,

phenols, flavonoids and terpenoids) and qualitatively (total phenolics and flavonoids content) of *Clitoria ternatea* var. *pleniflora*'s extract.

- iii. Antioxidants activity was measured using three assays which were ABTS, DPPH and FRAP. They were furthered from the the phytochemical analysis, to reveal their possibilities on holding their role against free radicals.



CHAPTER 2

LITERATURE REVIEW

2.1 Classification of morphology and taxonomy

Clitoria ternatea commonly known as ‘butterfly pea’ is a prominent white or blue of an appealing perennial climber flower (Figure 2.1). *Clitoria ternatea* is from South East Asia and abundantly found in Philippines, Madagascar and India (Manjula *et al.*, 2015). *Clitoria ternatea* has naturalized and disseminated widely at subtropical and tropical East and West Indies, South and Central America, China and India countries (Gomez & Kalamani, 2003; Lijon *et al.*, 2017).



Figure 2.1: Blue *Clitoria ternatea* (Jamil *et al.*, 2018).

Low land tropic and humidity are some of the determinants for their wide distribution, besides their adaptability in a wide range of rainfall, temperature and altitude (Chauhan *et al.*, 2012). Moreover, *Clitoria ternatea* species has a high growth rate, high adaptation to heavy clay soil and drought tolerance trait. Therefore, these traits will be useful to provide and help other species to survive and adapt to irrigation channels, disturbed sites, a margin of waterholes, grasslands and river banks (Staples, 1992).

Clitoria ternatea is commonly used by the Malaysians to give the blue colour to Nasi Kerabu. Additionally, it is also used to make the traditional dish known as ‘Kueh Tekan’ in Baba and Nyonya culture (Lijon *et al.*, 2017). In addition, other vernacular names of *Clitoria ternatea* in some of the western countries are blue pea and butterfly pea in English, Cunnã, Fula criqua in Portuguese, Cunha in Brazilian, and Mavi kelebek sarmaşığı in Turkish (Gupta *et al.*, 2010). Additionally, in most of the eastern countries, these attractive blue flowers are called as ‘Aparijita’ in Sanskrit and Bengali, ‘Buzrula’ in Arabic, ‘Lan hu die’ in Chinese, ‘Kajroti’ in India, ‘Tembang telang’ in Indonesian, ‘Dangchan’ in Thai, and ‘chi dâu biếc’ in Vietnamese (Kosai *et al.*, 2015). Table 2.1 shows the taxonomic classification of *Clitoria ternatea*.

Table 2.1: Taxonomy classification of the species of *Clitoria ternatea* (Sethiya *et al.*, 2009)

Taxonomy hierarchy	Description of <i>Clitoria ternatea</i>
Kingdom	Plantae
Sub-kingdom	Tracheobionta
Super-division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Sub-class	Rosidae
Order	Fabeles
Family	Fabaceae
Genus	<i>Clitoria</i>
Species	<i>ternatea</i>

This legume of Fabaceae family is an ornamental perennial climber, which has twinning fine stems with the vine’s length up to 1.5 to 5 m. Their leaves are pinnate with 5-9 foliolate, petioles of 2 until 2.5 cm long with 4 mm long of stipules (Kosai *et al.*, 2015; Liji, 2018). The flowers’ petals are blue or white, ranging from deep blue to blue mauve. The petals are orange colour at the centre, very short pedicelate, solitary and showy, style bearded below the stigma, and 4 to 5 cm long. Furthermore, the fruit

Pods are linear and compressed with a slightly pubescent of 6 to 10 seeds in each pod. They are flat and beaked with 6 to 12 cm long and 0.7 to 1.2 mm wide. The colours of the seeds are blackish and yellowish-brown, often mottled, oval or sub-globose in shape with 3 to 4 mm wide and 4.5 to 7 mm long (Gupta, Chahal *et al.*, 2010; Komaraiah & Rao, 2010; Lijon *et al.*, 2017).

Although the species of *Clitoria ternatea* is well-known for its ornamental value, the application in medical uses is also important, as discussed in the following section. Thus, this study will make a major contribution to research the species of *Clitoria ternatea* for the significant of its phytochemical productions when treated with various concentrations salt stress.

2.2 Application and uses of the species of *Clitoria ternatea* in medical prospect

Clitoria ternatea has been used by many traditional and herbal practitioners as one of the alternatives to help, cure and treat diseases. This is because medicinal properties lie on almost every part of this plant. It has been used to treat worm infestation, tonsillitis, cough, asthma, and infertility (Deka *et al.*, 2013). Then, it was found that *Clitoria ternatea*'s extracts have anticonvulsant activity, antidiabetic activity, antimicrobial activity, anti-inflammatory, antipyretic and analgesic activities, and able to enhance memory and improve the efficacy of learning (Sethiya *et al.*, 2009).

Consequently, the values of *Clitoria ternatea* have attracted researchers to conduct more studies to reveal their true potential. Their findings showed that the extract of *Clitoria ternatea* possessed anti-diabetic activity (Chayaratanasin *et al.*, 2015). It was focused on the advanced glycation end products (AGE) as an imperative approach to alleviate the complication of the diabetes. The results showed that the formation of advanced glycation end products was inhibited significantly with 0.25-1.00 mg/ml of *Clitoria ternatea* extract in a concentration-dependent manner (Chayaratanasin *et al.*, 2015). Moreover, the level of fructosamine has been reduced markedly and the decreasing protein carbonyl content leads to decreasing of protein's oxidation (Chayaratanasin *et al.*, 2015). Thus, the extract of *Clitoria ternatea* may have strong antioxidant and antiglycation properties and be able to prevent the AGE-mediated diabetic complication of therapeutic potentials.

A study has claimed and provided some evidence to prove the ethanolic extract of *Clitoria ternatea* might be a useful treatment for the obsessive compulsory disorder (OCD), a disabling psychiatric condition (Shende *et al.*, 2012). Marble-burying behaviour is a test to measure anxiety-related behaviour in rodent. 100, 200 and 400 mg/kg of ethanolic *Clitoria ternatea* extract reduced the marble-burying reading compared to the 5, 20 and 15 mg/kg of fluoxetine. In addition, 100 mg/kg of fluoxetine's sub-effective dose effects also have been studied with the sub-effective dose of 5 mg/kg of fluoxetine which significantly reduced the marble-burying behaviour of mice. Thus, the ethanolic extract of *Clitoria ternatea* has been suggested to modulate and serve as a potential herbal treatment for OCD (Shende *et al.*, 2012).

In sum, *Clitoria ternatea* possessed valuable phytochemicals in which making it as one of the important herb for practitioners. This is because every part of it is beneficial to consumers. However, the scientific advantages of *Clitoria ternatea* var. *pleniflora* are still unexplored (Lakshan *et al.*, 2019). Thus, the discovery of the benefits of a new variety of the species *Clitoria ternatea* is important.

2.3 Difference between *Clitoria ternatea* and *Clitoria ternatea* var. *pleniflora*.

Clitoria ternatea var. *pleniflora*'s morphology is different from *Clitoria ternatea*. Then, genus of *Clitoria* belongs to the family of Fabaceae or Leguminosae. The genus *Clitoria* means "clitoris", which refers to the shape of the flowers (Pendbhaje *et al.*, 2011). *Ternatea* means ternate, which is the epithet of the flowers known in Maluku Island, Indonesia (Pendbhaje *et al.*, 2011). *Pleniflora* means multi-petals, which refers to the flowers that are blue to purplish (Lakshan *et al.*, 2019). However, the difference between both varieties can only be seen by their flower morphology. Flowers of *Clitoria ternatea* (Figure 2.2A) are papilionaceous, which resemble the shape of a butterfly. *Clitoria ternatea*'s flower is also zygomorphic, which is bilaterally symmetrical (Acevedo-Rodriguez, 2005). On the other hand, flowers of *Clitoria ternatea* var. *pleniflora* (Figure 2.2B) are actinomorphic, that is radially symmetrical (Acevedo-Rodriguez, 2005). *Clitoria ternatea* var. *pleniflora* is recognized by five vexillary-like petals (Oguis *et al.*, 2019). In some cases, the flowers have five petals but rarely in four petals. Their flowers' morphology is important because it is the only way to differentiate their variations (Oguis *et al.*, 2019).



Figure 2.2A: The flower's morphology of *Clitoria ternatea*. Figure 2.2B: The flower's morphology of *Clitoria ternatea* var. *pleniflora* varieties. Both figures were retrieved from National Park for Flora and Fauna Web, A Singapore Government Agency Website

The variety of *Clitoria ternatea* var. *pleniflora* is important for new discoveries and provide new insights for future scientific uses and domestications of food supplies (Oguis *et al.*, 2019). On the other hand, the survival of most vegetative plants depends on fertile soils and lands. Salinity is one of the limitations for some plants to grow well and there is lacking in the studies of this salt stress on *Clitoria ternatea* var. *pleniflora* (Oguis *et al.*, 2019). Thus, it is important to learn how salt stress is triggering *Clitoria ternatea* var. *pleniflora* to produce phytochemicals as plant-self defence (Oguis *et al.*, 2019).

2.4 Salinity

Saline soils are soils containing neutral soluble salts that affect the growth of most crop plants. Sodium chloride and sodium sulphate are predominant soluble salts (Torres *et al.*, 2018). Chloride and sulphate of calcium and magnesium are present in substantial quantities in saline soils (Torres *et al.*, 2018). Therefore, the salt-affected soils not only differ in their chemical characteristics but also in their geochemical and geophysical distribution, as well as their biological and physical properties (FAO, 1988) (Table 2.2).

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