UNIVERSITI TEKNOLOGI MARA

ELICITATION OF ASIATIC ACID IN *CENTELLA ASIATICA* USING *KAPPAPHYCUS ALVAREZII*

PUTRI NUR HALIMAH BT ABDUL RAHMAN PUTRA

BACHELOR OF PHARMACY (Hons.)

FACULTY OF PHARMACY

2016

UNIVERSITI TEKNOLOGI MARA

ELICITATION OF ASIATIC ACID IN *CENTELLA ASIATICA* USING *KAPPAPHYCUS ALVAREZII*

PUTRI NUR HALIMAH BT ABDUL RAHMAN PUTRA

Dissertation submitted in partial fulfilment of the requirements for the Bachelor of Pharmacy (Hons.)

Faculty of Pharmacy

July 2016

APPROVAL FORM

I hereby recommend that the thesis prepared under my supervision by Putri Nur Halimah Bt Abdul Rahman Putra entitled 'Elicitation of Asiatic Acid in *Centella Asiatica* Using *Kappaphycus Alvarezii*' is accepted in partial fulfilment of the requirements for the degree of Bachelor of Pharmacy from the Faculty of Pharmacy, Universiti Teknologi Mara (UiTM).

……. ……

Date (Madam Noor Anilizawatima Bt Sulong)
Main Supervisor

……. ……

Date (Dr. Siti Alwani Bt Ariffin)
Co-Supervisor

……. ……

Date (Dr. Humera Naz)
Co-Supervisor

……. ……

Date (Prof. Dr. Aishah Bt Adam)
Dean Faculty of Pharmacy

ACKNOWLEDGEMENT

First and foremost, I would like to thank to the Almighty for His blessing toward accomplishing this project. His Protection and Guidance had enabling me to complete this thesis.

I was so indebted to many people who helped me toward accomplishing this project. It is impossible for me to acknowledge every one of them individually, but several in particular deserve recognition. I wish to deepest appreciation and thanks to Madam Noor Anilizawatima Bt Sulong as supervisor for my research project, for her invaluable concern, sustained guidance and diligent support which enable me to bring this project to complete. Her continuous review, guidance, ideas and suggestion has been precious to this project.

I also would like to take this opportunity to show my gratitude to Dr Humera Naz and Dr Alwani Bt Ariffin as my co-supervisor for their kind ideas and guidance. Next, I am particularly grateful to laboratory staffs of Plant Tisuue Culture Laboratory and Analytical Unit Laboratory for their guidance and effort to help in fulfilling my laboratory needs and supplies during this research.

I also would like to express my appreciation to my family and friends for their support during my research final project because their support had given me strength to continue and complete this research.

Lastly, special thanks to Faculty of Pharmacy, UiTM Puncak Alam for giving me the opportunity to learn and experience such a wonderful knowledge and provide a good research environment.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF GRAPHS

LIST OF ABBREVIATIONS

α	:	Alpha
α/β-AS	:	A/β-amyrin synthases
atm	:	Atmospheric pressure
β	:	Beta
BAP	:	6-benzylaminopurine
cm	:	Centimetre
^{o}C	:	Degree celcius
$CaCl_2.2H_2O$	:	Calcium chloride dehydrate
$CuSO_4.5H_2O$	:	Copper(II) sulfate pentahydrate
$COCl_2.6H_2O$	:	Cobalt (II) chloride hexahydrate
(-COOH)	:	Carboxy group
COX-2	:	Cyclooxygenase-2
DPP	:	Dimethylallyl diphosphate
DMSO	:	Dimethyl sulfoxide
DAD	:	Diode-array detector
ELSD	:	Evaporative light scattering setector
$FeSO_4.7H_2O$	:	Iron (II) sulfate heptaydrate
FPS	:	Farensyl diphosphate synthase
FPP	:	Farnesyl diphosphate
g	:	Gram
g/cm^2	:	Gram per centimetre square
g/mol	:	Gram per mol

g/L	:	Gram per litre
GA_3	:	Gibberellic acid
GC	:	Gas chromatography
H_3BO_3	:	Boric acid
HCL	:	Hydrochloride
$HgCl_2$	:	Mercuric chloride
HPLC	:	High performance liquid chromatography
IPP	:	Isopentenyl diphosphate
IAA	:	Indole-3-acetic acid
IBA	:	Indole butyric acid
iNOS	:	Nitric oxide synthase
IL-1	:	Interleukin-1
KI	:	Potassium iodide
Kn	:	Kinetin
KNO_3	:	Potassium nitrate
$kgcm^2$	:	Kilogram centimetre squared
$KHPO_4$	:	Potassium hydrogen phosphate
L	:	Litre
LC	:	Liquid chromatography
mL	:	Millilitre
ml/min	:	Millilitre per minute
mg/L	:	Milligram per litre
mm	:	Millimetre
mw	:	Molecular weight

mAU	:	Milli-absorbance unit
mAU*s	:	Milli-absorbance unit of seconds
$MgSO_4.7H_2O$	:	Magnesium sulfate heptahydrate
$MnSO_4.4H_2O$	:	Manganase (II) sulfate tetrahydrate
MS	:	Murashige and Skoog
nm	:	Nanometre
NAA	:	Naphthalene acetic acid
NaOH	:	Sodium hydroxide
NH_4NO_3	:	Ammonium nitrate
$Na_2MO_4.2H_2O$	:	Sodium molybdate dehydrate
Na_2 EDTA	:	Disodium ethylenediaminetatraacetate
OCS	:	Oxidosqualene cyclase
(-OH)	:	Hydroxyl group
ODS	:	Octadecylsilyl
PC	:	Paper chromatography
PEG	:	Polyethylene glycol
Rf	:	Response factor
ROS	:	Reactive oxygen species
SFC	:	Supercritical fluid
SiO_2		Silicon dioxide
SQE	:	Squalene epoxidase
SQS	:	Squalene synthase
SKALP	:	Akin anti-leuco protease
TLC	:	Thin-layer chromatography

TNF-α	:	Tumor necrosis factor alpha
UV	:	Ultraviolet
µm	:	Micrometer
µL	:	microlitre
VIS	:	Visible
w/v	:	Weight over volume
$ZnSO_4.7H_2O$	:	Zinc sulfate heptahydrate

ABSTRACT

Centella asiatica is an endangered medicinal herb which used in the preparation of herbal drugs mainly due to the presence of four pentacyclic triterpene which are asiatic acid, asiaticoside, madecassic acid and madecassoside. It's over exploitation necessitates the development of conservation strategies and enhanced the production of secondary metabolites. In present study, the effect of various concentration of seaweed *Kappaphycus alvarezii* elicitor was used to increase the amount of asiatic acid production in *C. asiatica*. Four difference concentration of seaweed elicitor was treated in *C. asiatica* which are 0 g/L, 2g/L, 4g/L and 8g/L. They are harvested at day 0, 7, 14 and 21 and soak with methanol to obtain crude brown extract. The amount of asiatic acid containing in the samples was analyzed by HPLC and area under the curve of retention peak was calculated by using a formula. The diameter of leaf, number of new shoots and flowers were measured during successive stages of development. In conclusion, *K. alvarezii* can increase the production amount of asiatic acid in *C. asiatica* at shorter time, which at day 14, concentration of 4g/L and 8 g/L are the best while the additional of certain concentrations of seaweed elicitor can induce flowering in *C. asiatica*.

CHAPTER ONE

INTRODUCTION

1.1 Background of study

Centella asiatica also known as 'Pegaga' or 'Gotu Kola' which come from the family of *Apiaceae*, a subfamily of the *Hydrocotyle* and genus of *Centella*. It is a slender creeping perennial herb that found in shady, moist area and usually as an annual plant in India, Sri Lanka, Malaysia, and other parts of Asia (Gandi & Giri, 2013). It also grows very well in sandy and clay soil rich in humus and organic matter (Jamil, Qudsia, & Salam, 2007). It produces a group of bioactive compounds collectively called 'Centellosides or Centelloids' which are ursane type of pentacyclic triterpenoid saponins and sapogenin that act as secondary metabolites. According to Gandi *et al.*, (2013), the Centellosides include asiaticoside, centelloside, madecassoside, brahmoside, asiatic acid, madecassic acid, thankuniside and others. However, the main bioactive compounds in *C. asiatica* are asiatic acid, asiaticoside, madecassic acid, and madecassoside (Kim *et al.*, 2009). It has been established that the climatic conditions, soil texture, and agronomic work will highly

influence the bioactive constituent contents of the *C. asiatica* (Govarthanan, Rajinikanth, Kamala-Kannan & Selvankumar, 2015).

Centella asiatica is well known for its medicinal herb with a long history of therapeutic uses (Govarthanan *et al.,* 2015). The pharmacological uses of *C. asiatica* is claimed to possess a wide range of applications such as wound healing, leprosy, skin diseases, microangiopathy, venous insufficiency, rheumatism, inflammation, syphilis, antibacterial, antifungal, mental illness, memory enhancement, neuroprotective, antidepressant, hepatoprotective, anticancer and antidiabetic (Chaturvedi & Joshi, 2013; Govarthanan *et al.,* 2015). Owing to its high medicinal value, there is overexploitation from its natural habitat cause the plant species are endangered or threatened with extinction leading to severe depletion of biodiversity. Thus, this plant has been listed under highly endangered species by the International Union for Conservation of Nature (IUCN) (Halladay & Gilmour, 1995).

Through the development of science and biotechnology, there are many ways that had been discovered to conserve and enhance the valuable medicinal plants. One of the most successful ways is by plant tissue culture technology. Plant tissue culture is the technique of growing the plant cells, tissue, and organs in an artificial nutrient medium under aseptic condition (Qadry, 2010). This technique was conceived and enunciated by Gottlieb Haberlandt in 1902. The advantage of plant tissue culture are the production is in rapid

biomass, the nutritional and environmental condition can be controlled for optimum growth and the media can be easily manipulated by adding of elicitors which can produce more desirable quantities of secondary metabolite. The production of Centelloside by plant tissue culture give an alternative way to get the medicinal value of this plant which also help to reduce the depletion of wild C. asiatica. However, plant tissue culture technique cannot be continued in this research since the plantlets in *in-vitro* culture was not grown enough to the expected time which is 21 days (Gandi & Giri, 2013) and was continued with the observation in natural *C. asiatica* plant that be supplemented with several difference concentration of seaweed elicitor.

Rapid exploration in approach and technique of model systems that concerned with the physiology of nutrient growth results to the rise of different formulation in artificial nutrient media. One of the most commonly used artificial media for plant cell culture work is Murashige and Skoog (MS) which have proved effective for growth of a variety of plants (Qadry, 2010). According to Trigiano & Gray (2005), the plant elicitors can be added to the culture media, which function to stress the plant, leading to the formation of a large amount of secondary metabolite. Plant elicitors can be grouped to three categories which are biotic, abiotic and endogenous elicitors (Fu, Singh, & Curtis, 2012). In this study, a seaweed of red algae species that known as *Kappaphycus alvarezii* or the synonym name is *Eucheuma cottonii* will be used as biotic elicitor. Biotic elicitor usually comes from polysaccharides which presence in seaweed as carrageenans that have the ability to protect the plants by inducing the systemic resistance and give beneficial of

biostimulant effect (Stadnik & Freitas, 2014). Various concentrations of *K. alvarezii* is used to find the most suitable concentration that enhances the greatest production of centellosides in *C. asiatica.*

After the plants have enough exposed to various concentration of elicitor, the secondary metabolites profiling will be compared between elicited plants with the control plant by High-Performance Liquid Chromatography (HPLC). HPLC is used because it is more rapid, sensitive and accurate method. Reverse-phase C18 of HPLC column can be used for the identification and qualification of *C. asiatica* secondary metabolites (Thongnopnua, 2008).

1.2 Problem Statement

The problem statements related to this study are the plant has been listed under highly threatened species. However, nowadays it has been overharvested because of its precious medicinal value. Next, a large quantity of the plants must be sacrificed in order to get a huge amount of secondary metabolites. This is because the desired secondary metabolite usually have a small amount in a natural plant and also presence only at the certain part of the plant. Conventionally, the whole plant is overharvested causes a threat towards their extinction. Lastly, the weather in the natural environment and other conditions such as

fertilizer, water presence, and the temperature are not in a standard condition which can affect the production of secondary metabolites in the plant.

1.3 Objective of study

This study aims to increase the amount of secondary metabolite in a *C. asiatica* by manipulating various concentrations of biotic elicitor in culture media. There is much evidence has been conducted so far that proof the biotic elicitor such as seaweed capable of increasing the production of secondary metabolite (Sharma, Chauhan, & Sood, 2015).. Therefore, the objectives of this study is to increase the amount of secondary metabolite in natural plant of *C. asiatica* by biotic elicitor (seaweed) and to compare the secondary metabolite profiles between elicited plants and the control plants using chromatography technique.

1.4 Hypothesis of study

In this research, there are several hypothesis that can be identified which there is certain concentration of the plant elicitor (seaweed) that will enhance the production of the secondary metabolite of the plant. Lastly, the amount of secondary metabolites of elicited plant can be higher than control plant if given in the right culture environment.

1.5 Significance of study

In this research, all the procedure will be done in the laboratory, thus, it will produce a uniform set of the plant because all the environmental standard condition such as light, temperature and nutrient will be control which give the same effect to the production of secondary metabolite in the plant. Besides, the desired secondary metabolites can be produced in a huge amount instead only small amounts of them that presence in a natural plant by manipulating the concentration of biotic elicitor. Since the secondary metabolite can be accumulated in higher amount in elicited plant, it can act as an alternative way to produce the desired compound without a need to sacrifice a large amount of its natural plant.

1.6 Limitations

There are several limitation that must be encountered in this study. Firstly, the first exposure to the new technique knowledge such as plant tissue culture technique and the lack of experience in handling tools causes the extra care must be taken by the researcher when handling the tools. Next, the sterilization and aseptic technique must be applied in each procedure which will make the work take a longer time to finish. Besides, the duration provided for students to complete this research are very limited. So, the research procedure must be done carefully in order to avoid errors. Other than that, the risk of contamination from bacteria or fungus in the media can contribute to delay in the project

because the contaminated media containing plant must be subculture to another media. Since the plant tissue culture plantlets are not grown enough to expected time, these research had been change by observe the elicited plants in natural condition. Lastly, HPLC gradient condition had been repeated several times in order to obtain better retention peak.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Biology and Characteristics of *Centella asiatica.*

Centella asiatica is a well-known and important medicinal herbs that being used all over the world since the prehistoric times. Usually, the active ingredients contain in the aerial and the whole plant parts (Singh & Singh, 2002). It has many synonyms name such as *Hydrocotyle asiatica, Hydrocotyle lunata, Trisanthus cochinchinensis, Centella coriacea, Centella cordifolia, Centella dusenii, Centella floridana, Centella repanda, Centella triflora* and lastly, *Centella uniflora* (HMPC, 2010). It belongs to the family of *Apiaceae* or *Umbelliflorae* which the taxonomy classification of *C. asiatica* can be seen in Table 2.1. In African only, there are 20 different types of *C. asiatica* species were found in there (Tiwari, Gehlot, & Gambhir, 2011).

Usually, different people in different place will have a different type of common or vernacular names for this plant which can be seen in Table 2.2. The table consist of the various name in the region of India and also in other countries (Singh, Gautama, Sharma,

& Batra, 2010). However, there is an earlier confusion of vernacular name occurred in India which the plant of Bacopa monnieri Wettst is being sold together with C. asiatica in the market that have been called as "Brahmi" but the case is resolved by concluded "Brahmi" is B. monnieri while "Mandookaparni" is C. asiatica (Singh *et al.*, 2010)

Table 2.1: Taxonomy classification of *C. asiatica*
(Singh *et al*., 2010)

Classification	**Name**
Kingdom	*Eukaryota*
Subkingdom	*Embryophyta*
Division	*Spermatophyta*
Subdivision	*Angiospermae*
Class	*Dicotyledoneae*
Subclass	*Rosidae*
Superorder	*Aralianae*
Order	*Araliales (Umbelliflorae)*
Family	*Apiaceae or Umbelliferae*
Subfamily	*Hydrocotyle*
Genus	*Centella*
Species	*Centella asiatica*

Table 2.2: Vernacular name of C. asiatica in India
(Singh *et al*., 2010)

Region/Language	**Vernacular Name**	**Region/Language**	**Vernacular Name**
Hindi	Bemgsag, Gotukola, Khulakhudi, Mandookaparni	**Sanskrit**	Bhekaparni, Bheki, Brahmamanduki, Divya, Mandukaprnika
Malayalam	Kodagam, Kondangal, Muttil, Muthal	**Kanarese**	Bramisoppu, Urage, Vondelaga
Telugu	Bekaparnamu, Bokkudu, Saraswati plant	**Gujrati**	Barmi, Moti Brahmi
Marathi	Karinga, Karivana	**Tamil**	Babassa, Vallarai
Tripura	Thankuni, Thunimankuni	**Bengal**	Tholkuri
Assam	Manimuni	**Deccan**	Vallarai
Bihar	Chokiora	**Meghalaya**	Bat-maina
Oriya	Thalkudi	**Sinhalese**	Hingotukola
Urdu	Brahmi	**English**	Marsh/Indian pennywort,
Vietnamese	Rau má	**French**	Hydrocotyle asiatique
Philippines	Yahong yahong	**Indonesian**	Pegagan, Daun Kaki Kuda
Korean	(byeongpul)	**Malay**	Pegaga
Hawaii	Pohe Kula	**Chinese**	(Bēng dà wǎn)
Fiji	Totodro	**Cook Island**	Kapukapu
Tahiti	Tohetupou	**Nepal**	Ghod tapre

2.1.1 Morphological Characteristics of *Centella asiatica*

Centella asiatica can be identified by their macroscopic and also microscopic characteristics which will differentiate them from other types of plants. They can be considered as perennial, creeper, prostrate and also stoloniferous herbs which can reach 15 cm to 6 inches height. According to European Pharmacopiae (2011), the characters that can be seen in *C. asiatica* are the leaf have a variety of size and the petiole has 5-15 times longer than lamina that have 10-40 mm long with 70 mm wide. Usually, this plant will grow wildly under different types of climatic conditions, but the most preferred to grow is in the shady, marshy and moist areas, or at the paddy fields and also at the river banks to form a dense green carpet. Seevaratnam *et al.*, (2012) states that the most fertile and suitable soil for *C. asiatica* regeneration is at sandy loam containing 60% of sand rather than grow in clayey soil. Thus, the climatic conditions, soil texture, and agronomic work have been established to highly influencing the bioactive constituent contents of the *C. asiatica* (Govarthanan, *et al.*, 2015).

2.1.1.1 Macroscopic Description

Macroscopic description consists the shape, length, colour, consistency of leaves and other characteristics of the plant which can be observed by the naked eyes or magnifying glass (Weber, 2015). Based on the European Pharmacopiae (2011), the leaves are green, alternate and grouped together with 1-3 leaves at each node by up 20 cm of a long petiole.

This plant also has 1.5-5 cm of wide orbicular-reniform or oblong-elliptic leaves shape with palmate nervation. The leaf size and margins may have a different range from smooth, crenate or slightly lobed with the rounded apex (Singh *et al.*, 2010; GlobinMed, 2015). They also have a few trichomes in young leaves on the lower surface while adult leaves are glabrous. The stem of this plant is striated and rooting at the nodes. The rootstock consist of rhizomes that grow vertically down and the stolon grow horizontally in order to interconnect one plant to another (GlobinMed, 2015).

The flower of this plant consists of white to purple or pink to reddish petals. The inflorescence of the flower has a single fascicled umbel consisting of 3 flowers rarely 2 or 4 with a small size of 2 mm (European Pharmacopiae, 2011). It has a pentamerous shape with hermaphrodite characteristics consisting 4-6 stamens with 2 styles and has an inferior ovary (GlobinMed, 2015). It will be flowering between the month of April and June throughout the growing seasons (Singh *et al.*, 2010). Fruit of *C. asiatica* has a colour of brownish-grey and the size is 2 inches long with laterally flattened, globular and oblong in shape. It also has 7-9 of prominent curved ridges with a strong thickened pericarp (Vohra *et al.*, 2011). The simple description and the parts of the plants such as leaves, flower, fruit, stolon and rootstock can be seen in the Figure 2.1 and Table 2.3.

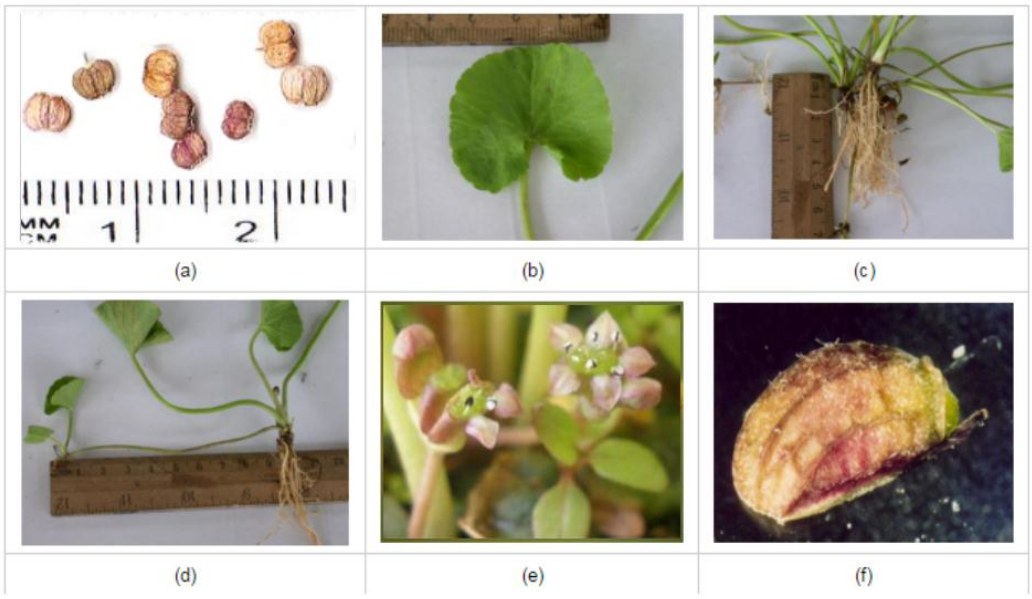


Figure 2.1 : Macroscopic parts of *Centella asiatica*
(a) Seeds; (b) Leaves; (c) Root & Rhizomes; (d) Stolon; (e) Flower; (f) Fruit
(GlobinMed, 2015)

Table 2.3: Simplified macroscopic description of *C. asiatica* parts.
(European Pharmacopiae, 2011)

Part	**Description**
Leaves	- Alternate & group at nodes. - Reniform (kidney shape)/orbicular/oblong elliptic with palmate nervation (7 veins) - Crenate margin - Young → few trichomas on lower surface - Adult → glabrous (free from trichomas)
Flower	- Inflorescence → single umbel (3 flower) - Very small (2mm) - Pentamerous - Inferior ovary - Reddish petal & hermaphrodite
Fruit	- Brownish-grey - Orbicular cremocarp (5 mm long) - Flattened laterally - 7-9 prominent curved ridges
Stolon	- Grow horizontally - Interconnecting one plant to another
Rootstock	- Rhizome grow vertically down

2.1.1.2 Microscopic Description

The microscopic characteristics can be obtained by observing under the microscope in 400X magnification. The important histological characters to be observe are the presence and the shapes of fibres, calcium oxalate crystal, hairs or trichomes and also special secretory organs like glands, laticifers and resin ducts (Weber, 2015).

In *C. asiatica*, it has polygonal cells of leaf epidermis with paracytic stomata that have the size of 28 μm on both surfaces of the leaf but more numbers of stomata at the lower epidermis. Paracytic stoma is a parallel cell type which the stoma has on each side of the long axis of the pore and guard cells (European Pharmacopiae, 2011). The epidermis of petiole has elongated parenchymal cells containing the single large prisms of calcium oxalate and macles which are presence up to 40 μm. While the stem consists of bundles of narrow septate fibres and the fruits have parenchyma cells containing starch granules (Vohra *et al.*, 2011). The simplified microscopic description of *C. asiatica* parts can be referred to Table 2.4 while the picture of the plant cells under the microscope with magnification 400X can be seen in Figure 2.2.

Table 2.4: Simplified microscopic decription of *C. asiatica* parts (European Pharmacopiae, 2011)

Parts	Description
Leaf epidermis	- Polygonal cells - Paracytic stomata
Petiole epidermis	- Elongated cell - Calcium oxalate prisms & macles
Stem	- Bundles of narrow septate fibres
Fruits	- Parenchyma cells containing starch granules

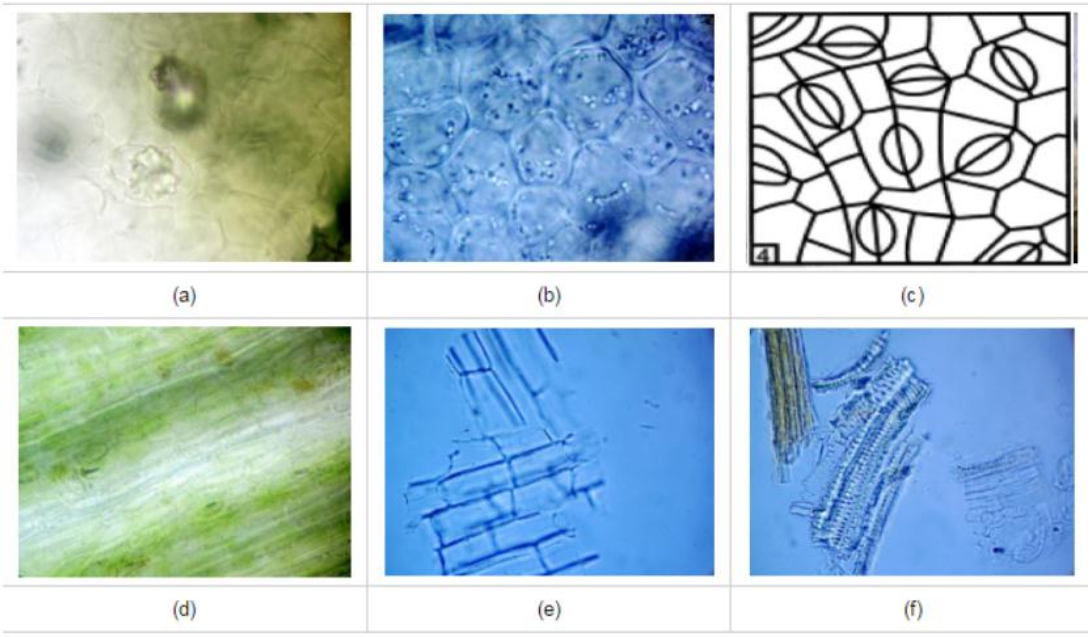


Figure 2.2: Microscopic characters of *Centella asiatica* in magnification (400X)
(a) calcium oxalate macles in Parenchyma cells; (b) Polygonal cells in fragment of leaf epidermis; (c) Drawing of paracytic stomata; (d) Paracytic stomata on the leaf epidermis; (e) Petiole containing elongated parenchyma cells; (f) Bordered pitted vessels fragment. (GlobinMed, 2015)

2.1.2 Organoleptic properties

Organoleptic is an acting or involving the organs sense while the organoleptic properties is a property of the substance that can be detected by organ sense like the taste, sight, smell and touch. In *C. asiatica*, it has a greyish green colour and has slightly bitter and sweet in taste (Vohra *et al.*, 2011). However, Islam, *et al.*, (2003) states that *C. asiatica* has a salty taste and umami taste which detect by the sensor using the array of electrodes that composed of different lipid-polymer membranes. Other than that, this plant has a fragrance smell because of the essential oil or resin that presence in all of the plant organs (Das, 2011).

2.2 Metabolites in plants

Plants are just like animals which are composed of many chemical compounds known as constituents or metabolites. Small molecules are usually restricted for the metabolites term. Metabolites are the intermediates and the products of metabolism reactions that occur naturally in the cells (Harris, 2008). They have many function including for signalling, fuel, stimulatory and inhibitory effects on the enzyme, interaction with other organism and also for defence.

These metabolites consist of inert constituents and also active constituents. They are important in plants because it is essential for proper growth and also for the survival of plants. The inert constituent of plants that do not possess any therapeutic value are comprised of water, inorganic compounds, cell wall constituents and primary metabolites. While, the active principle or known as secondary metabolites is responsible for the therapeutics effects of the plant material and ecological function (Kar, 2007).

2.2.1 Primary Metabolites

Primary metabolites are one of the component in inert constituents which also known as the central metabolite. It is directly involved in development, normal growth and reproduction that make it as essential metabolites in plants. However, these metabolites

do not directly affect the therapeutic mechanism but it will influence the bioavailability and provide the optimum effect of pharmacokinetics and pharmacodynamics of active metabolites. It also will give stability and minimise the possible side effect of the plants (Stough & Scholey, 2013).

Primary metabolites consist of lipids, carbohydrates, proteins and chlorophyll which can be seen in Figure 2.3 shows that they are products of primary metabolism. Then, primary metabolism will undergo secondary metabolism to produce secondary metabolites that consist of alkaloids, terpenoids, cyanogenic glycosides and phenolic.

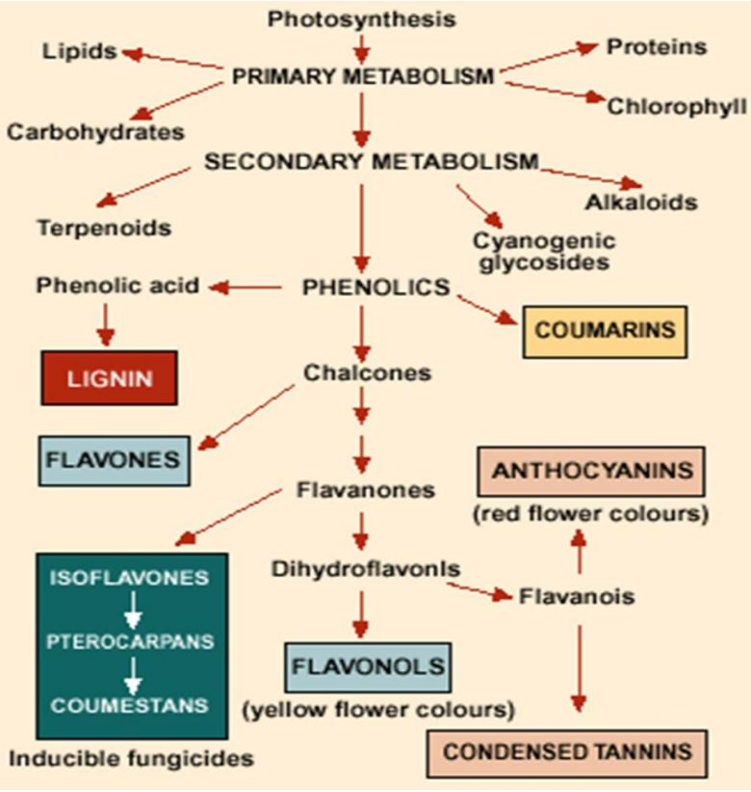


Figure 2.3: Inter-relationship between primary and secondary metabolism in plants

The distinction of primary and secondary metabolites are not easily be made because they share many of the same intermediates at the biosynthetic level and are derived from the same metabolic pathways (DifferenceBetween, 2013). However, some of the distinction between primary metabolites and secondary metabolites can be seen at Table 2.5.

Table 2.5: Difference between primary and secondary metabolites

Primary Metabolites	Secondary metabolites
- Identical in the most of plants.	- Have numerous and widespread in one plant.
- Widely distributed in nature.	- Idiosyncratic which only found in a few species or genera of plants.
- They are accumulated by plant cells in large quantities	- They are accumulated by plant cells in very small quantities
- Trophophase is the growth phase where the primary metabolites are produced.	- Idiophase is the phase during the secondary metabolites are produced.
- Examples are proteins, carbohydrates, and lipids.	- Examples are alkaloids, phenolic, triterpenes and essential oils.

2.2.2 Secondary Metabolites

Secondary metabolites are non-nutritive plant chemical that contain protective and disease preventing compounds (Qadry J. S., 2010). They are also called as phytochemicals, plant constituents, active principles or natural products that are responsible for medicinal properties of plants (Kabera, Semana, Mussa, & He, 2014). They are derived from compounds generated by the primary metabolism in which they are diverted from the metabolic cycles and follow specific biogenetic pathways with the presence of the specialised enzyme (Weber, 2015). Therefore, secondary metabolites are reflected as the end products of primary metabolites but it does not continuously produce these metabolites (DifferenceBetween, 2013).

There are many scientific sources state that their role is not crucial for plant living cell in normal growth, development and reproduction, but they act as a defence purpose in order to protect a plant from any possible harm in the ecological environment and also interspecies protection (Stamp, 2003). These characteristics give a natural defence mechanism to the host plants by the presence of astringent in shrubs, a poisonous alkaloid in plants and pungent volatile oil in trees. They are also crucial as a tool for communication with another organism like the neighbouring plants, pollinators and herbivores avoidance (Kabera *et al.,* 2014). Usually, the production is increased when the plants are attacked by herbivores and also it will be released into the air which attract the predators to kill the herbivores (Schultz, 2015).

Secondary metabolism is also important to agriculture that act as agents of plant-plant competition and plant-microbe symbioses. The compounds produced are very defensive which increase the reproductive capability of plants by repelling the fungi, bacteria, herbivores and also humans by make them undesirable to consume (Vince, Zoltán, Egyetem, Egyetem, & Egyetem, 2011). The production of secondary metabolites which gives a negative impact to other organism leads to the hypothesis that they have evolved because of their protective value. However, they also give benefit to the humans which it usually used as industrial materials, flavours, colouring, poisons and medicinal drugs. This active principle can be classified into three main groups that is based on their chemical structure, composition, solubility in various solvents or the synthesized pathway

in the plants. The three main groups are alkaloids, phenolics and terpenoids (Kabera *et al.*, 2014).

2.2.2.1 Alkaloids

Alkaloids are nitrogenous secondary metabolites which have properties of nitrogen basicity, but some neutral and weakly acid properties also included in alkaloids (Kabera *et al.*, 2014). This group consist of carbon, hydrogen and nitrogen and also may contain oxygen or sulfur (Nicolaou, Chen, & Elias, 2011). They are biosynthesized from amino acid like tyrosine which will produce morphine by the phenol coupling reaction involve a benzylisoquinoline alkaloid. However, compounds such as amino acids, proteins, nucleic acid, amines and peptides are not alkaloids.

There are several effect to the human body when consuming it in overdose condition which it can block the ion channels, inhibit the enzymes, interfere with neurotransmission, produce hallucinations, loss of coordination, convulsions, vomiting, and lastly is death (Schultz, 2015). Alkaloids also famous with their toxicity and medicinal properties which it act as a defense molecules against herbivores especially the mammals (Labster, 2014). Example of well-known alkaloid is morphine that come from *Papaver somniferum* and

poppy derivatives which act on central nervous system, acute pulmonary edema and reduce the shortness of breath (Takita, Herlenius, Yamamoto, & Lindahl, 2000)

2.2.2.2 Phenols

Phenolic compounds are the largest group of the secondary metabolite that synthesize by fruits, vegetables, teas and other plants. They can be characterized by the presence of hydroxylated aromatic rings in structure and is polymerized into larger molecules such as lignin and condensed tannins (Kabera *et al.,* 2014). Actually, a compound is said to be a phenolics when its physicochemical and pharmacological properties are related to the presence of phenol. Other conditions are from the biogenesis from phenolics which have two pathways of shikimate and polyacetate pathways (Weber, 2015).

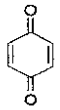
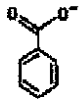
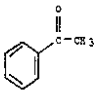
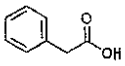
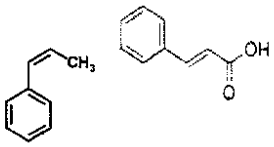
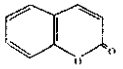
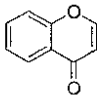
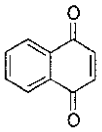
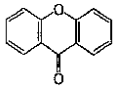
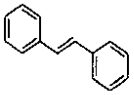
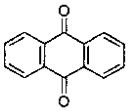
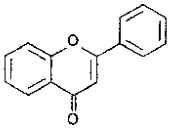
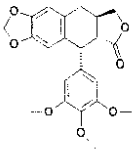
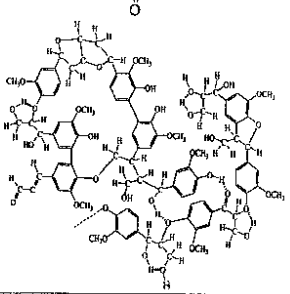
The classification of this group depends on a number of hydroxyl groups, chemical composition and the substituent in carbon skeleton. Phenolic can be divided into four main groups which are phenolic with one aromatic ring, two aromatic ring, quinones and polymers (Kabera *et al.,* 2014). Theis classification can be seen in Table 2.6 with various classes of phenols. From the tables, the example of phenolic with one aromatic ring is simple phenols while two aromatic rings are xanthones. Other examples for quinones is anthraquinones and for the polymers are tannin and lignin (Giada, 2013).

They have certain benefits in terms of antioxidant, anti-inflammatory, anti-carcinogenic, simple phenol act as bactericidal and antiseptic and also used as a standard for other antimicrobial agents (Gao & Hu, 2010). One of the largest classes of phenolic compounds is the flavonoids. They presence on the yellow pigment of citrus fruits and also abundant in berries, red wine and dark chocolate. The process of phenolic formed flavonoids with the subgroup of flavonols, flavones and anthocyanins is shown in the Figure 2.2 page 16. Flavonoids have powerful antioxidant properties which give important roles in anti-inflammatory, anti-allergic and anti-cancer capabilities (Labster, 2014). However, some of the phenolics will interfere with digestion, slow the growth, block enzyme activity and cell division and also have an awful taste (Schultz, 2015).

2.2.2.3 Terpenoids

Terpenoids is a polymeric isoprene derivative which it is synthesized from the acetate via mevalonic acid pathways. They are also called as isoprenoid and contain functional groups that are attached to a highly nonpolar terpene backbone which makes them insoluble in water. Terpenoids can be classified into five groups which are monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes. Monoterpenes consist of C_{10} that derived from two isoprene units while sesquiterpenes consist of C_{15} which derived from three isoprenyl units. Diterpenes, triterpenes and tetraterpenes have a C_{20}, C_{30}, and C_{40}

Table 2.6: The main classes of phenolic compounds regarding their carbon chains.
(Kabera et al., 2014)

Class	Basic skeleton	Basic structure
Simple phenols	C_6	
Benzoquinones	C_6	
Phenolic acids	C_6-C_1	
Acetophenones	C_6-C_2	
Phenylacetic acids	C_6-C_2	
Hydroxycinnamic acids	C_6-C_3	
Phenylpropenes	C_6-C_3	
Coumarins, isocoumarins	C_6-C_3	
Chromones	C_6-C_3	
Naphthoquinones	C_6-C_4	
Xanthones	C_6-C_1-C_6	
Stilbenes	C_6-C_2-C_6	
Anthraquinones	C_6-C_2-C_6	
Flavonoids	C_6-C_3-C_6	
Lignans and neolignans	$(C_6$-$C_3)_2$	
Lignins	$(C_6$-$C_3)_n$	

.which derived from four, six and eight isoprenyl units respectively (Shah, 2015). The example with some effect and uses of classified terpenoids are shown in Table 2.7.

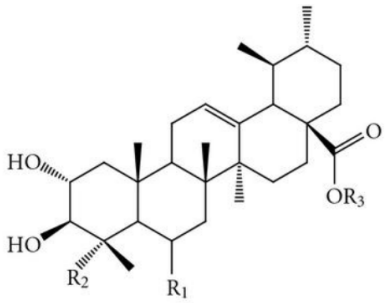
Most of them are biologically active and protect the plant from parasites and predators. According to Beaulieu & Baldwin, (2002) the Lamiaceae family has the most abundant diterpenes and has antimicrobial and antiviral properties. They also have volatile properties which under groups of monoterpenes and sesquiterpenes that have essential oil. It is usually used for plant interaction like inhibit the germination of seeds from other species which can be seen in the pine tree that inhibit another species to germinate around them and as results produce pine tree forest (Shah, 2015). They are also used to signal between symbiotic organisms and attraction of pollinators (Kabera *et al.*, 2014). Another function of this volatile properties is used as the method of pest control and the production of flavours and fragrances (Maffei, 2010) and also as the spices in food (Gershenzon & Dudareva, 2007). This is because they have a strong scent and are specially used for aromatic purposes (Labster, 2014).

Table 2.7: Class of Terpenoids with the example of compounds and effect.
(Shah, 2015)

Class	Example Compounds	Example Sources	Some Effects and Uses
TERPENOIDS			
Monoterpenes	Menthol linalool	Mint	Interfer with neurotransmission, block ion transport
Sesquiterpenes	Parthenolid	Parthenium (Asteraceae)	Contact dermatitis
Diterpenes	Gossypol	Cotton	Block phosphorylation
Triterpenes	Digitogenin	Digitalis (foxglove)	Stimulate heart muscle
Tetraterpenoids	carotene	Many plants	Antioxidant & orange colour
Terpene polymers	Rubber	Dandelion	Gum up insect, airplane tires
Sterols	Spinasterol	Spinach	Interfere with animal hormone action

2.2.2.3.1 Terpenoids in *Centella asiatica*

The secondary metabolite of *C. asiatica* is in a group of triterpenes which consist of 6 isoprene units with C_{30}. Triterpenes have two main types which are tetracyclic and pentacyclic but in *C. asiatica* is classified under pentacyclic rings with the molecular formula of $C_{30}H_{48}$ (James & Dubery, 2009). This plants accumulate a large amount of asiaticoside, madecassoside, brahmoside, brahminoside, thankuniside, sceffoleoside, centellose, asiatic acid, brahmic acid, centellic acid and madecassic acid. Among them, the four major compounds of pentacyclic triterpene or called as centellosides are asiaticoside, madecassoside, asiatic acids and madecassic acids as shown in Figure 2.4 (Orhan, 2012)**.** Since the Centellosides have many auxochrome substituents like hydroxyl group (-OH) at the position of carbon 2, 3, 23 or 6 makes them as UV active molecule.



	R1	R2	R3
Asiatic acid	H	CH2OH	H
Madecassic acid	OH	CH2OH	H
Asiaticoside	H	CH2OH	Glu-Glu-Rham
Madecassoside	OH	CH2OH	Glu-Glu-Rham

Figure 2.4: The major of pentacyclic triterpene derivatives found in *C. asiatica*. (Orhan, 2012)

They have two types of structure which are sapogenin which known as triterpenes without glycoside or aglycoside and also saponins that known as triterpenes with glycoside. In

other words, saponin is the sapogenin that combine with glucose. Saponins can be characterised as a surfactant which when it dissolve in water, it will form foamy solutions that can be seen in asiaticoside and madecassoside. While, sapogenin have two types of a skeleton which are ursane or known as α-amyrin and oleane or known as β-amyrin. The difference between this structures of the compound is the position of Methyl-29 whether it attached at carbon 19 or at carbon 20 which can be seen in Figure 2.5. This acidic sapogenin group of *C. asiatica* can be seen in asiatic acid and madecassic acid. In *C. asiatica* the structure of the centelloside is under the ursane group which can differentiate between them by the presence of glycoside at the position of carbon 23 and also the presence of OH group at the position of carbon 6.

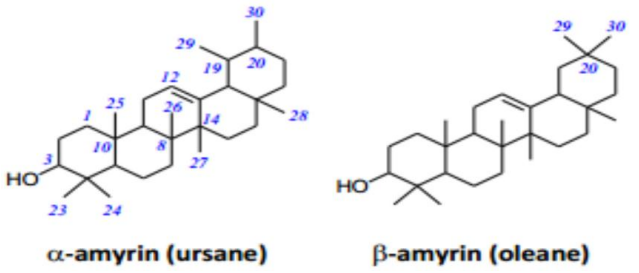


Figure 2.5: Difference position of Methyl-29 in ursane and oleane (Orhan, 2012)

Centellosides can be synthesized via the isoprenoid pathway in order to produce a hydrophobic sapogenin triterpenoid structure which are asiatic acid. They also can form a hydrophilic saponin triterpenoid structure which are asiaticoside and madecassoside by the addition of two molecules of glucose and one molecule of rhamnose (Kumar, *et al.*, 2013). The biosynthesis of triterpenoid production in *C. asiatica* can be seen in Figure 2.6. The first step of biosynthesis is the isomerization of isopentenyl diphosphate (IPP)

with dimethylallyl diphosphate (DPP) in the presence of Farensyl diphosphate synthase enzyme (FPS) to produce farnesyl diphosphate (FPP). Then, FPP is converted to squalene by enzyme squalene synthase (SQS). Next, the presence of squalene epoxidase (SQE) oxidises the squalene into to 2,3-oxidosqualene. This oxidosqualene formed a cyclic Dammarenyl cation or known as cationic intermediates that produce one or more cyclic triterpene skeletons by the presence of oxidosqualene cyclase (OCS). This step is the precursors step in the production of *C. asiatica* pentacyclic triterpenoid sapogenins. The cyclic triterpene skeleton is Oleanyl cations and Lupenyl cation but focusing on the Oleanyl cations which have been undergone ring expansion, rearrangement and also deprotonation will produce α-amyrin and β-amyrin by the presence of α/β-amyrin synthases (α/β-AS). α-amyrin and β-amyrin are produced to act as the precursor of sapogenin which the α-amyrin or ursane have a product of asiatic acid.

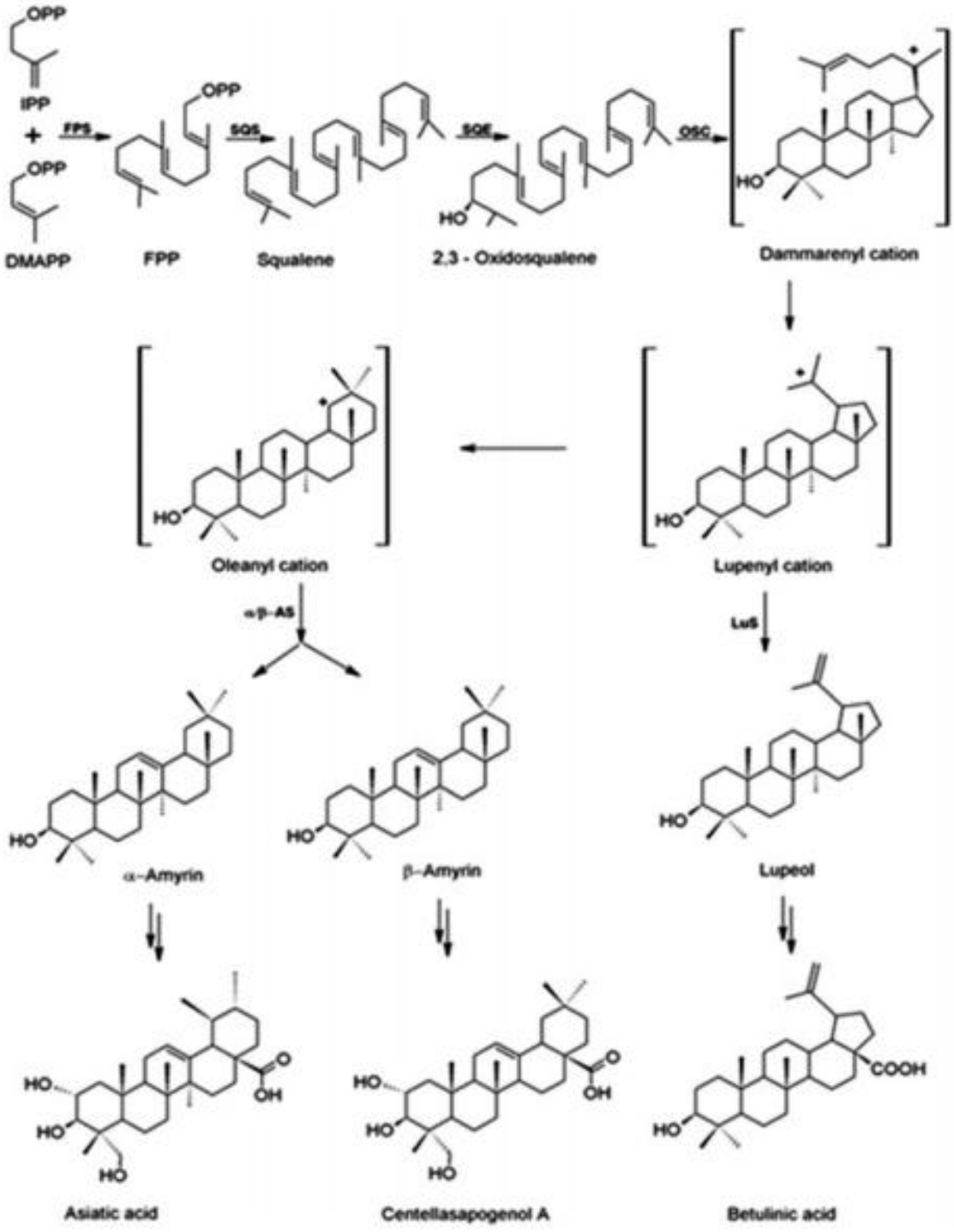


Figure 2.6: Simplified process of triterpenoid biosynthesis in *C. asiatica*
(Kumar, *et al.*, 2013)

2.2.2.3.1.1 Asiatic Acid

Asiatic acid or known as Dammarolic acid is one of the major secondary metabolites in *C. asiatica* that has a pentacyclic triterpenoid sapogenin under an ursane group. It has a substitute of a carboxy group (-COOH) at the position of carbon 28 and hydroxy groups (-OH) at positions of carbon 2, 3 and 23 (ChEBI, 2015). In this research, asiatic acid is the main focus of secondary metabolites in the micropropagation of *C. asiatica*. The molecular formula and weight are C_{30} H_{48} O_5 and 488.69912 g/mol respectively (PubChem, 2015). While, this compound is soluble in Dimethyl sulfoxide (DMSO) and sparingly soluble in water at 25°C which indicates this compound are non-polar molecule (Selleckchem.com, 2013). It must be protected from light and moisture which can be stored at +4°C in the short term while for long term at least two years must be in -20°C (Caymanchem, 2015). *C. asiatica* that originate from Madagascar has been found to contain the highest level of asiatic acid (TreatingGlioblastoma.com, 2008). The structure of asiatic acid can be seen in Figure 2.7 below which have the same structure as asiaticoside but lack of sugar molecules. Asiaticoside is the functional parents of asiatic acid.

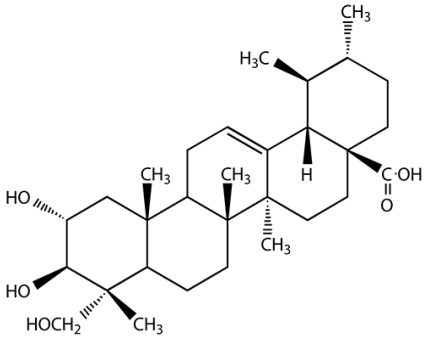


Figure 2.7: Asiatic acid
(ChEBI, 2015)

This compound has many beneficial function to human which act as anti-inflammatory actions and ulcer healing by inhibiting the synthesis of nitrogen oxide (NO) (Guo, Cheng, & Koo, 2004) and also stimulates the wound healing by increase the production of collagen in the recovering patient who recently undergo brain tumour surgery. This condition shows that Asiatic acid crosses the blood brain barrier with low risk of adverse effect by providing the protection against neurodegeneration, lowering the blood pressure and also reduce leg oedema (TreatingGlioblastoma.com, 2008). It also induce the apoptosis and arrest the cell cycle in breast cancer (Hsu, Kuo, Lin, & Lin, 2005) which prove it has a cytotoxic property to many cancer cell lines and also it would prevent the process of angiogenesis of glioblastoma in cells and tumours (Kavitha, Agarwal, & Agarwal, 2011).

2.2.2.3.2 Madecassic Acid

Madecassic acid is an ursane triterpenoid group which have the substitute of carboxy group (-COOH) at position carbon 28 and hydroxy groups (-OH) at position carbon 2, 3, 6 and 23. The difference of molecular structure of Asiatic acid is just at position carbon 6 which in Madecassic acid has –OH group attached to it. The structural of Madecassic acid can be seen in Figure 2.8 below. This molecule also called as Brahmic acid and it have the molecular weight of 504.69852 g/mol with the molecular formula of $C_{30}H_{48}O_6$ (PubChem, 2015). It also soluble in DMSO and also in methanol which indicates this

molecule is non-polar but is more polar than Asiatic acid. This compound must be stored at the room temperature for at least 12 months and it is active *in vivo* (Abcam, 2015). This molecule and also Asiatic acid can act as Bronsted acid which it can donate a hydrogen to an acceptor molecule or Bronsted base. The parent derivative of Medacassic acid is Medacassoside (ChEBI, 2015).

This molecule has an ability to stimulate the collagen synthesis of types I and III (ChemicalBook, 2010) in order to restructure the extracellular matrix of the dermis. Activity of keratinocytes and enzyme matrix metallo proteins (MMP) is reduced by this molecule which can lower the breakdown of skin's matrix and framework (Centesia, 2013). It also inhibits the Nitric Oxide Synthase (iNOS), Cyclooxygenase-2 (COX-2), Tumor Necrosis Factor-α (TNF-α), Interleukin-1 (IL-1), and Interleukin-6 (IL-6) that cause inflammation. It function as an antioxidant which opposed the oxidation process and also inhibit the reaction of peroxide and dioxygen (ChEBI, 2015). This molecule usually used in cosmetic which suitable for the skin that damage by harsh environmental condition, very sensitive skin, premature aged skin caused by sun exposure or dry room, and also for dry skin (Centesia, 2013).

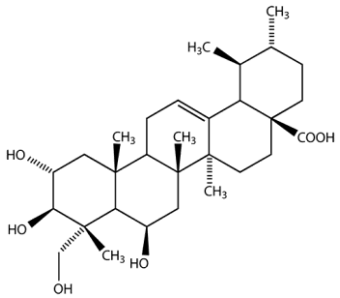


Figure 2.8: Madecassic acid
(ChEBI, 2015)

2.2.2.3.3 Asiaticoside

Asiaticoside is the main ursane triterpenoid saponin in *C. asiatica which have a similar name as* Madecassol or Emdecassol. It has a molecular formula and weight of $C_{48}H_{78}O_{19}$ and 959.12152 g/mol respectively (PubChem, 2015). Usually, it has a same skeleton structure of Asiatic acid but this molecule have an addition of sugar moieties of glucose and rhamnose that attached to the oxygen at carbon 28 which can be seen in Figure 2.9. It must be stored at 4°C with 2 years of stability (Caymanchem, 2015). This molecule is active *in vivo* and *in vitro* (Abcam, 2015). It is very soluble in propylene glycol, and ethoxydiglycol with water in the ratio of 1:1. While, it is soluble in ethanol, glycerin, butylene glycol and also polyethylene glycol (PEG) 400 and 600 (Indena, 2013).

It acts as a parent to its sapogenin molecule which is asiatic acid by undergoing an acid hydrolysis reaction. This reaction causes a deglycosylation process which the sugar molecule from the saponin leaves the skeleton structure and produce aglycone form which can be seen in Figure 2.10 below. The acid hydrolysis in anhydrous methanol is the preferable method to produce sapogenin because it has the highest percentage to produce aglycones rather than alkaline hydrolysis with 5% sodium hydroxide (NaOH) in anhydrous methanol which produce a partial hydrolysis that is prosapogenin (Hostettmann & Marston, 2005)

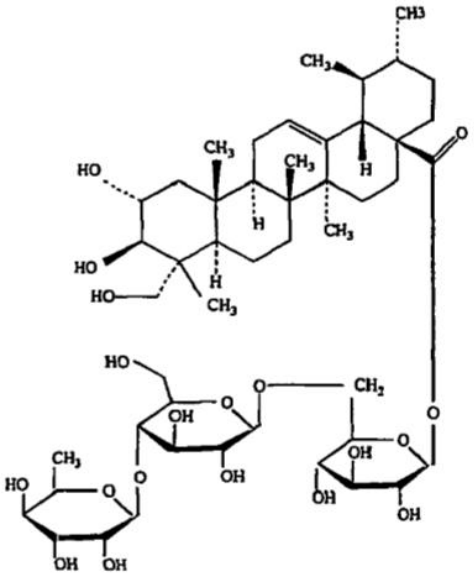


Figure 2.9: Asiaticoside
(ChEBI, 2015)

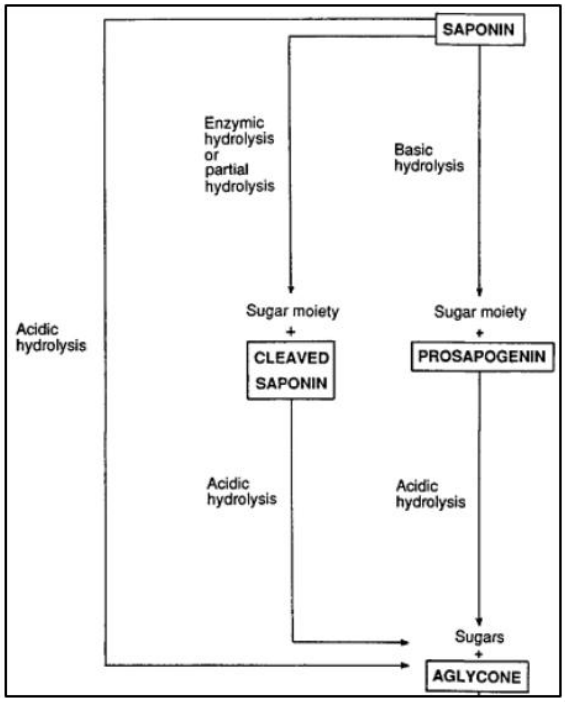


Figure 2.10: Structure elucidation of saponins
(Hostettmann & Marston, 2005)

The function of Asiaticoside is important in wound healing because it stimulate the synthesis of glycosaminoglycan and collagen types I and III remodelling matrix, increase the hydroxyproline content by improve its tensile strength and also promote epithelialization process (Caymanchem, 2015). This molecule also found to has an activity against herpes simplex virus type 1 and 2 and also mycobacterium tuberculosis (Centesia, 2013). It also have an anti-wrinkle properties by reinforcing the biomechanic properties of mature skin especially on the lips which can be used as a lip balm (Indena, 2013). It also functions to regulate epidermis homeostasis and also balance the renewal and differentiation of keratinocytes (Centesia, 2013).

2.2.2.3.4 Madecassoside

Madecassoside is an ursane triterpene saponin of *C. asiatica* and acts as the parent of Madecassic acid molecule which undergo acid hydrolysis to form its sapogenin. It has a molecular formula of $C_{48}H_{78}O_{20}$ and the molecular weight is 975.12092 g/mol (PubChem, 2015) which can be seen in Figure 2.11 below. It is soluble in water and should be stored at 2-8°C (Sigma-Aldrich, 2015).

The function of this molecule act as anti-inflammatory to the chronic skin inflammation which help to clear chronic skin lesion by regulates the IL-1, IL-8 and skin anti-leuco protease (SKALP). It also will disturb the proliferation of keratinocytes which can reduce the development of keloid scar on the skin and protect from further inflammation. It has a potential to increase the production of collagen III and also have a stronger strength of deposited collagen. The specialized function is it can be used for skin treatment like psoriasis (Centesia, 2013)

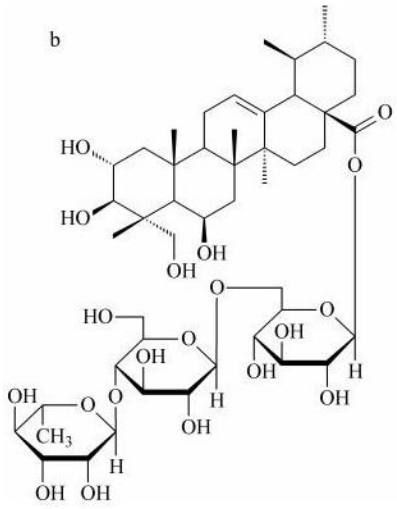


Figure 2.11: Madecassoside

2.3 The Uses of *Centella asiatica*

C. asiatica is already known for its precious nutritional and medicinal values in all over the world. *C. asiatica* are seemed to be important in Asian countries like Bangladesh, Malaysia, Indonesia and Sri Lanka since they are using this medicinal herb in hundreds of years ago. According to Vohra *et al,* (2011), Monograph of this plant has been listed in various pharmacopeias such as Indian Herbal Pharmacopeia, Pharmacopeia of the People's Republic of China German, European Pharmacopeia and also in Homeopathic Pharmacopeia (GHP) which they are listed as a drug (Singh *et al.,* 2011).

2.3.1 Traditional Uses of *Centella asiatica*

In Malaysia, Malay and Javanese people eat the *C. asiatica* as a green leafy vegetable in the form of salad or known as "ulam" which rich in micronutrients such as vitamins and mineral elements. Usually, ulam are eaten together with the main meal and also can act as an appetizer. Besides being eaten raw, this plant also can be cooked as soup or main vegetable with a presence of mild bitterness due to Vallarin that can be seen especially in China at Yunnan and Guangxi. However, in Southeast of Asia the bitter taste is mask by the cook and serve the plant together with coconut milk or shredded coconut and also with sweet potatoes (Centesia, 2013). For example, people in Sri Lanka used the *C. asiatica* leaves as their traditional curry called "mallung" and also as the porridge known as "kola kenda".

People also used this plant as beverages, tonic and teas. This can be seen in several countries like Malaysia, India and Thailand. In Malaysia, it is used as the health tonic, cordial drinks and also ready to drink juice. However, Kadazandusun communities used it as herbal tea which made by pouring boil water in a cup containing *C. asiatica* materials either using dried or fresh materials and then let it brew for a few minutes before drinking. This herbal tea also can be prepared by using a mixture of many different herbal plants. In India, it act as Indian summer drinks or known as "thandaayyee" which very important for brain tonic (Indianmirror, 2015). In Thailand, the popular used of C. asiatica is by freshly prepared juice which available in the restaurants and also sold by the roadside. Thai and Chinese people, used it as a cooling drink by reducing the "inner heat" and also to assist in healing and curing of aphthous ulcers (Centesia, 2013).

2.3.2 Pharmacological Uses of *Centella asiatica*

C. asiatica consists of many phytoconstituents present that exert their own pharmacological properties which contribute in their medicinal values. The major active principle that give pharmacological effect is from four molecules of triterpenoids saponins and sapogenin which are asiaticoside, madecassoside, asiatic acid and madecassic acid. Other chemical constituents that presence in this plant are volatile oil, fatty acids, alkaloids, glycosides, flavanoids, amino acid, aspartic acid, glycine, glutamic acid, α-allanine and phenylallanine.

All the parts of the plant mostly have medicinal values, but the leaves are useful to treat abdominal disorder due to dysentery (Kasture *et al.*, 2014). This plants also have a wide range of applications in memory enhancement, wound healing, immunomodulatory antidepressant, neuroprotective, autoimmune, venous insufficiency, antidiabetic and anticancer (Chaturvedi & Joshi, 2013) which can be seen in Table 2.8. It also may treat certain diseases like dermatosis, elephanthiasis, psoriasis, diabetes, syphilis, cough, epilepsy, anaemia and also dyspnoea.

Table 2.8: Pharmacological application in *C. asiatica*

Applications/Citation	**Description**
Memory enhancement (Chaturvedi & Joshi, 2013)	- Effect in memory enhancing cause by Brahmic acid, brahminoside and brahmoside that present in *C. asiatica* extract - Aqueous extract of *C. asiatica* leaves are revitalized the nervous system and brain by increasing the level of serotonin, norepinephrine and dopamine in the brain that give significant effect on memory and learning.
Wound healing (Chaturvedi & Joshi, 2013)	- Madecassol extract contains asiatic acid, madecassic acid and asiaticoside responsible to facilitate the wound healing. - Asiaticoside induces antioxidant activity at the initial stage of wound healing. -This compound cause increasing the effect of cellular proliferation, collagen synthesis, angiogenesis and epithealization at the site of the wound.
Immunomodulatory (Das, 2011)	- The methanol extract of *C. asiatica* can increase the lymphocyte cell counts which act as the first line of defense by invading the bacteria.
Antidepressant (Singh *et al.*, 2010)	- Triterpenoid saponins cause the corticosterone level in serum to reduced.
Neuroprotective (Chaturvedi & Joshi, 2013)	- Protect the neurons against oxidative damage by destroying the excess of free radicals - Potentiate the cellular oxidative defense mechanism in order to keep the oxidative stress state in balance.
Autoimmune (Chaturvedi & Joshi, 2013)	- Madecassol gives an efficacious effect in the treatment of advanced focal scleroderma and also to the chronic or subchronic systemic scleroderma.
Venous insufficiency (Chaturvedi & Joshi, 2013)	- Triterpenoid saponins improved the alterations wall in chronic venous hypertension which will strengthen the weakened veins and protect the venous endothelium. - Stabilized the connective tissue growth by stimulating the production of chondriotin sulfate and hylauronidase which gives a balancing effect on connective tissue.
Antidiabetic (Dave & Katyare, 2002)	- Methanolic and ethanolic extracts can reduce the blood glucose levels to normal. - Brahmoside and brahminoside give sedative and hypoglycemic effect. - Polyphenolic polymers act as antioxidants and also potentiate insulin effect.
Anticancer (Chaturvedi & Joshi, 2013)	- Methanol extract reduced the development solid tumors. - Inhibit the proliferation of ascites tumors which involve the inhibition of DNA synthesis.

2.4 Plant Tissue Culture Technique

Natural products for health care have increasing demands which approximately 80% of the population still use the traditional medicine that derived from the plants. This higher demands also include the *C. asiatica* herbs which it has many medicinal values and have lower side effects compared to the synthetic drugs. The precious medicinal value cause the overexploitation of *C. asiatica* thus it has been endangered or threatened with extinction. Thus, the most successful alternative ways to conserve and enhancement the valuable medicinal plants is by plant tissue culture technology. Plant tissue culture can be defined as the technique of growing the plant cells, tissue and organs in an artificial nutrient medium by aseptic condition (Qadry J. S., 2010).

There are many different types of plant tissue cultures include embryo culture, organ culture, callus culture and cell culture (Jamiepighin, 2003). Organ culture or organogenesis is used in this research which refers to the *in vitro* culture and the maintenance of some or whole part of plant organ such as roots, shoots and leaves that allow differentiation and preservation of structure and function (Qadry, 2010). They also might be taken directly from the meristem or callus which known as undifferentiated cell masses. This process can be seen example in Figure 2.12, which the meristem tip is excised from a plant and undergo organogenesis in order to generate a whole new plant. The advantage of plant tissue culture technique are the production is in rapid biomass, the environmental and nutritional condition is controlled at the optimum growth and also, the

media used can be easily manipulated by adding of elicitors and hormones which can produce the more desirable quantity of secondary metabolite.

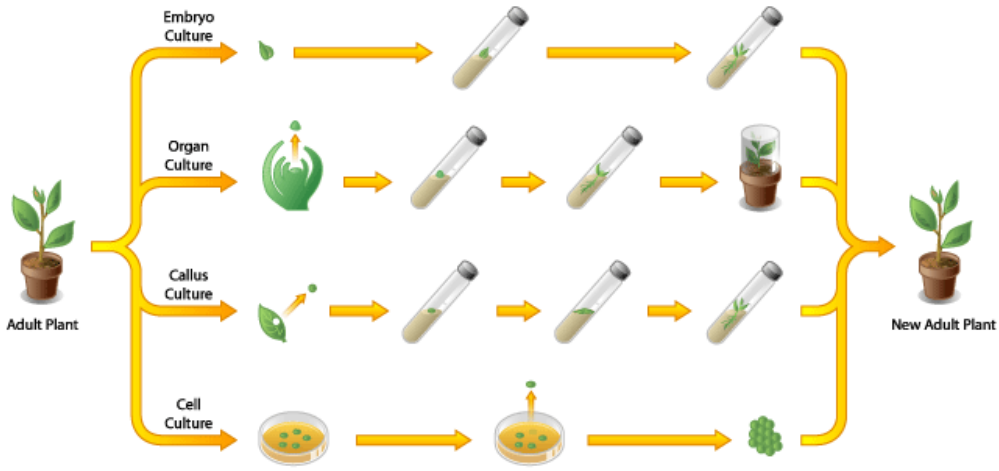


Figure 2.12: Different methods of cell culture.
Embryo culture: embryo cells grown on agar media and develop into plants.; Organ culture: specialized tissues will be regenerate into the whole plant.; Callus culture: specific tissues are excised from any part of the plant and form a undifferentiate callus to produce plants.; Plant cell culture: dissociation of cells into a single cell suspension which can generate new plants (Jamiepighin, 2003).

2.4.1 Micropropagation Technique

Micropropagation or known as *in vitro* rapid clonal propagation is a plant tissue culture technique which the plants are rapidly reproduced to thousands of copies by asexually manners. Usually, the explant must be select from a healthy adult plant which can be anything that can reproduce like a tissue or organ part or from a piece of differentiated tissue that has been cut out (Jamiepighin, 2003). The common example of explant material is apical meristem that consist of meristem tissue containing undifferentiated cells that usually presence in growing zones. The organ that can be produced by these meristematic cells are the leaves, flowers and also the growth of the root.

The process of a single cell or an explant to produce a full genome by cell division is called as totipotency of plant cells. This concept was developed by Haberiandt who is a German biologist in 1902. The process of totipotency is only presence in plant cells and can be retained even they have undergone final differentiation as long as the membrane system and a viable nucleus are in intact with the plant body (Jamiepighin, 2003). The phenomenon of totipotency can be seen in Figure 2.13 which the isolated carrot cell is cultured in nutrient and agar medium in order to grow a mature carrot.

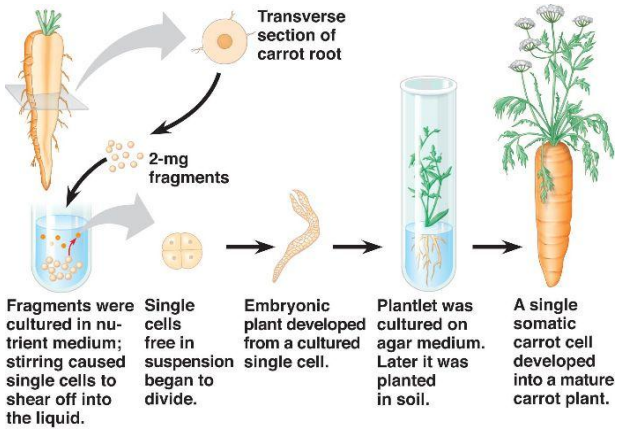


Figure 2.13: Totipotency process of isolated carrot cells (Jamiepighin, 2003)

2.4.1.1 Stages of Micropropagation

Micropropagation consist of four stages which are sterilization, multiplication, hardening and transferring *in vitro* culture to the soil (Hussain *et al.*, 2012). The first stage of the sterilization process is the surface of explant must be sterilized first before transfer it into the culture medium. The sterilization of explant surface is crucial to remove any contaminants presence without damage the plant cells. The combination of bactericide and the fungicide products give a synergistic effect to the sterilization process. The

common chemical solutions used as disinfectants in sterilization process are calcium hypochlorite, sodium hypochlorite, mercuric chloride ($HgCl_2$) and ethanol. However, the selection of disinfectant must depend on the type of explant to be introduced into the media which can be seen in Table 2.9.

Table 2.9: Disinfectant used in sterilization procedure depends on type of explant parts (Street, 1973).

Explant parts	**Procedure**		
	Pre-sterilization	**Sterilization**	**Post-sterilization**
Seeds	- Submerged in absolute ethanol (10 seconds) - Rinse in sterile distilled water.	- Seeds with intact testas submerge in:- →10% w/v calcium hypochlorite (20-30 min) or →1% w/v bromine water solution (5 min).	- Washed five times with sterile distilled water
Fruits	- Absolute ethanol used to rinse briefly.	- Submerge in 2% (w/v) sodium hypochlorite (10 min)	- Wash repeatedly with sterile water - Dissect out seeds or interior tissue
Pieces of Stem	- Scrub clean in running tap water - Rinse with absolute ethanol.	- Immerse in 2% (w/v) sodium hypochlorite (15-30 min) - Removes ends	- Wash three times in sterile water
Leaves	- Absolute ethanol used to rub the surface briefly	- Immerse in 0.1% (w/v) mercuric chloride (1 min)	- Wash repeatedly with sterile water

The second stage of micropropagation is known as multiplication process which the tissue samples produced from the first stage are taken to increase the number of small plants or plantlets. In this research, the plantlets are micropropagate with different concentration of elicitor in the MS media. The plantlets will produce many small offshoot which it can be removed and recultured to another MS media until the number of desired plants are reached which can be seen in Figure 2.14. This stage is very crucial because the plantlets will interact with media containing elicitor to produce higher secondary metabolites that can be identified by chromatography technique.

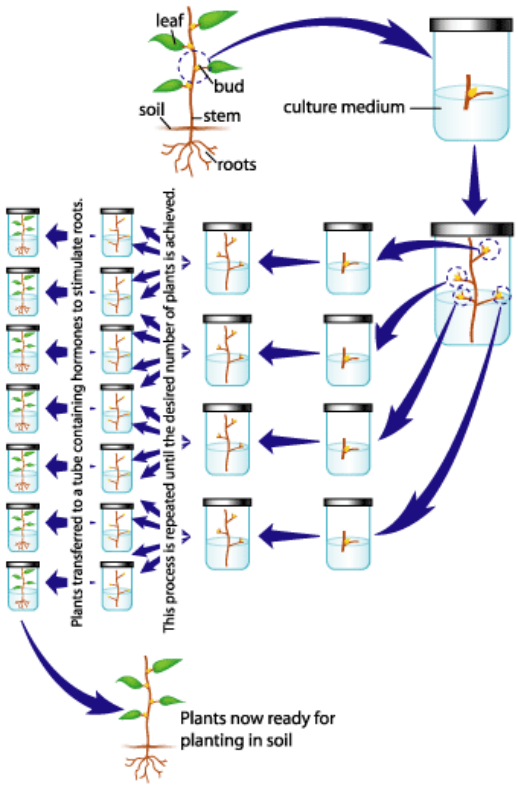


Figure 2.14: Plantlets multiplication stage (Hussain *et al.*, 2012)

The next stage is the hardening stage which involves the process of treating the plantlets by encouraging it to grow the roots which act as a pre-transplant process. In other words, this process makes the plants prepare itself before transferred to natural growth environment. The culture media used during the explant multiplications has an important role in order to enhance the rapid growth of plantlets and also the growth of strong root development (Hussain *et al.*, 2012).

Lastly, the last stage of micropropagation is the plantlets that are transferred to the soil under the greenhouse. Usually, the greenhouse has an environment with higher light intensity, and also lower relative humidity which give a pressure condition to the plantlets

rather than *in vitro* conditions (Kumar & Rao, 2012). So, there are some number of micropropagated transferred plants cannot survive in the greenhouse or natural environment but if the acclimatization stage is successful, the plants can sufficiently survive and can grow in the soil. However, in this research, the third and fourth stage are not applied because they will be harvested from the media for identification of the secondary metabolite production after the stage two are completed in certain time.

2.4.2 Plant Tissue Culture Media

The culture media are important in plant tissue culture because it will provide the nutrients required for the growth and development of plantlets. There are several types of culture media such as White's, MS, B5 (Gamborg), N6 (Chu) and Nitsch medium that available commercially in the form of powders which contain all components required except growth regulator or elicitor, sucrose and agar (Qadry, 2010) which can be seen in Table 2.16. Usually, the composition and selection of culture media depends on two parameters that are the particular species of plant and also the type material used for the culture which it will be formulated by consider the requirement of culture system and also the state of media whether it is in solid or liquid form. In this research, MS medium is used because it is the most widely use medium and function to induce organogenesis and also the regeneration of plants under controlled environment in plant tissue culture (Qadry, 2010).

According to Hussain *et al.*, (2012), culture media usually composed mainly of macronutrients, micronutrients, other organic components, vitamins, plant growth

regulators or elicitors, carbon source and some gelling agents. There are many elements are needed by a plant for its nutrition and physiological functions. Thus, elements such as nitrogen, calcium, zinc and others must be supplied to the culture medium in order to give an adequate growth of *in vitro* cultures which can be seen in Table 2.15 that provide the elements needed with the function in plants. pH of the medium can affect the growth of the plant in plant tissue culture which usually the optimal pH is in the range of 5.0-6.0. While preparing the medium, pH can be adjusted to the required optimal level which in this research has been adjusted to pH of 5.4 – 5.8. However, at pH higher than 7.0 or lower than 4.5 will stop the growing and at pH 5.0 will not allow the gelling of the medium while pH above 6.0 give the hard appearance to the medium (BiologyDiscussion, 2013).

Table 2.15 List of the elements and their functions in plants
(Qadry, 2010)

Element	*Function(s)*
Nitrogen	Essential component of proteins, nucleic acids and some coenzymes. (Required in most abundant quantity)
Calcium	Synthesis of cell wall, membrane function, cell signalling.
Magnesium	Component of chlorophyll, cofactor for some enzymes.
Potassium	Major inorganic cation, regulates osmotic potential.
Phosphorus	Component of nucleic acids and various intermediates in respiration and photosynthesis, involved in energy transfer.
Sulfur	Component of certain amino acids (methionine, cysteine and cystine, and some cofactors).
Manganese	Cofactor for certain enzymes.
Iron	Component of cytochromes, involved in electron transfer.
Chlorine	Participates in photosynthesis.
Copper	Involved in electron transfer reactions, Cofactor for some enzymes.
Cobalt	Component of vitamin B_{12}.
Molybdenum	Component of certain enzymes (e.g., nitrate reductase), cofactor for some enzymes.
Zinc	Required for chlorophyll biosynthesis, cofactor for certain enzymes.

Table 2.16: Composition of commonly used plant tissue culture media
(Qadry, 2010)

Components	Amount (mg l^{-1})				
	White's	Murashige and Skoog (MS)	Gamborg (B5)	Chu(N6)	Nitsch's
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	—	0.025
$CoCl_2.6H_2O$	—	0.025	0.025	—	0.025
KI	0.75	0.83	0.75	0.8	—
$FeSO_4.7H_2O$	—	27.8	—	27.8	27.8
$Na_2EDTA.2H_2O$	—	37.3	—	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thiamine HCl	0.01	0.5	10	1	0.5
Pyridoxine (HCl)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	—	100	100	—	100
Others					
Glycine	3	2	—	—	2
Folic acid	—	—	—	—	0.5
Biotin	—	—	—	—	0.05
pH	5.8	5.8	5.5	5.8	5.8

2.4.3 Plant elicitors

Elicitation can be defined as the production of the secondary metabolite that induce by the molecules or treatments called elicitors in order to ensure their survivability and competitiveness. According to Hashemi (2015), elicitors can be grouped into three categories which are biotic elicitor, abiotic elicitor and endogenous elicitor which can be seen in Table 2.17. Biotic elicitor is usually from microbe-derived molecules such as glycoproteins, polysaccharides, cell walls and low molecular weight organic compound while abiotic elicitor or stress comes from UV radiation, chemicals and heavy metal salts. While, endogenous elicitor is chemical that produce within the cell that act as secondary messengers like methyl jasmonate (Fu *et al.*, 2012).

The elicitors used in plant tissue culture can give much advantageous in various area of research which it can increase the yield of target substance like the secondary metabolite, can investigate the regulation and enzymology of secondary metabolite production towards the elicitors and also study the plant defense mechanism (Patel & Krishnamurthy, 2013). The application of elicitors in plant tissue culture is the focus of this research which it has been a hypothesis to be the most effective methods to improve the synthesis of secondary metabolites in *C. asiatica.* Accumulation of this metabolites usually occurs in plants that are subjected to stresses including various elicitors or signal molecules for example like seaweed extract. The previous successful of the usage of elicitors which is methyl jasmonate by cell suspension technique can be seen in Paclitaxel compound that come from the bark of Yew tree which purposely used for the cancer treatments (Yukimune Y, 2015).

Table 2.17: Classification of elicitors for plant cells
(Hashemi, 2015)

<table>
<tr><td colspan="10">Elicitors</td><td></td></tr>
<tr><td colspan="9">Chemical</td><td></td><td>Physical</td><td>Endogenous</td></tr>
<tr><td colspan="9">Biotic</td><td>Abiotic</td><td rowspan="2">Wound</td><td rowspan="2">Methyl Jasmonate</td></tr>
<tr><td colspan="7">Defined composition</td><td>Complex composition</td><td rowspan="3">Metal ions (lanthanum, europium, calcium, silver, cadmium), Oxalate</td></tr>
<tr><td colspan="3">Carbohydrate</td><td>Proteins</td><td>Lipids</td><td>Glycoprotein</td><td>Volatile</td><td rowspan="2">Yeast cell wall, Fungal extract or spore, Mycelia cell wall</td></tr>
<tr><td>Polysaccharides</td><td>Oligosaccharides</td><td>Peptides</td><td rowspan="2">Cellulas, Elicitins, Oligandrin</td><td rowspan="2">Lipopolysaccharides</td><td rowspan="2">Not characterize</td><td rowspan="2">C6-C10</td></tr>
<tr><td>Alginate, Pectin, Chitosan, Guar Gum, Seaweed</td><td>Galacturonides</td><td>Glutathione</td></tr>
</table>

2.4.3.1 Seaweed as Biotic Elicitors

Biotic elicitor used in this research are seaweed which under the carbohydrate groups containing polysaccharides sources. They can be classified into three groups which based on their pigmentation that are Phaeophyta, Rhodophyta and Chlorophyta or known as brown, red and green algae respectively (Khan *et al.*, 2009). There are different types of unusual and complex polysaccharides constituent that can be found in each type of algae (Table 2.18) which not present in land plants. They are proving that seaweeds have benefits as a source of organic matter and fertilizer nutrients which provide the nutrient supplements and also act as biostimulant to enhance the plant growth and yield.

Actually, the beneficial effect of seaweed to the plant growth is caused by the presence of more than one of plant growth-regulatory hormones such as cytokinins and auxins that will promote the growth effect in the treated plants. Usually, the cytokinins is detected on the fresh seaweed while seaweed extract of marine algae rich in auxin (Khan *et al.*, 2009). They also will affect the chemical, physical and biological properties of the soil by

improving the moisture-holding capacity and also promoting the growth of roots development. They also act as a source of plant defense elicitors (Cluzet *et al.*, 2004) which the plants protect themselves by detecting the alien molecules such as polysaccharides, lipids and proteins that presence on the cell wall of the pathogen. However in this research, the presence of seaweed rich of polysaccharides in plant tissue culture media which alien to the plant will enhance the production of secondary metabolites of *C. asiatica.*

Table 2.18: Polysaccharide constituents found in green, red and brown seaweeds. (Khan *et al.*, 2009).

Polysaccharides in green seaweeds (Chlorophyceae)	**Polysaccharides in red seaweeds (Rhodophyceae)**	**Polysaccharides in brown seaweeds (Phaeophyceae)**
Amylose. Amylopectin	Agars	Alginates
Cellulose	Carrageenans	Cellulose
Mannans	Cellulose	Fucoidans
Inulin	Furcellaran	Laminarans
Xylans	Mannans	Fucose containing glucose
Pectin	Rhodymannan	Lichenan-like glucan
Laminarans	Xylans	Complex sulfated heteroglucans

2.4.3.1.1 Biotic Elicitor of *Kappaphycus alvarezii*

Kappaphycus alvarezii is in a group of Rhodophyta or red algae. This alga also known as *Eucheuma cottonii* and commercially called as "cottonii". Their cell walls are rich in carrageenans that are from sulfated linear galactan family which useful in the overproduction of secondary metabolites in plants. According to Patel & Krishnamurthy (2013) states that the carbohydrates elicitor can induce the signal transfer in tobacco cell by calcium influx and peroxide production and also activate the signalling pathways that

mediated by ethylene, jasmonic acid and salicylic acid which some of the effects are last for at least a week (Mercier *et., al*, 2001).

The enhancement of growth plant by the addition of *E. cottoni* extract is because it contain indole acetic acid, zeatin, kinetin and gibberellic acid (GA_3) but there is no study yet reported on the interactions of this constituents and when they are autoclaved all the growth hormones is destroyed (Mondal *et al.*, 2015). This red alga, has been used as the foliar spray in tomato which improved the nutrient uptake, increase the fruit quality and the number and size of fruits per plant, the content of macro and micro elements are increased over control and also the plant is resistance to leaf curl, fruit borer and bacterial wilt (Zodape *et al.*, 2011).

2.5 Chromatography

Chromatography derives its name from two words as"chromo" means colour and "graphy" means writing (Kumar *et al.*, 2015). It can be defined as an analytical technique based on the separation of molecules due to differences in their structure or composition (Kupiec, 2004). It involves the movement of the sample by mobile phase through an immobilized stationary phase. According to Robards *et al.*, (2012), mobile phase can be a liquid, gas or supercritical fluid whereas stationary phase can be a solid or liquid which

the uses depend on the classification of chromatography in Figure 2.15. The sample compounds will have different affinity and interaction toward the stationary phase which it will move more slowly if the affinity is strong (Kupiec, 2004). Different compounds are separated into individual component during their travel through the column.

2.5.1 Technique in Chromatography

The technique of chromatography can be classified into two broad categories which are the column and planar chromatography (Robards *et al.,* 2012). Planar chromatography has a stationary phase that is supported on a two-dimensional planar surface which can be seen in Paper Chromatography (PC) and Thin Layer Chromatography (TLC). Stationary phase for PC consists of a sheet of paper while TLC consists of a thin layer of solid spread uniformly over a flat sheet of glass, plastic or aluminium that often comes with the binder. Planar chromatography alternatively has a stationary phase that is packed in a closed tube called column. This can be achieved by coating or chemically bonding the liquid stationary phase to an inert solid support which is then packed in the column. The column material that usually used is fused silica for capillary columns, glass, stainless steel and also plastics. This planar technique can be seen in the uses of Liquid Chromatography (LC), Gas Chromatography (GC) and Supercritical Fluid Chromatography (SFC) in Figure 2.19.

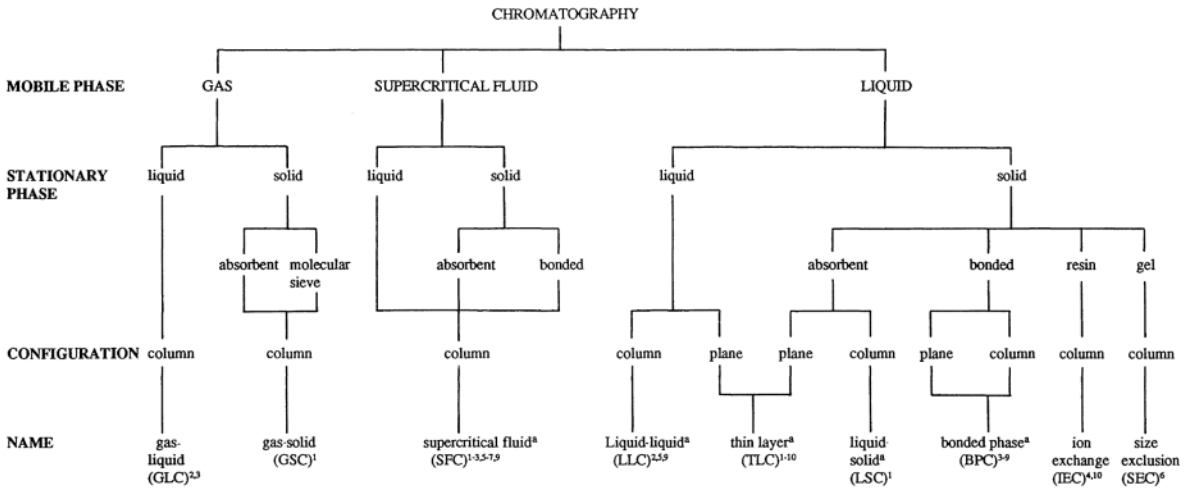


Figure 2.19: Classification of chromatographic system (Robards, et al., 2012).

2.5.2 Mode of Operation in Chromatography

Mode of operations of chromatography have two different types which are development chromatography and elution chromatography. According to Robards *et al.,* (2012), the development mode is referred as the movement manner of the sample and the mobile phase that is applied to stationary phase bed which the flow will be stopped before reaching the end of the stationary phase. TLC is one example of chromatography that using this mode. While, elution mode involve the sample is being applied as a compact band to the mobile phase followed by a continuous flow of fresh mobile phase. The mobile phase itself known as eluent while the sample that emerge with the mobile phase which function to carry them known as an eluate. The sample will undergo solute migration and the individual component of sample moves in separate zones which the zones is detected

by detector once it emerges from the column. An example of chromatography that undergo elution mode is column chromatography.

2.5.3 Mechanism of separation in chromatography

The interaction of sample component with the two phases has a certain mechanism of separation by the various physicochemical process that occur in the system. It is crucial to know the knowledge of separation mechanism because it will enable the predictions of system behaviour and also can give a guide for choosing the best combination of mobile and stationary phase in order to get the desired separation. The classified mechanisms are adsorption, partition, size exclusion, ion exchange, affinity, ion-exchange, ion-interaction, micellar, complexation and countercurrent (Robards *et al.*, 2012). However, the most common use mechanism in chromatography are adsorption and partition mechanism.

Adsorption known as the surface phenomenon that the interaction occurs only on the surface of one substance. This mechanism, consist of adsorbent or stationary phase of solid silicon dioxide gel (SiO_2) that usually covered by a monolayer of mobile phase molecule. The separation of the sample depends on the differences of adsorption affinities of sample component to the surface of adsorbent. The sample component has a competitive reaction with the mobile phase for adsorption to the surface of adsorbent by

van der Waals force, dipole-dipole interaction and hydrogen bonding. Then, the solute retention occurs by a competitive displacement of mobile phase molecule from the adsorbent surface (Robards *et al.*, 2012). Figure 2.20 shows the mechanism of adsorption of aspirin as an analyte to the silica gel adsorbent which it displaced two molecules of water as a monolayer of mobile phase that covered the adsorbent (Weber, 2015).

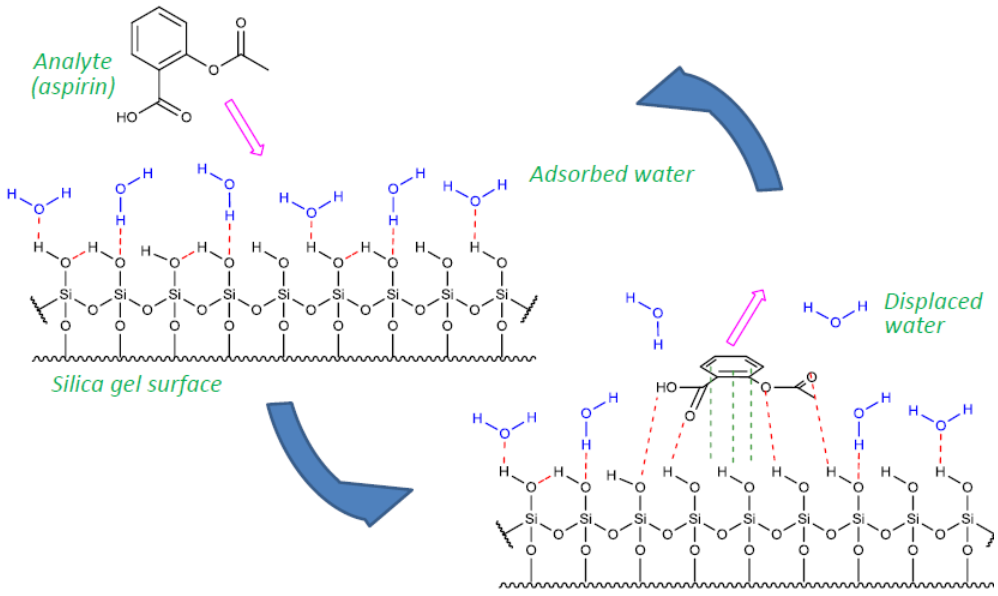


Figure 2.20: Mechanism of solute adsorption to the adsorbent.
(Weber, 2015).

Partition chromatography is a process of mixture separation in columns that based on the partition of a solute between the two immiscible liquids solvents which one of the solvent is immobilized on a solid support in the column (Merriam-Webster, 2015). According to Robards *et al.*, (2012), the example of solid support is silica gel, cellulose, diatomaceous earth, polystyrene or polytetrafluoroethylene (PTFE) which can provide a large surface area for the stationary phase to retain and is chemically inert. In LC, commonly methanol or acetone are used as the stationary phase which it is held by hydrophobic silica

backbone. The most popular silica backbone is Octadecylsilyl (ODS) bound chain that also known as C18 (Weber, 2015). The partition action occurs when a solute is in contact with two immiscible phases which it will distribute itself between them according to its partition coefficient (Robards *et al.,* 2012). The analyte that have greater partition coefficient to the stationary phase will have higher affinity to it so it will travel slower and vice versa (Kumar *et al.,* 2015). The intermolecular forces involved in this mechanism are the dispersion, induction, donor-acceptor interaction and also hydrogen bonding. Figure 2.21 shows the partitioning of solute to the stationary phase of methanol hold by C18 silica backbone through the water as mobile phase in the column chromatography (Weber, 2015).

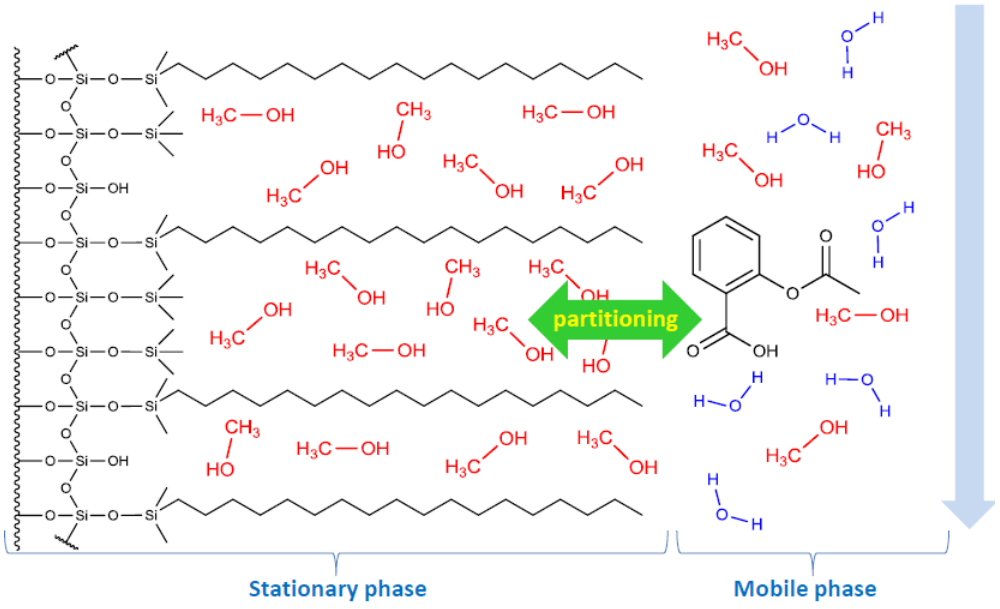


Figure 2.21: Partitioning of the analyte to the stationary phase (Weber, 2015).

2.6 High-Performance Liquid Chromatography (HPLC).

High-performance liquid chromatography (HPLC) is called based on their high efficiency in the analysis of compound. It also known as high-pressure liquid chromatography because of the uses of high pressure at 400 atm through a column instead of allowing the solvent drip by the influence of gravity which makes the process a lot faster (Kumar *et al.,* 2015). It has a highly improved performance than column chromatography in terms of separation efficiency, sample throughput, analytical precision and ease of use by being fully automated through the control of a personal computer (Robards *et al.,* 2012). It is the most commonly used technique for quantitation of drugs in formulation (Watson, 2005), clinical and biochemical research, industrial quality control and for the assay of molecules from mixtures of biological, plant and medical importance (Kumar *et al.,* 2015).

HPLC is classified under LC mobile phase which have a column technique that undergo elution mechanism by partition operation of chromatography. This chromatography technique has a liquid mobile phase that is pumped under a high pressure through a stainless steel column that contain column packing material which consist very small particles size of spherical silica gel with a diameter of 5 or 4µm (Watson, 2005). The large surface area of stationary phase gives the greater interaction of partition between the mixtures of compounds which gives a much better separation to its individual components. Usually, HPLC system that can be seen in Figure 2.22 consists of solvent or

mobile phase in the reservoir, a pump for the solvent delivery system, a column containing stationary phase that connecting to the injector that used as sample introduction which operate manually or automatically. The detector used to generate a signal from the detection of eluted compound which is recorded as a chromatogram that consist of retention time on a data system.

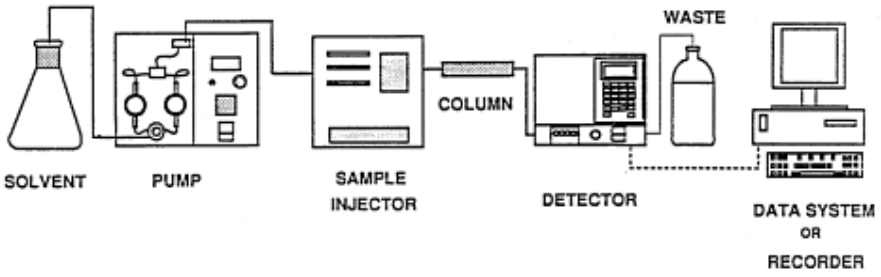


Figure 2.22: Components of a basic HPLC system
(Watson, 2005)

There are two types of stationary phase which are the normal phase and reverse phase considering in adsorption and partition. Normal phase is considered as the stationary phase that is polar in nature and has non or less polar mobile phase. According to Kumar *et al.,* (2015), the column is filled with tiny silica particles and the solvent is nonpolar hexane. Therefore, the compounds that have higher polarity will stick longer to the polar silica and will elute out last while the non-polar will come out first. However,the normal phase is not commonly used in HPLC but the reversed phase of the C18 column is used in the separation of secondary metabolite in *C. asiatica*. Reversed phase is the stationary phase that is non-polar in nature such as C18 which the silica is modified by attaching long hydrocarbon chains to its surface (Kumar *et al.*, 2015). The polar mobile phase is the combination of methanol and acetonitrile in ultrapure water (Thongnopnua, 2008). So, the

polar molecule in the mixture will spend their time moving with the solvent and travel through the column quickly. While the nonpolar compound will elute slower because it will form an attraction with hydrocarbon group by van der Waals forces. It also less soluble in the solvent because it needs to break the hydrogen bonds as they squeeze through the water or methanol molecules (Kumar *et al.*, 2015).

2.6.1 High Performance Liquid Chromatography (HPLC) in *C. asiatica*

According to European Pharmacopiae (2011), the reverse phase of HPLC is used for the assay of *C. asiatica* herb. The non-polar stationary phase is ODS silica gel which have a diameter of 5µm in the column. While, the polar stationary phase consist of the combination of two mobile phase which are mobile phase A consist of acetonitrile while mobile phase B consist of 3 ml of phosphoric acid diluted with 1000 ml of deionized water. The diluted phosphoric acid act as a buffer in the system (Shimadzu, 2015). The detector used is spectrophotometer at 200 nm and the flow rate is at 1.0 ml/min with gradient elution shown in Table 2.10.

Table 2.10: Gradient elution of Mobile phase A and Mobile phase B (European Pharmacopiae, 2011)

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0-65	22	78
65-66	55	45
66-76	95	5
76-85	22	78

The retention time for madecassoside is about 5.8 minutes, asiaticoside is about 8.1 minutes, madecassic acid is about 17.6 minutes and asiatic acid is about 21.7 minutes. This shows that the most polar compound in *C. asiatica* is madecassoside because it is eluted faster than the others while asiatic acid is the most non-polar compound because it is the compound that have large interaction with the ODS silica gel hence it is eluted last from the column.

CHAPTER THREE

METHODOLOGY

3.1 Materials

3.1.1 Plant materials

Centella asiatica plant for *in vitro* plantlets were taken from Plant Tissue Culture Laboratory, Department of Pharmacology, Faculty Pharmacy, UiTM Puncak Alam. However, the healthy and young natural *C. asiatica* plant were purchased from the nursery at Puncak Alam, Selangor Darul Ehsan, Malaysia.

3.1.2 Preparation of *in vitro C. asiatica* plantlets

First step, these natural plants were put in a 1000 mL of conical flask and then washed under running tap water throughly in two hour. Then, dettol was used to rinse them and treated with 100mL of clorox and 900mL of sterilized distilled water which the percentage were 10% clorox and 90% sterilized distilled water and also two drops of Tween 20 were

added into the flask. The plants was soaked and shaked vigorously for 15 to 30 minutes. This action provides the plants to have a better surface contact with the fungicide. Lastly, they had been washed four or five times with sterilized distilled water before being transferred into the sterile agar medium.

3.1.3 Preparation of natural *C.asiatica* with seaweed elicitor

The natural plant were washed with running tap water thoroughly. Then, the plant were placed into four different containers with distilled water labelled 0g/L, 2g/L, 4g/L and 8 g/L. The plant were being familiarize with the surrounding for a week. After a week, three of the plant containers was filled with different concentration of seaweed elicitor which were 2g/L, 4g/L and 8g/L and one container act as a control known as 0g/L.

3.1.4 Pre-sterilized tools

All tools and apparatus prior used to culture the plantlets had been wrapped with aluminium foil and then they had been sterilized in an autoclaved at 121 ^{o}C for twenty minutes.

3.1.5 Culture media for *in vitro C. asiatica* plantlets

In this research, the full strength solid of Murashige and Skoog (MS) culture media was used. MS stock solutions were prepared with the basal medium which include of macro (100 mL), micro (10 mL), ferum (10 mL) and vitamin (10 mL). Next, it was supplemented by myoinositol (0.1 g) and 3 % sucrose (30 g). The distilled water was topped up to one liter and a mixture of plant growth regulators which were 1.0 mg/L naphthalene acetic acid (NAA) and 1.0 mg/L benzyl amino purine (BAP) were added into the preparation and was heated by using the hot plate to allow them mixed thoroughly. Then, 1 g of 0.2% Gelrite agar with a strength of 550-850 g/cm^2 was added into the preparation and the pH of media was adjusted to 5.6–5.8 using 0.1 M NaOH. Next, 20 mL was dispensed in each 2.15 x 15cm plant tissue culture bottles which one litre of media can obtain approximately 25 plant tissue culture bottles. Those bottles containing media was autoclaved at 1.06kgcm^2 in 121 ^{o}C for 15 minutes. Methods were repeated by added other combination of plant growth regulators concentrations mixture which were 1.0 mg/L NAA + 2.0 mg/L BAP.

3.1.5.1 Murashige and Skoog (MS) stock preparation

The stock solutions will be stored at -4 ^{o}C which consists of macro, micro, vitamin and ferum ingredients. The MS Stock Preparation summary has been attached in Appendix I.

3.1.5.1.1 Macro

Macro stock preparations contain compounds of ammonium nitrate (NH_4NO_3) in 16.5 g, potassium nitrate (KNO_3) in 19.0 g, calcium chloride dehydrate ($CaCl_2.2H_2O$) in 4.4 g, potassium hydrogen phosphate ($KHPO_4$) in 1.7 g of and magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) in 3.7 g of. All these compounds will be diluted in one litre of distilled water.

3.1.5.1.2 Micro

Micro stock preparations contain compounds of manganese (II) sulfate tetrahydrate ($MnSO_4.4H_2O$) in 2.2 g, zinc sulfate heptahydrate ($ZnSO_4.7H_2O$) in 0.86 g, boric acid (H_3BO_3) in 0.62 g, potassium iodide (KI) in 0.083 g, sodium molybdate dehydrate ($Na_2MO_4.2H_2O$) in 0.025 g, copper(II) sulfate pentahydrate ($CuSO_4.5H_2O$) in 0.025 g and cobalt (II) chloride hexahydrate ($COCl_2.6H_2O$) in 0.0025 g. All these compounds will be diluted in one litre of distilled water.

3.1.5.1.3 Ferum

Compounds containing in ferum solution consist of disodium ethylenediaminetatraacetate (Na_2 EDTA) in 3.73 g and iron (II) sulfate heptaydrate ($FeSO_4.7H_2O$) in 2.7 g. All these compounds will be diluted in one litre of distilled water. Ferum prone to undergo an oxidation and degradation process, thus the bottle containing ferum must be wrapped with aluminium foil or using the amber bottle.

3.1.5.1.4 Vitamin

Compounds containing in vitamin solution consist of glycine (0.2 g), nicotinic acid (0.05 g), pyridine HCl (0.05 g) and Thiamine HCl (0.01 g). All these compounds will be diluted in one litre of distilled water.

3.2 Media cultivation containing different concentrations of plant growth regulators for *in vitro C. asiatica*

In overall, there were two different concentrations of plant growth regulators concentrations. The plant regulators used were 6-benzylaminopurine (BAP) and naphthalene acetic acid (NAA) with combination concentration of 1.0 mg/L NAA and

1.0 mg/L BAP or 2.0 mg/L BAP. Three replicates of the explant were cultivated in each concentration.

3.3 Duration of harvesting the *in vitro C. asiatica*

All of the explant was grow up until two month and the observation of the growth was recorded. The morphology of the *in vitro* plantlets was observed by the naked eye after two month.

3.4 Duration of harvesting the natural *C. asiatica*

The five replicates of plant in each concentration were harvested at day 0, day 7, day 14, and day 21. During harvested day, the morphology of the plants were observed and recorded such as the diameter of leaf, the number of new shoots and flowers. Then, the plants were proceed to the extraction process for measuring the amount of secondary metabolites contain in it.

3.5 Extraction process of natural *C. asiatica*

The harvested *C. asiatica* samples were dried by the oven at 50 ^{o}C for seven days. Then, the dried sample was grounded with mortar and pestle to produce more powdered form and increase its surface area. Next, 1 g of grounded sample was extracted in 20 mL of absolute analytical grade methanol thrice which they were being kept for one week at the room temperature under dark condition. Lastly, the extracted samples was filtered out and the filtrate was evaporated to dryness by the laminar flow that produce an extract of dark brown crude (Mohammadparast, Rasouli, Rustaiee, Zardari & Agrawal, 2014).

3.6 High performace liquid chromatography (HPLC) analysis

5-10 mg/ml of the dried crude extract was dissolved in 1.0 mL of absolute HPLC grade methanol. Prior to run a sample on the HPLC system, the samples extract was filtered through a 0.45 µm filter. The column used was Column-ZORBAX 300SB-C18 (particle size 5-Micron, size 250 mm X 4.6 mm; from Agilent Technologies). While, detector used in the HPLC system were Diode Array Detector (DAD) at wavelength of 206 nm and evaporative light scattering detector (ELSD). The gradient elution mechanism was performed using the mixture of two mobile phase which were mobile phase A that consist of acetonitrile while mobile phase B consist ultrapure water. The flow rate was at 1.0 mL/min. Post-run was 5 minutes. A 20 µL aliquot of the extract was automatically injected into the column which the analysis was controlled by Chemstation for LC systems

software from Agilent Technologies (2001-2012). The best gradient condition for HPLC analysis can be seen in Table 3.1.

Table 3.1: Gradient condition

Time (min)	Pump A, ultrapure water (%)	Pump B, acetonitrile (%)
0	80	20
15	30	70
25	10	90
35	5	95
45	80	20

CHAPTER FOUR

RESULTS

4.1 Observation of *in vitro C. asiatica* growth with plant growth regulators.

Plant tissue culture technique was being used to grow *C. asiatica* in an artificial nutrient medium which were MS media containing a combination of naphthalene acetic acid (NAA) and benzyl adenine (BAP) hormones in *in vitro* by aseptic condition. There were 56 plant tissue culture glass bottles with plastic lid had been cultured with petioles, roots and leaves of *C. asiatica* explants on MS media with a concentration of 1.0 mg/mL NAA and difference concentration of BAP which were concentration of 1.0 mg/L BAP and 2.0 mg/L BAP.

The observation was carried out for two months, which there was a progression growth in petioles while roots and leaves are not growth. The results showed that, the combination of 1.0 mg/L NAA with 2.0 mg/L BAP was the best results than a combination of 1.0 mg/L NAA with 1.0 mg/L BAP for *C. asiatica* petioles growth. Based on figure 4.1, four bottles

cultured with petioles which are bottle 5, bottle 8, bottle 10 and bottle 37 containing a combination of 1.0 mg/L NAA and 2.0 mg/L BAP MS medium show a good growth of shoot progression after two months exposed to the medium.

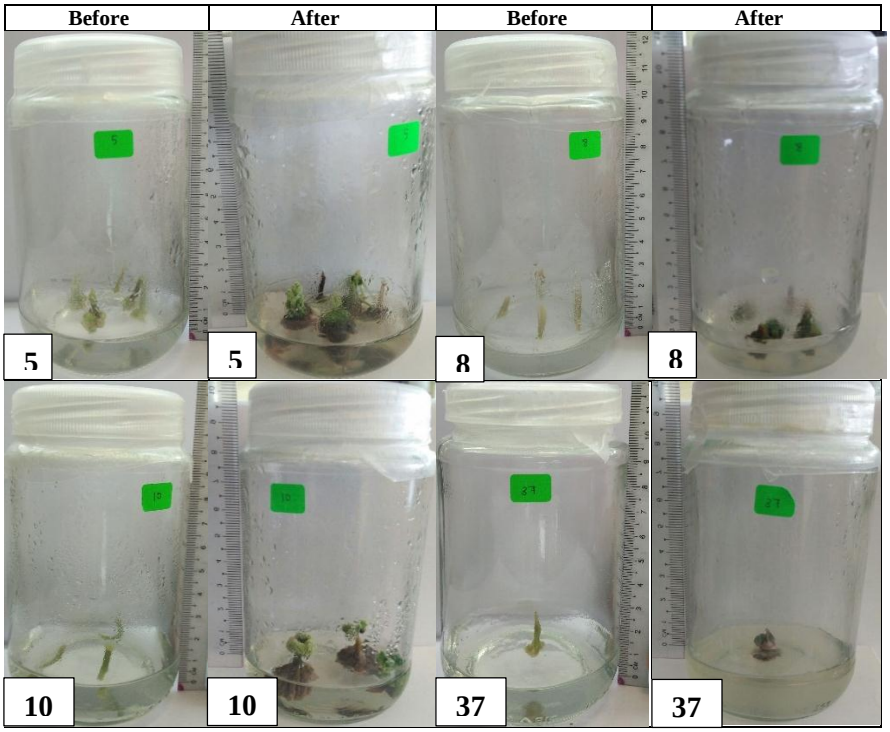


Figure 4.1: Comparison of *in vitro C. asiatica* plantlets before and after 2 month exposed to plant growth regulators.

4.2 Observation of natural *C. asiatica* growth with seaweed elicitor.

Natural *C. asiatica* was taken from nursery to the laboratory and was separated into four groups in trays which containing distilled water and let the plants acclimatized with surrounding for a week. Then, the plants in trays had been treated with three difference concentrations of seaweed elicitor which were 2 g/L, 4g/L and 8 g/L and also one control which known as 0 g/L.

Four trays of *C. asiatica* with difference concentration of seaweed elicitor were being observed and harvested at day 0, day 7, day 14 and day 21 for extraction and determination amount of secondary metabolites by HPLC that can be seen in Figure 4.2. Their macroscopic morphological characteristics such as diameter of leaves, number of flowers and number of new shoots were also recorded in order to know the effects of seaweed elicitors to the physical state of the plants.

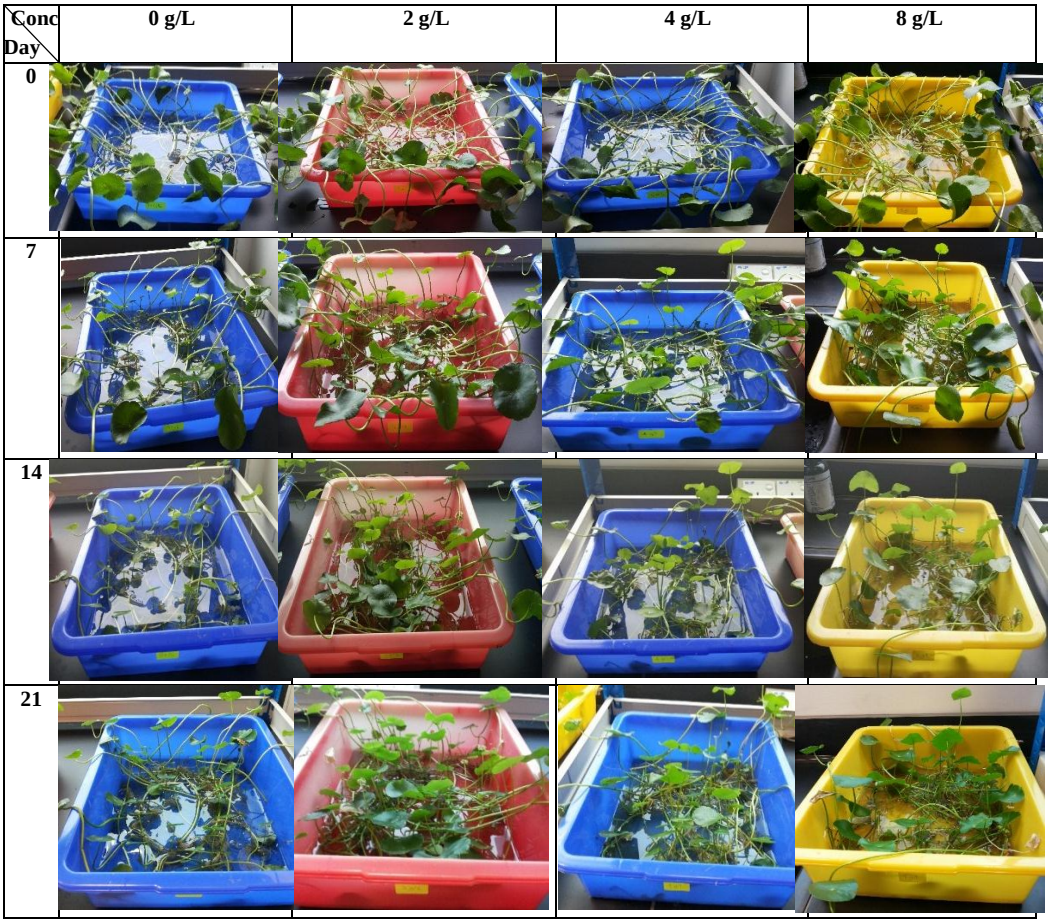


Figure 4.2: Progression of natural *C. asiatica* in different concentrations of seaweed elicitor at day 0, 7, 14 and 21

4.2.1 Diameter of leaves

One of the macroscopic morphological characteristics of the plants can be seen through the diameter of leaf, which each plants from different concentration of seaweed elicitor was being measured using centimetre ruler at day 0, day 14 and day 21 that can be seen in Figure 4.3. The observation that can be seen on the leaves were green in colour, glabrous and have orbicular-reniform shape with palmate nervation. It also consist of smooth and crenate margins with rounded apex which can be seen in Figure 4.3.

Three measurement was taken to determine the average diameter of leaves and the standard deviation was calculated shown in Table 4.1. The results showed that, when the concentration of seaweed elicitor was higher and the time exposure of the plants to the elicitor was longer, the higher the diameter of the leaves. The lowest reading diameter of leaf was 3.43±0.379 at the concentration of 0 g/L at day 0 while the highest reading was 6.70±0.173 at the concentration of 8 g/L at day 21.

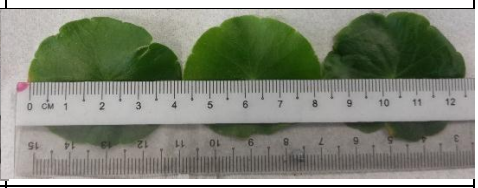
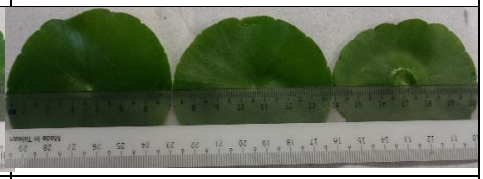
Conc / Day	0 g/L	2 g/L
0		
Average diameter	3.43 cm	4.17 cm
14		
Average diameter	4.30 cm	5.03 cm
21		
Average diameter	4.70 cm	5.83 cm
Conc / Day	**4 g/L**	**8 g/L**
0		
Average diameter	4.96 cm	5.17 cm
14		
Average diameter	5.87 cm	6.53 cm
21		
Average diameter	6.37 cm	6.70 cm

Figure 4.3: Average diameter of leaf in different concentration of seaweed elicitor at day 0, 14 and 21

Table 4.1: Diameter of leaf in different concentration of seaweed elicitor at day 0, 14 and 21

Day	Diameter of leaf (cm)	Concentration of seaweed elicitor (g/L)			
		0	2	4	8
0	1	3.6±0.379	4.2±0.153	5.0±0.0577	5.0±0.289
	2	3.7±0.379	4.0±0.153	5.0±0.0577	5.0±0.289
	3	3.0±0.379	4.3±0.153	4.9±0.0577	5.5±0.289
	Average	**3.43±0.379**	**4.17±0.153**	**4.96±0.0577**	**5.17±0.289**
14	1	4.3±0.100	4.7±0.306	6.0±0.416	7.0±0.404
	2	4.2±0.100	5.3±0.306	6.2±0.416	6.3±0.404
	3	4.4±0.100	5.1±0.306	5.4±0.416	6.3±0.404
	Average	**4.3±0.100**	**5.03±0.306**	**5.87±0.416**	**6.53±0.404**
21	1	5.1±0.289	5.8±0.0577	6.9±0.462	6.8±0.173
	2	4.6±0.289	5.8±0.0577	6.1±0.462	6.8±0.173
	3	4.6±0.289	5.9±0.0577	6.1±0.462	6.5±0.173
	Average	**4.7±0.289**	**5.83±0.0577**	**6.37±0.462**	**6.70±0.173**

4.2.2 Number of new shoots

The other macroscopic morphological description that can be observed was the number of new shoots growth in different concentration of seaweed elicitor at day 0, 7, 14 and 21. The new shoots of *C. asiatica* were usually grow at the nodes together with the rootstock as shown in Figure 4.4.

Figure 4.4: *C. asiatica* new shoot

The number of new shoots growth were taken three times and the average of the number was calculated with standard deviation as shown in Table 4.2. Based on the results, there were no new shoots growth being observed at day 0 for all concentrations while the highest average value for the growth of new shoots was 6.6±0.577 at day 14 in concentration 0 g/L.

Table 4.2: The number of new shoots produce in different concentration of seaweed elicitor at day 0, 7, 14 and 21

Day	Number of new shoots	Concentration of seaweed elicitor (g/L)			
		0	**2**	**4**	**8**
0	**1**	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	Average	**0**	**0**	**0**	**0**
7	**1**	1±0.577	1±0.577	2±0.577	0±0.577
	2	1±0.577	1±0.577	2±0.577	0±0.577
	3	2±0.577	0±0.577	1±0.577	1±0.577
	Average	**1.3±0.577**	**0.6±0.577**	**1.6±0.577**	**0.3±0.577**
14	**1**	7±0.577	5±0.577	4±0.577	4±0.577
	2	7±0.577	6±0.577	3±0.577	4±0.577
	3	6±0.577	5±0.577	4±0.577	5±0.577
	Average	**6.6±0.577**	**5.3±0.577**	**3.6±0.577**	**4.3±0.577**
21	**1**	2±0.577	3±0.577	3±0.577	4±0.577
	2	2±0.577	2±0.577	3±0.577	4±0.577
	3	1±0.577	2±0.577	4±0.577	3±0.577
	Average	**1.6±0.577**	**2.3±0.577**	**3.3±0.577**	**3.6±0.577**

4.2.3 Number of flowers

Observation on the flower of *C. asiatica* consist of white purple petals which the inflorescence of the flower had a single or more fascicled umbels with 2 mm of size. It also had a pentamerous shape with hemophrodite flowers consisting 4-6 stamens with styles and has inferior ovary which can be seen in Figure 4.5.

The number of flowers was taken three times in different concentration of seaweed elicitor at day 0, 7, 14 and 21 which the average of the results was being calculated with standard deviation as shown in Table 4.3. There was no flowers being observed at day 0 for all concentration and the highest average number of flower was 3±0.000 at day 21 in concentration of 0 g/L.

Figure 4.5: *C. asiatica* flowers

Table 4.3: Number of flower produce in different concentration of seaweed elicitor at day 0, 7, 14 and 21

Day	**Number of flower**	**Concentration of seaweed elicitor (g/L)**			
		0	**2**	**4**	**8**
0	**1**	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	Average	**0**	**0**	**0**	**0**
7	**1**	0	0±0.577	0±0.577	1±0.577
	2	0	0±0.577	0±0.577	1±0.577
	3	1	1±0.577	1±0.577	0±0.577
	Average	**0.3**	**0.3±0.577**	**0.3±0.577**	**0.6±0.577**
14	**1**	1±0.577	2±0.577	3±0.577	2±0.577
	2	0±0.577	1±0.577	3±0.577	2±0.577
	3	1±0.577	1±0.577	2±0.577	3±0.577
	Average	**0.6±0.577**	**1.3±0.577**	**2.6±0.577**	**2.3±0.577**
21	**1**	3±0.000	3±0.577	2±0.577	2±0.577
	2	3±0.000	2±0.577	2±0.577	1±0.577
	3	3±0.000	3±0.577	1±0.577	1±0.577
	Average	**3±0.000**	**2.6±0.577**	**1.6±0.577**	**1.3±0.577**

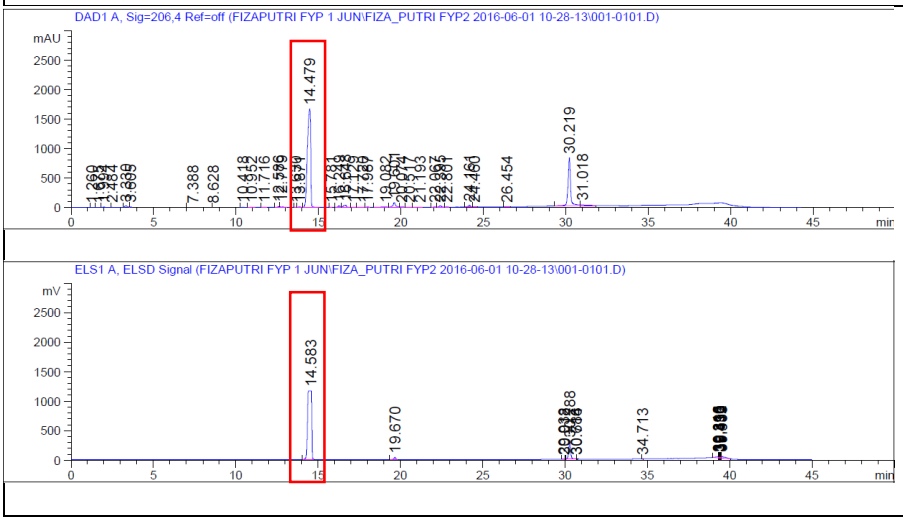
4.3 HPLC chromatogram of asiatic acid

Asiatic acid was the major secondary metabolites in *C. asiatica* which it had a pentacyclic triterpenoid sapogenin in ursane group. It was a non-polar molecules since it was soluble in methanol and sparingly soluble in water at 25˚C. To determine the amount of asiatic acid contain in each samples, reverse phase C18 column silica gel was used in HPLC and the mobile phase were acetonitrile and ultrapure water. The gradient elution mechanism was used and the detectors detect the elution of asiatic acid was by diode array detector (DAD) at wavelength of 206 nm and evaporative light scattering detector (ELSD).

4.3.1 HPLC chromatogram reference of asiatic acid

Reference of asiatic acid was dissolved in absolute HPLC grade methanol to produce 1.0 mg/ml and had been filtered through a 0.45 µm filter. The reference solution was transferred into HPLC vial and being automatically injected 20 µL into the HPLC for the detection of asiatic acid by retention time as shown in Table 4.4 below. The retention time for asiatic acid reference in DAD was 14.479 min while in ELSD was 14.583 min.

Table 4.4: Chromatogram and retention time of asiatic acid reference in DAD and ELSD detector

Reference	Retention Time
	DAD = 14.479 ELSD = 14.583

4.3.2 HPLC chromatogram of asiatic acid samples in natural *C. asiatica*

The chromatogram and retention time of asiatic acid detected by DAD and ELSD for each concentration at day 0, day 7, day 14 and day 21 were listed in Table 4.5, Table 4.6, Table 4.7 and Table 4.8 respectively.

Table 4.5: Chromatogram and retention time of asiatic acid at day 0 in DAD and ELSD detector

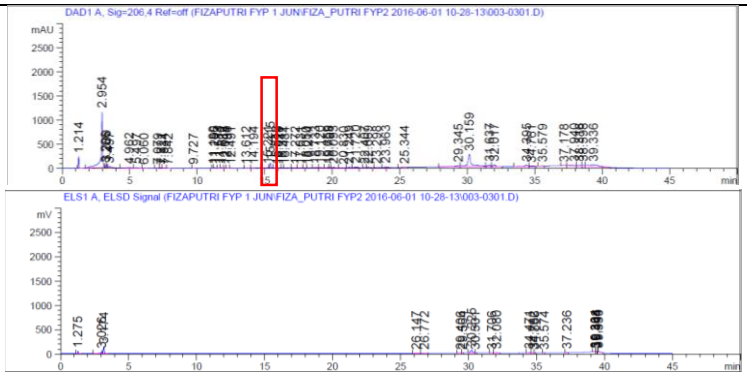
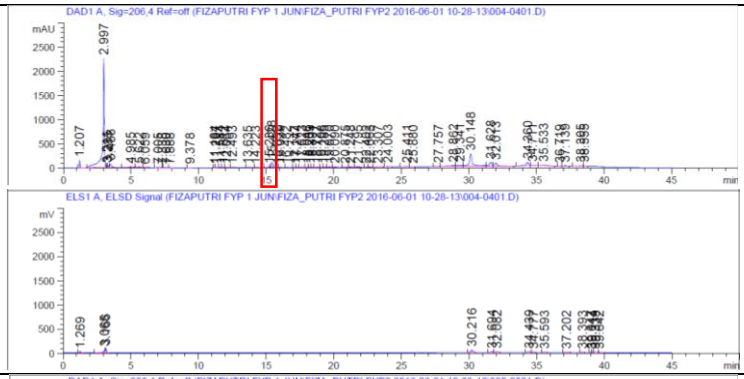
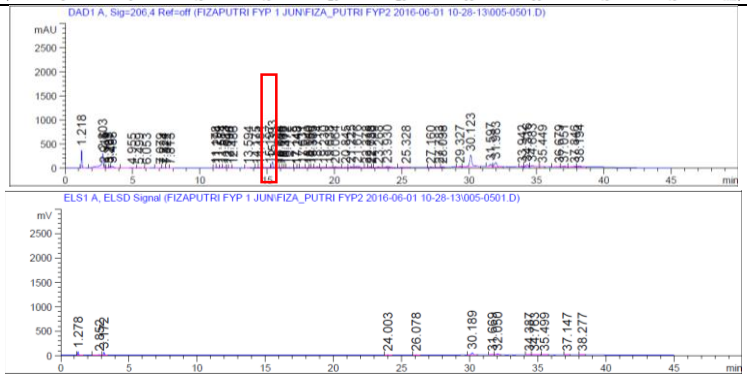
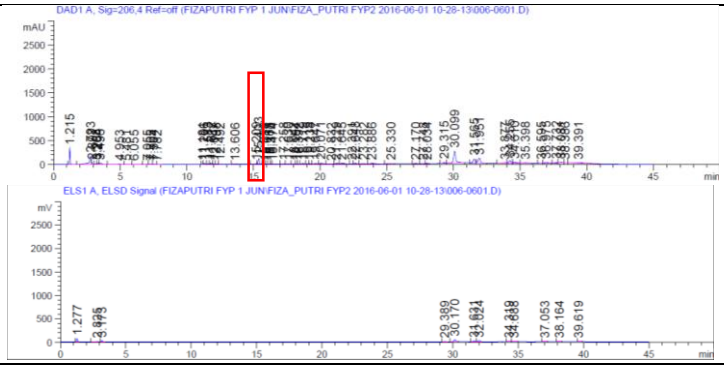
Day / Conc	Day 0	Retention Time
0 g/L		DAD = 15.415 ELSD = -
2 g/L		DAD = 15.428 ELSD = -
4 g/L		DAD = 15.393 ELSD = -
8 g/L		DAD = 15.403 ELSD = -

Table 4.6: Chromatogram and retention time of asiatic acid at day 7 in DAD and ELSD detector

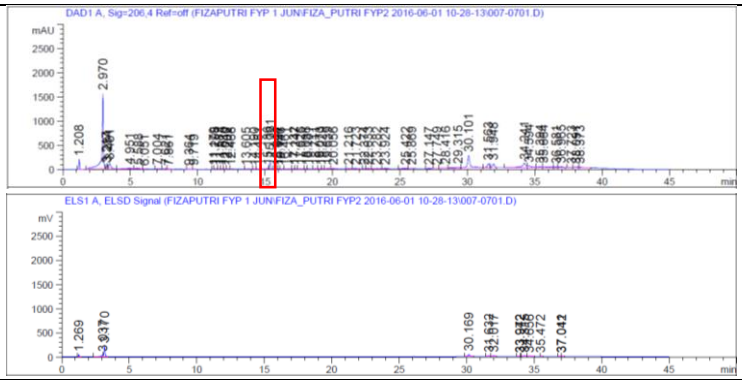
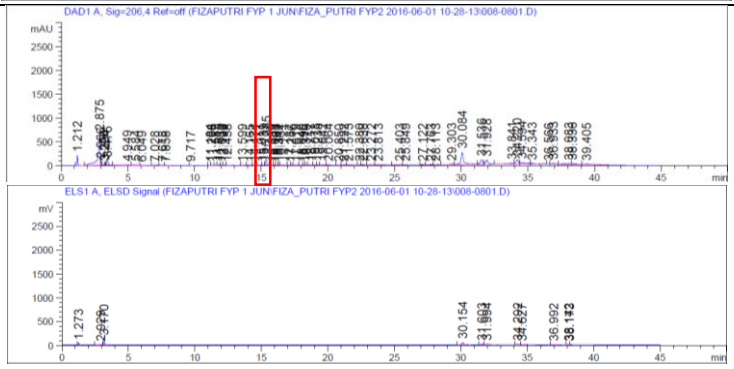
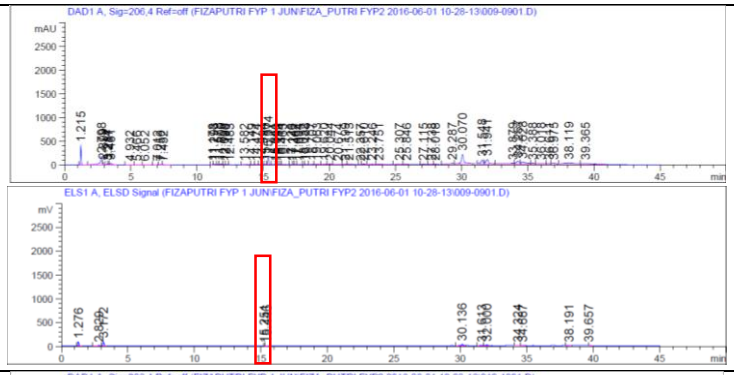
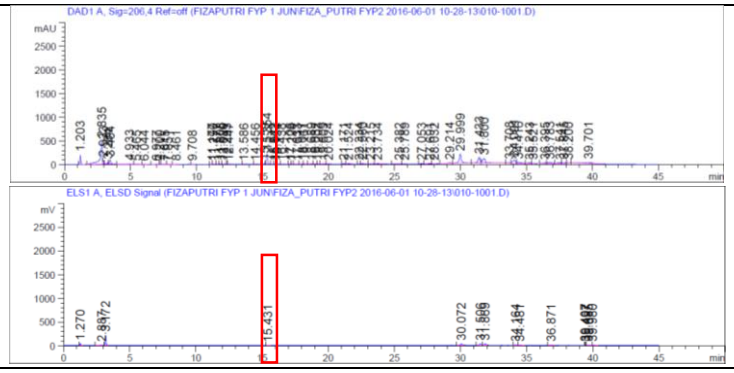
Day / Conc	Day 7	Retention Time
0 g/L		DAD = 15.381 ELSD = -
2 g/L		DAD = 15.385 ELSD = -
4 g/L		DAD = 15.374 ELSD = 15.446
8 g/L		DAD = 15.354 ELSD = 15.431

Table 4.7: Chromatogram and retention time of asiatic acid at day 14 in DAD and ELSD detector

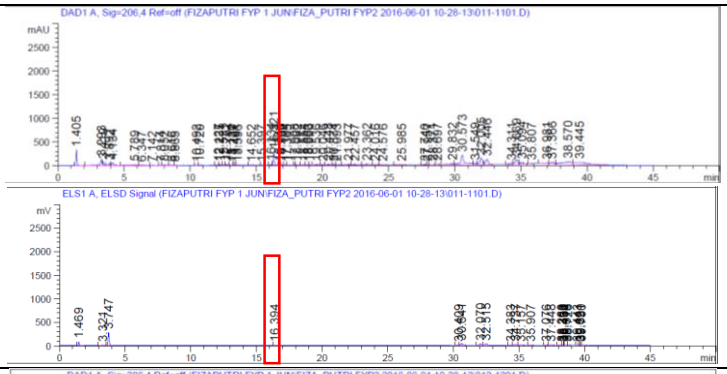
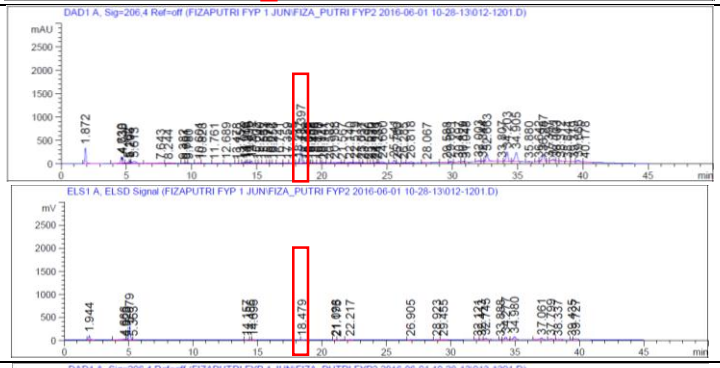
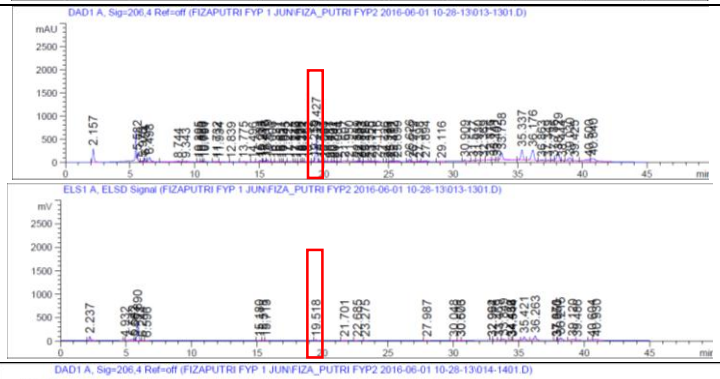
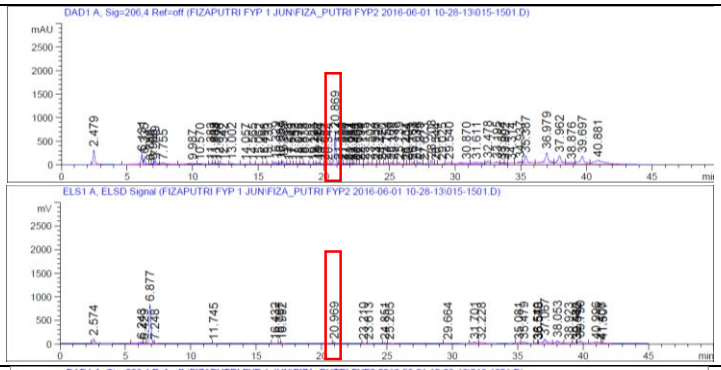
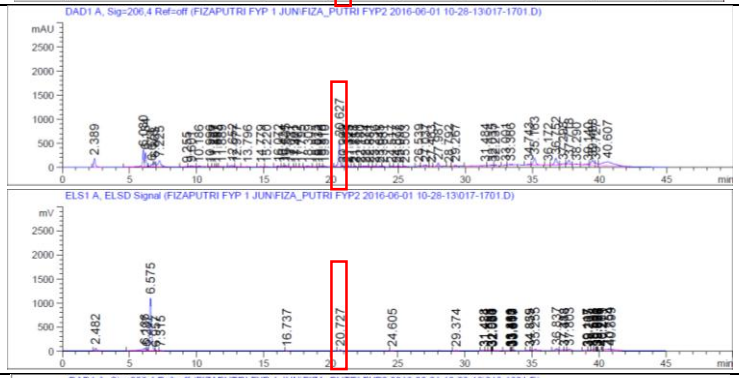
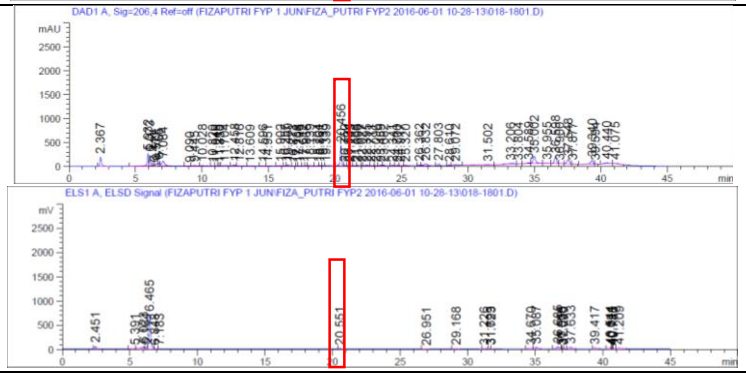
Day / Conc	Day 14	Retention Time
0 g/L		DAD = 16.321 ELSD = 16.394
2 g/L		DAD = 18.397 ELSD = 18.479
4 g/L		DAD = 19.427 ELSD = 19.518
8 g/L		DAD = 20.482 ELSD = 20.580

Table 4.8: Chromatogram and retention time of asiatic acid at day 21 in DAD and ELSD detector

Day / Conc	Day 21	Retention Time
0 g/L		DAD = 20.869 ELSD = 20.969
2 g/L		DAD = 20.822 ELSD = 20.914
4 g/L		DAD = 20.627 ELSD = 20.727
8 g/L		DAD = 20.456 ELSD = 20.551

CHAPTER FIVE

DISCUSSION

5.1 Observation of *in vitro C. asiatica* growth

Tissue culture technique can play an important role to multiply rapid elite clones and conservation of *C. asiatica* germplasm. Despite all of these efforts of the last 30 years, the *in vitro* of tissue culture systems have an objective to improve the production of secondary metabolites of a plant which in *C. asiatica* the major secondary metabolites compound was asiatic acid (Bourgaud, Gravot, S, & Gontier, 2001). In this research, full strength of MS basal medium with different concentrations and combinations of BAP with NAA were used for studying the *in vitro* multiplication responses which the concentration were 1.0 mg/L NAA combine with 1.0 mg/L BAP or 2.0 mg/L BAP.

Based on the results on Figure 4.1 (page 67), the MS media containing the best combinations of auxin and cytokinin were 1.0 mg/L NAA combine with 2.0 mg/L BAP. These hormones function best in the explant petioles which capable to directly develop

multiple shoots (Das, Hasan, Hossain, & Rahman, 2008). This can be justified by Stiff (1998), which the BAP hormone was a cytokine growth regulator that function to induce shoots while NAA hormone was an auxins that tends to induce root growth. The effect of higher BAP concentration in plant tissue culture were adventitious shoot formation and stimuli the outgrowth of axillary buds while the combination of BAP and NAA had a synergistic effect to induce cell division and the formation of callus (Duchefa, 2012).

The high frequency of multiple shoot regeneration were achieved on previous study conducted in MS media combination with 4.0 mg/L BAP with 0.1 mg/L NAA which it was the best treatment for multiple shoot induction and also give a maximum number of shoots per explant (Das, Hasan, Hossain, & Rahman, 2008). Rao, Rao, & Sadanandam (1999), stated that the stolon and the stem or the petioles were the best for callus induction followed by leaf explants. In this research, petiole was used and were the best among other parts of the plant. However, research done by Thangapandian, Suganya, & Theresa (2012) showed that 2 mg/L of BAP combined with 0.5 mg/L of NAA had a lower shoot induction than the combination of 2 mg/L of BAP with 0.5 mg/L of Indole-3-acetic acid (IAA). Since the *in vitro* plantlets were not grow enough to the expected time at 21 days (Gandi & Giri, 2013), the micropropagation stages with seaweed elicitor cannot be done. Thus, this research were continued using the natural plant that were supplemented with several different concentrations of seaweed elicitor.

5.2 HPLC chromatogram of asiatic acid

Monograph for *C. asiatica* in European Pharmacopeia (2011), stated that the reverse phase of HPLC was being used which the stationary phase was an ODS C18 silica gel and the mobile phase were acetonitrile and phosphoric acid buffer. Since the phosphoric acid was not available in faculty, acetonitrile and ultrapure water were being used as mobile phase in asiatic acid qualification (Rafamantana, *et al*., 2009). The gradient elution mechanism was being used and the gradient condition had been changed for several times to obtain the required chromatogram results which can be seen in Table 3.1 (page 65)

There was two types of detector being used in this HPLC which were DAD and ELSD. ELSD was a universal detector which can detect substance that was UV active and UV inactive. The principle of ELSD was the components was targeted and converted to a fine spray by nebulizer and was heated so that only the mobile phase was evaporated. Then, the light was directed to the remaining substance and the scattered light was detected which it will detect most of the components that are less volatile than mobile phase (Shimadzu, 2016). While, DAD detects absorption of substance in UV to VIS region and have much advantages which it allows the best wavelength to be selected for analysis for example 206 nm of wavelength was being used in this research (Kazakevich, 2010).

5.2.1 HPLC chromatogram reference of asiatic acid

Asiatic acid reference was dissolved in absolute HPLC grade methanol and detected by using HPLC detectors which were DAD at wavelength 206 nm and ELSD. The results on Table 5.1 shows the peak retention time of asiatic acid reference detect by DAD was 14.479 min while ELSD was 14.583 min. The area of the peak was 22800 mAU*s in DAD while ELSD was 19550 mAU*s.

Table 5.1: Results of retention time, area and height of asiatic acid reference

REFERENCE / SIGNAL	MeOH	
	206	**ELSD**
Retention Time (min)	14.479	14.583
Area (mAU*s)	2.280e4	1.955e4
Height (mAU)	1666.551	1161.246

The peak of retention time in asiatic acid reference act as guidance for peak of retention time in the *C. asiatica* sample. The results in Table 5.3 page 86 showed the peak of retention time in *C. asiatica* samples were progressively shift slightly to the right when the plant being exposed to higher concentration of seaweed elicitor and in the longer time. This can be seen, the retention time of the asiatic acid reference was 14.479 minutes (Table 5.1) while retention time of the samples at day 0 in concentration of 0 g/L was 15.415 minutes (Table 5.3). Thus, method validation was being conducted to clarified the retention peak of the sample was same with the retention peak of asiatic acid reference. This method was done by compared the retention time of three samples at day 0 in concentration of 0 g/L, 4 g/L and 8 g/L were compared with the retention time of asiatic acid reference and also mixture of asiatic acid reference+day 0 in concentration of 0g/L.

This can be seen in Table 5.2 that shows the retention time for the three samples was about 17 minutes while the retention time for reference was about 16 minutes. However, when one of the sample which at day 0 in concentration of 0 g/L was mixed with asiatic acid reference, the retention time was detected at 16 minutes. This can clarified that the slightly shift to the right of the retention time in the samples was an asiatic acid.

Table 5.2: Method validation by comparing retention time of samples with reference

DAY		0		REFERENCE			
				MeOH		MeOH + DAY 0 0 g/L	
SIGNAL / CONC (g/L)		206	ELSD	206	ELSD	206	ELSD
0	Retention Time (min)	17.974	-	16.554	16.662	16.436	16.516
	Area (mAU*s)	633.121		2.265e4	1.918e4	1.035e4	9314.587
	Height (mAU)	100.579		1476.117	1160.743	970.026	998.193
4	Retention Time (min)	17.966	-				
	Area (mAU*s)	766.298					
	Height (mAU)	123.181					
8	Retention Time (min)	17.975	-				
	Area (mAU*s)	671.37134					
	Height (mAU)	106.111					

5.2.2 HPLC chromatogram of asiatic acid in natural *C. asiatica*

Natural *C. asiatica* treated with difference concentration of seaweed elicitors was harvested at day 0, 7, 14 and 21 and the extract of each sample was being analysed through HPLC which can be seen in Table 5.3. Initially, the retention time at day 0 and day 7 for all concentration showed the peak at about 15 min, which it was the same retention time of the reference and the validation method that had slightly shift the peak to the right. However, when the plant was exposed to the elicitor at longer time, the retention time also being increased and shift to the right. This can be seen at day 14, when the concentration

of seaweed elicitor was increased, there was a progressive increase in retention time. Based on the results, at day 14, the retention time in concentration of 0 g/L was 16 min, the retention time in concentration of 2 g/L was 18 min, at concentration 4 g/L the retention time was 19 min and lastly, at concentration 8 g/L the retention time was 20 min. In addition, the retention time at day 21 for all concentrations were fixed to 20 min.

The slightly shifted to the right of the retention time in asiatic acid samples can be defined as the structure of asiatic acid molecule had been changed to less polar molecules than asiatic acid by the elicitor. This is because, the reverse phase system consist of non-polar stationary phase which the less polar molecule will have a large interaction with C18 silica gel. Hence, the less polar molecule was eluted late than the asiatic acid molecule. The changes of structure in asiatic acid molecule might due to esterification of carboxy group (-COOH) at carbon 28 position in asiatic acid to form ester which was acetyl methyl asiatic acid. Since ester molecule are less polar than carboxylic acid (Ophardt, 2003), molecule of acetyl methyl asiatic acid were eluted late than asiatic acid. Further study need to be carried out in order to elucidate the structure and isolate the compound to check the changes in asiatic acid structure.

Table 5.3: Results of retention time, area and height of asiatic acid samples in different concentration of seaweed elicitor at day 0, 7, 14 and 21

DAY		0		7		14		21	
SIGNAL / CONC (g/L)		206	ELSD	206	ELSD	206	ELSD	206	ELSD
0	Retention Time (min)	15.415	-	15.381	-	16.321	16.394	20.869	20.969
	Area (mAU*s)	601.795		980.387		1660.730	17.414	5252.202	154.474
	Height (mAU)	97.755		164.404		276.280	3.255	684.292	23.873
2	Retention Time (min)	15.428	-	15.385	-	18.397	18.479	20.822	20.914
	Area (mAU*s)	664.234		1002.904		2520.325	48.311	4913.647	138.217
	Height (mAU)	111.671		167.255		396.108	8.404	636.097	20.865
4	Retention Time (min)	15.393	-	15.374	15.446	19.427	19.518	20.627	20.727
	Area (mAU*s)	754.514		951.979	75.420	3503.806	88.175	4134.544	106.703
	Height (mAU)	126.253		161.275	5.112	527.506	14.455	549.731	15.844
8	Retention Time (min)	15.403	-	15.354	15.431	20.482	20.580	20.456	20.551
	Area (mAU*s)	686.584		1356.713	11.576	3249.371	71.833	3094.667	61.386
	Height (mAU)	115.903		221.509	1.894	438.131	10.397	418.809	9.471

Based on the Table 5.3, there was no reading for ELSD at day 0 for all concentration and at day 7 for the concentration of 0 g/L and 2 g/L. This might because the temperature was not control during analysing the asiatic acid samples which the temperature must be set at drift tube since it was an important consideration during performing high-sensitivity analysis with an ELSD. This can be seen, when analysing a substance with low boiling points, the intensity of the signals depends on the temperature. For example, when the temperature was too high, the substance which was asiatic acid may be partially or completely evaporated together with the mobile phase that consequently make the quantity of minute solid particles decreased the scattered light. This may lead to a reduction in sensitivity detection or make the detection completely impossible (Shimadzu, 2016).

5.2.3 Determination amount of asiatic acid from chromatogram

Peak of retention time for asiatic acid from the chromatogram can be used to determine the amount of asiatic acid by using the area under the curve of the peak. Chromacademy, (2016), states that to obtain amount of sample, response factor (Rf) must be calculated first from the peak area of reference divided by the concentration of reference. The peak area of reference can be obtain in Table 5.1 page 83, which the value of peak reference are 22800 mAU*s and the concentration of reference was in 1 mg/ml. The Rf value was calculated based on formula 5.1 and the value was 22800.

Response factor (Rf) = $\frac{\text{Peak area of reference}}{\text{Concentration of reference } \left(\frac{\text{mg}}{\text{ml}}\right)}$ .....(5.1)

$$Rf = \frac{22800\ mAU*s}{1\ mg/ml}$$

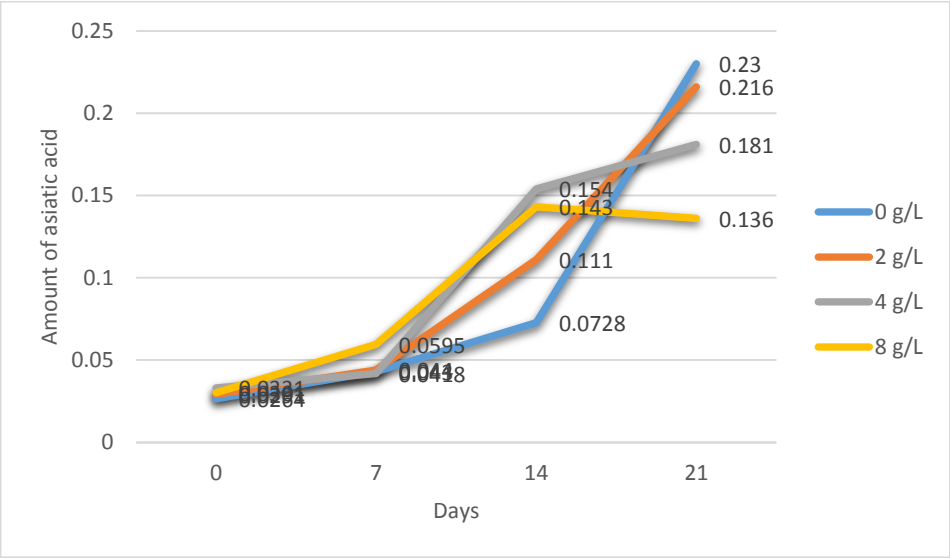
$$= 22800$$

Since the Rf value was known, the concentration of asiatic acid can be obtain by the peak area of sample dividing with Rf value that can be seen in formula 5.2 (Chromacademy, 2016). Based on Table 5.4, the area of peak of each concentration was calculated using this formula which the peak area of sample was divided by 22800 to obtain concentration of asiatic acid in mg/ml unit. The total concentration of each sample in difference concentration of seaweed elicitor was plotted on line graph against days which can be seen in Figure 5.1.

Concentration of Asiatic acid (mg/ml) = $\frac{\text{Peak area of sample}}{\text{Response factor}}$ .....(5.2)

Table 5.4: Area of the peak and total concentration of asiatic acid in different concentration of seaweed elicitor at day 0, 7, 14 and 21

CONC (g/L) \ DAY		0	7	14	21
0	**Area (mAU*s)**	601.795	980.387	1660.730	5252.202
	Total Conc (mg/ml)	**0.0264**	**0.0430**	**0.0728**	**0.230**
2	**Area (mAU*s)**	664.234	1002.904	2520.325	4913.647
	Total Conc (mg/ml)	**0.0291**	**0.0440**	**0.111**	**0.216**
4	**Area (mAU*s)**	754.514	951.979	3503.806	4134.544
	Total Conc (mg/ml)	**0.0331**	**0.0418**	**0.154**	**0.181**
8	**Area (mAU*s)**	686.584	1356.713	3249.371	3094.667
	Total Conc (mg/ml)	**0.0301**	**0.0595**	**0.143**	**0.136**



Graph 5.1: Amount of asiatic acid in different concentration of seaweed elicitor at day 0, 7, 14 and 21

Based on the Graph 5.1, initially at day 0 there is slightly different in amount of asiatic acid for all concentration of seaweed elicitor which the highest amount was in concentration of 4 g/L which was 0.0331 mg/ml while the lowest amount was in control concentration which was 0.0264 mg/ml. This is because, the plant was being introduced in the morning and was harvested on the same day at the evening so the plant do not interact yet with the seaweed elicitor.

At day 7, there is significant different between the concentration which the highest amount was 0.0595 mg/ml at concentration of 8 g/L while the lowest reading amount of asiatic acid was 0.0418 mg/ml at concentration of 4 g/L. This results show that, higher concentration of elicitor can enhance the production of asiatic acid in shorter time which

8 g/L was the best among others. However, all the concentrations were increased than the amount of asiatic acid from day 0.

The results at day 14 shows a large increasing and difference between elicited plant and the control plant. The amount of asiatic acid in elicited plant were higher than the control which when the concentration of seaweed elicitor was increase, the amount of asiatic acid production also increase. The best results at day 14 were concentration of 4 g/L which was 0.154 mg/ml while the second best result was 0.143 in concentration of 8 g/L. To harvest at shorter time with large amount of secondary metabolite, the concentrations at 4 g/L and 8 g/L were the best.

Lastly, at day 21 the control plant had the highest amount of asiatic acid than the elicited plants. All concentration were increasing than the amount of asiatic acid at day 14 except concentration of 8 g/ml which the production of asiatic acid was decreasing. This trends were good because the limitation of 8 g/ml was known which the highest production of asiatic acid in this concentration was limit to day 14. So, further study need to be conducted for longer time in order to know the limit of the other concentration.

5.3 Factor contribute variation in chemical composition of plant materials

Natural *C. asiatica* was considered as a plant material originates from the same species, but it does not necessarily contain the same composition. This was because, within a single plant species, there can be a large quantitative as well as qualitative differences in the composition of bioactive secondary metabolites. For example, Schlag and McIntosh, (2006) states that American ginseng or scientifically known as *panax* quinquefolius there must be at least two distinct chemotypes differing in relative composition of two therapeutic protopanaxatriol ginsenoside. This conditions was due to two main factor which are genetic variability and differences in growing condition.

The first factor was genetic variation between plants which can be a major source of variation in plants secondary metabolites. It can effect the amount and the type of metabolite produced thus hindering the reproducibility in test (Poulev *et al.*, 2003). The differences between cytotypes were being observed not only quantitatively which had varying amount of bioactive compounds, but also qualitatively with some of the compounds being present only in some plants and absent in others. However, this variation can be eliminated through the generation of uniform plant material by the usage of plant tissue culture micropropagation of selected plants in order to ensure that the consistency levels of secondary metabolites (Gorelick & Bernstein, 2014).

The other factor that contribute to variation in chemical composition of plant materials was environmental variation such as light, temperature, relative humidity, water availability, heavy metal and salinity were all affect the plants secondary metabolites. Gorelick & Bernstein (2014), states that the changes may be happen due to the adaptive mechanism or damage mechanism which induce the secondary metabolites by exposure it to specific growing conditions.

5.4 Mechanism of action in biotic elicitor

Biotic elicitor induce the defence response by specific chemical compound which was the elicitor trigger a reaction in the threatened plant that stimulate the beneficial effects. A number of biotic elicitor can increase the production of secondary metabolite in medicinal plant cell culture by using biological mixtures. This can be seen in *taxus chinensis* cells that was treated with an endophytic fungus found in the bark of *T. chinensis* tree produced a third fold of taxol compared to non-elicited cells (Wang *et al.*, 2001)

The mechanism of action in biotic elicitor was by signalling pathways of elicitation which the perception of elicitor was discovered as "elicitor receptors" (Gorelick & Bernstein, 2014). This theory can be supported by the discovery of a number of high affinity binding sites for peptides, glycopeptides and oligosaccharides on plant cellular surface (Mithofer

et al., 2000). After initial perception of elicitor was done, a number of signalling pathways may be activated by the plants which involves G-proteins, protein kinases, and ion channels. Signallers transmit the elicitor signal to the downstream of cascade which lead to the formation of reactive oxygen species (ROS), hormonal signal such as jasmonate and consequently produce secondary metabolites in plants (Blume *et al.*, 2000).

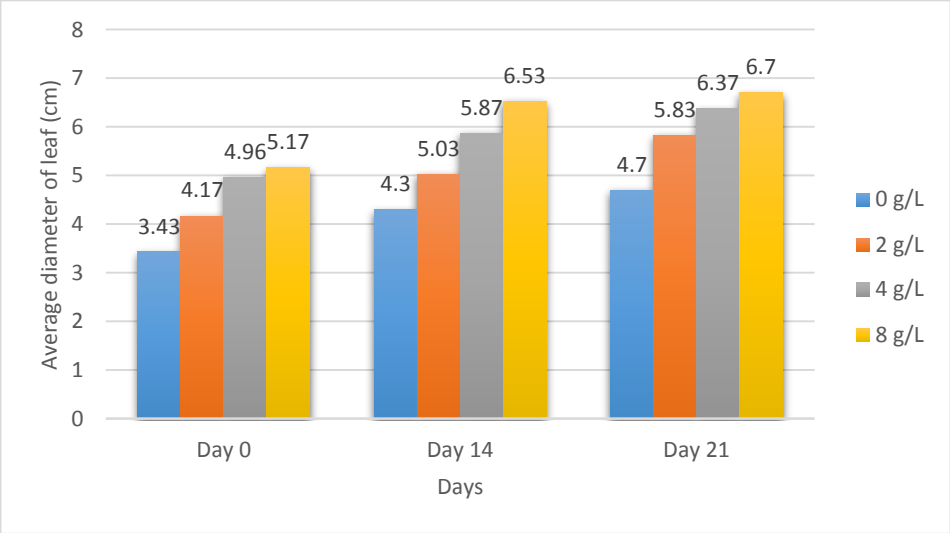
5.4.1 Mechanism of action of seaweed elicitor in *C. asiatica*

Natural *C. asiatica* was being treated with different type of seaweed elicitor which it was contain polysaccharides of carrageenan from red seaweed. Carrageenan was a naturally-occurring family of polysaccharides extracted from red seaweed (Duchefa, 2012). In previous study conducted by Sharma, Chauhan, & Sood (2015), the first reported demonstrating the potential of seaweed extract act as an elicitor was the enhancement of growth and secondary metabolites production in *Picrorhiza kurroa*. The mechanism of action of seaweed extract was the expression of 1-deoxy-D-xylulose 5-phosphate synthase and 3-hydroxy-3-methylglutaryl-CoA reductase that act as the key enzymes of non-mevalonate and mevalonate pathways which being up regulated the metabolite content. Thus increased much higher amount of secondary metabolites and the growth of *Picrorhiza kurroa*.

5.5 Observation of natural *C. asiatica* growth with seaweed elicitor

The natural C. asiatica was being observed in different concentration of seaweed elicitor at day 0, 7, 14 and 21. The macroscopic morphological characteristics such as diameter of leaf, number of flower and number of new shoots was being observed and can be related to the production of secondary metabolites elicit by the seaweed elicitor in *C. asiatica.*

5.5.1 Diameter of leaf

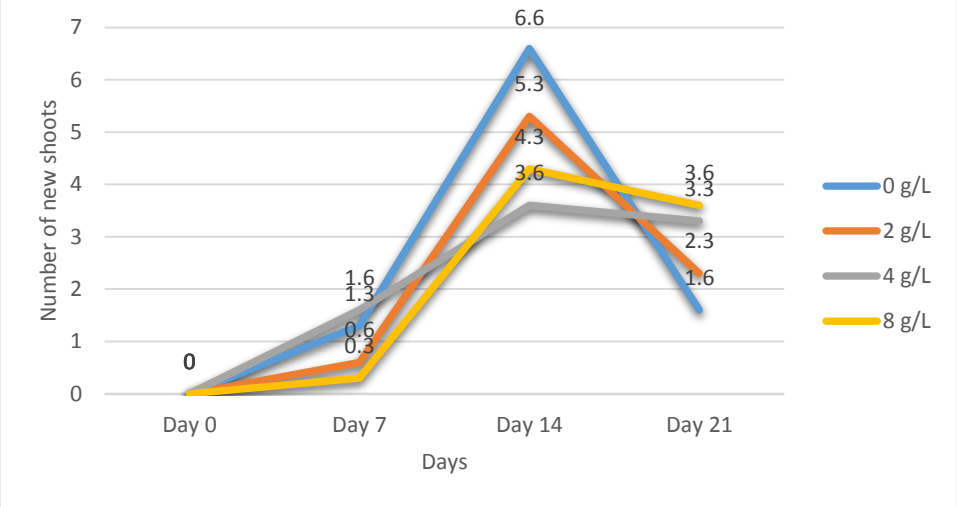


Graph 5.2: Average diameter of leaf (cm) in difference concentration of seaweed elicitor at day 0, 14 and 21

The average diameter of leaf in Graph 5.2 was increase when the concentration and the time of exposure to the seaweed was increase. This was because the elicitation of carrageenan seaweed give a good results on growth of leaf characters which can be seen

in chickpea and maize plants were conducted on previous study by using *K. alvarezii* act as elicitor by Bi, Iqbal, Arman, Ali, & Hassan (2011).

5.5.2 Number of new shoots

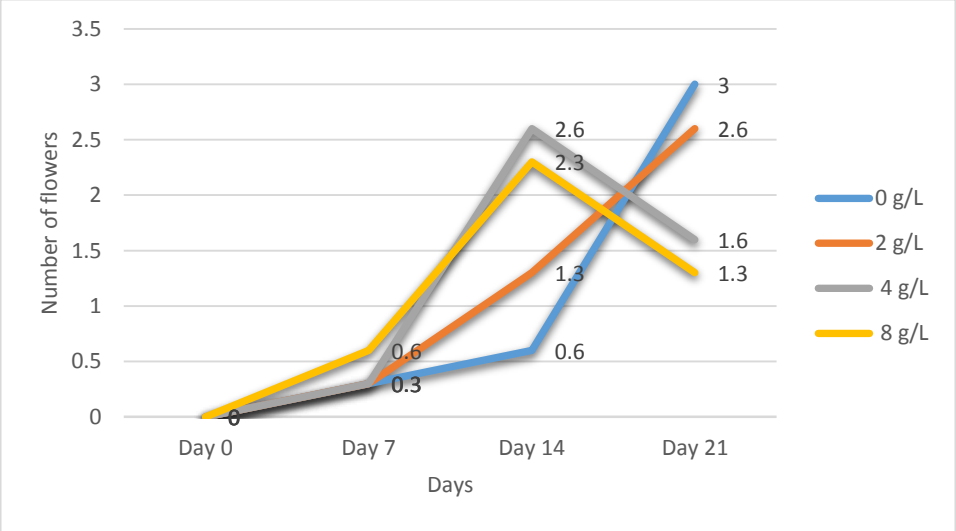


Graph 5.3: Number of new shoots in difference concentration of seaweed elicitor at day 0, 7, 14 and 21

The number of new shoots were observed from day 0 until day 21 which at day 0 there was no observation of new shoots growth. At day 7 the highest value was at concentration of 4 g/L while the lowest value was at concentration of 8 g/L. While, at day 14, the highest value was 6.6 at concentration 0 g/L and the lowest value was at concentration of 4 g/L. Lastly, at day 21 the highest value was at concentration of 8 g/L while the lowest value was at concentration of 0 g/L.

The trends show that when comparing this graph in Graph 5.3 with the graph amount of asiatic acid produced in different concentration of seaweed elicitor in Graph 5.1 page 88, the opposite trends were produced. In other words, when the stress were low, asiatic acid production also low so the plants focus to grow the new shoots that lead to increase the production of new shoots. Other than that, *K. alvarezii* contain cytokinin that induce the growth of shoot such as kinetin and zeatin (Zodape *et al*., 2011).

5.5.3 Number of flowers



Graph 5.4: Number of flowers in different concentration of seaweed elicitor at day 0, 7, 14 and 21

The number of flowers produce in different concentration of seaweed elicitors at day 0, 7, 14 and 21 was being observed. Initially at day 0, there was no flower being produced for all concentration. While at day 7, the highest value was at concentration of 8 g/L. In

addition, the highest value at day 14 was at concentration of 4 g/L and the lowest was at concentration of 0 g/L. Lastly, at day 21, the highest concentration was at concentration of 0 g/L while the lowest was at concentration of 8 g/L.

Since the research were conducted on April to June 2016, the flowering season of *C. asiatica* flower was within the time (Singh *et al.*, 2010). So, it influenced the number of flower being produced during the research. Furthermore, the elicitation of *K. alvarezii* towards *C. asiatica* induced the number of flowering when compared to the amount of asiatic acid produced. The trend of this graph in Graph 5.4 when compared to the graph amount of asiatic acid produced in different concentration of seaweed elicitor in Graph 5.1 page 88, shows the same trends. In other words, when the stress were high, the production of asiatic acid also high and induce the production of new flower. It also shows the flowering time start earlier in elicited plants (Bi *et. al.*, 2011). The normal C. asiatica flower consist a single fascicled umbels consisting 3 flowers and rarely 2 or 4 but flower produce by *C. asiatica* that being induced with elicitor consist more than single umbel which can be seen in Figure 4.5 page 73

CHAPTER SIX

CONCLUSION

Biotic elicitor like *K. alvarezii* seaweed elicitor can induced the amount of secondary metabolite in *Centella asiatica* by manipulating various concentration of elicitor. Initially, plant tissue culture technique was used in this research but the plantlets were not grown enough in expected time and the project was changed to observe the elicit plants in natural condition. However, the *in vitro* plantlets containing plant growth regulator BAP and NAA was also being observed for their growth in two month.

It can be concluded that *K. alvarezii* can increase the production amount of asiatic acid in *C. asiatica* at shorter time. This can be seen, at day 7, the best concentration was concentration of 8 g/L while at day 14, the concentration of 4 g/L and 8 g/L were the best. However, until day 21, concentration of 0 g/L showed the best. So to harvest the plant in shorter time with higher concentration of secondary metabolite, 4g/L and 8 g/L were the best. The graph line pattern of amount of asiatic acid in concentration of 8 g/L was the

best since the limit of asiatic acid production were known but to induce secondary metabolite in longer time others were better. The *in vitro* plantlets showed the combination of 1.0 mg/L of NAA with 2.0 mg/L of BAP gave a good results in growth of petiole explants.

HPLC was used in this study to determine the retention peak and amount of asiatic acid presence in the sample by calculate area under the curve of the peak by using a formula. However, method validation was being conducted in order to clarified the retention peak of the sample was same with the retention peak of asiatic acid reference because there was slightly right shift of sample retention peak. Less polar asiatic acid molecules was being produced when the plant being exposed to higher concentration and longer time since the retention peak was progressively shift to the right in the chromatogram.

There were two suggestion for this research, firstly, the research need to be further investigated for longer time of period in order to get better promising results due to time constrain such as to know the limit production of secondary metabolite in each concentration. Lastly, further study need to be carried out for structure elucidation and isolation of compound in order to check the changes in structure of asiatic acid in C. *asiatica* when exposed to the higher concentration of elicitor in a longer time.

BIBLIOGRAPHY

Abcam. (2015). *Madecassic acid, Asiaticoside*. Retrieved Disember 11, 2015 from Abcam discover more: http://www.abcam.com/madecassic-acid-ab141952-references.html

Beaulieu, J. C., & Baldwin, E. A. (2002). Flavor and Aroma of Fresh-Cut Fruits and Vegetables. In *Fresh-Cut Fruits and Vegetables: Science, Technology, and Market* (pp. 391-425). CRC Press.

Bi, F., Iqbal, S., Arman, M., Ali, A., & Hassan, M.-u. (2011). Carrageenan as an elicitor of induced secondary metabolites and its effects on various growth charactersof chickpea and maize plants. *Journal of Saudi Chemical Society*, 269-273.

BiologyDiscussion. (2013). *Plant Tissue Culture Media: Types, Constituents, Preparation and Selection*. Retrieved Disember 13, 2015 from Biology Discussion: http://www.biologydiscussion.com/plants/plant-tissue-culture/plant-tissue-culture-media-types-constituents-preparation-and-selection/10656

Blume B, Nürnberger T, Nass N, Scheel D (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 12: 1425–1440

Bourgaud, F., Gravot, A., S, M., & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science, 161*(5), 839-851.

Caymanchem.com (2015). Asiatic acid, Asiaticoside. Retrieved Disember 11, 2015 from Cayman Chemical: https://www.caymanchem.com/app/template/Product.vm/catalog/11818

Centesia. (2013). *Madecassic Acid, Asiaticoside, Madecassoside, Food*. Retrieved Disember 11, 2015 from Centesia by nature: http://centella-extract.com/

Chaturvedi, P., & Joshi, K. (2013). Therapeutic Efficiency of *Centella asiatica* (L.) Urb. An Underutilized Green Leafy Vegetable : an Overview. *International Journal of Pharma and Bio Sciences*, 135-149.

ChEBI. (2015). *Asiatic acid, Madecassic acid, Asiaticoside, Madecassoside*. Retrieved Disember 8, 2015 from ChEBI: http://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI:73058

ChemicalBook. (2010). *Madecassic acid*. Retrieved Disember 8, 2015 from Chemical Book: http://www.chemicalbook.com/ChemicalProductProperty_EN_CB7280283.htm

Chromacademy. (2016). *Internal standard theory of HPLC quantitative and qualitative hplc*. Retrieved from LC GC's CHROMacademy: www.chromacademy.com

Cluzet, S., Torregrosa, C., Jacquet, C., Lafitte, C., Fournier, J., & Mercier, L. (2004). Gene expression profiling and protection of Medicago truncatula against a fungal infection in response to an elicitor from the green alga Ulva spp. *Plant Cell Environ*, 917–928.

Das, A. J. (2011). Review on Nutritional, Medicinal and Pharmacological Properties of *Centella asiatica* (Indian pennywort). *Journal of Biologically Active Products from Nature*, 216-228.

Das, R., Hasan, M., Hossain, M. S., & Rahman, M. (2008). MICROPROPAGATION OF Centella asiatica L. AN IMPORTANT MEDICINAL HERB. *Progress. Agric*, 51-56.

Dave, K. R., & Katyare, S. S. (2002). Effect of alloxan induced diabetes on serum and cardiac butrylcholinesterases in the rat. *Journal of Endocrinology*, 241-250.

DifferenceBetween. (2013). *Difference Between Primary and Secondary Metabolites*. Retrieved Disember 7, 2015 from Difference Between.com: http://www.differencebetween.com/difference-between-primary-and-vs-secondary-metabolites/

Duchefa. (2012). Plant Hormones. In *Plant Cell and Tissue Culture Phytopathology Biochemicals* (pp. 18-22). Netherlands: DUCHEFA BIOCHEMIE B.V.

European Pharmacopiae. (2011). Centella asiaticae herba. In *European Pharmacopoiea 7.0* (pp. 1096-1097). Conseil de l'Europe.

Fu, T.-J., Singh, G., & Curtis, W. R. (2012). Elicitation-Manipulating and Enhancing Secondary Metabolite Production . In G. Singh, *Plant Cell and Tissue Culture for the Production of Food Ingredients* (p. 102). Springer Science & Business Media.

Gandi, S., & Giri, A. (2013). Production and quantification of Asiatic acid from in vitro raised shoots and callus cultures of *Centella asiatica* (L.) Urban. *Annals of Phytomedicine*, 95-101.

Gao, S., & Hu, M. (2010). Bioavailability Challenges Associated with Development of Anti-cancer Phenolics. *Mini Reviews in Medicinal Chemistry*, 67-550.

Gershenzon, J., & Dudareva, N. (2007). The Function of Terpene Natural Products in the Natural World. *Nature Chemical Biology 3*, 408-14.

Giada, M. d. (2013). Food Phenolic Compounds: Main Classes, Sources and Their Antioxidant Power. In J. Morales González, *Oxidative stress and chronic degenerative diseases.* Rijeka: InTech.

GlobinMed. (2015). *Centella Asiatica*. Retrieved Disember 12, 2015 from GlobinMed Global information Hub on Integrated Medicine: http://www.globinmed.com/index.php?option=com_content&view=article&id=102002:centella-asiatica-l-urban&catid=209&Itemid=143

Gorelick, J., & Bernstein, N. (2014). Elicitation: An Underutilized Tool in the Development of Medicinal Plants as a Source of Therapeutic Secondary Metabolites. *Advances in Agronomy*, 201-219.

Govarthanan, M., Rajinikanth, R., Kamala-Kannan, S., & Selvankumar, T. (2015). A comparative study on bioactive constituents between wild and in vitro propagated Centella asiatica. *Journal of Genetic Engineering and Biotechnology*, 25–29.

Guo, J., Cheng, C., & Koo, M. (2004). Inhibitory effects of *Centella asiatica* water extract and asiaticoside on inducible nitric oxide synthase during gastric ulcer healing in rats. *Planta Med*, 4-1150

Halladay, P., & Gilmour, D. A. (1995). *Conserving Biodiversity Outside Protected Areas: The role of traditional agrp-ecosystems.* Retrieved from International Union for Conservation of Nature: https://portals.iucn.org/library/efiles/documents/FR-013.pdf

Harris, E. D. (2008). *Biochemical Facts behind the Definition and Properties of Metabolites.* Retrieved November 28, 2015 from U.S. Food and Drug Administration: http://www.fda.gov/ohrms/dockets/ac/03/briefing/3942b1_08_Harris%20Paper.pdf

Hashemi, S. M. (2015). Mechanism of Elicitors' activity in Plant Cell and Tissue Culture for Production of Secondary Metabolites. University of Tehran, Iran.

HMPC. (2010). Assessment report on Centella asiatica (L.) Urban, herba. *European Medicines Agency*, 4.

Hostettmann, K., & Marston, A. (2005). Acidic Hydrolysis. In *Saponins* (p. 177). Cambridge University Press.

Hsu, Y.-L., Kuo, P.-L., Lin, L.-T., & Lin, C.-C. (2005). Asiatic Acid, a Triterpene, Induces Apoptosis and Cell Cycle Arrest through Activation of Extracellular Signal-Regulated Kinase and p38 Mitogen-Activated

Protein Kinase Pathways in Human Breast Cancer Cells. *The Journal of Pharmacology and Experimental Therapeutics*, 333-344.

Hussain, A., Qarshi, I. A., Nazir, H. & Ullah, I. (2012). Plant Tissue Culture. *Current Status and Opportunities*, 1-28.

Indena. (2013). *Asiaticoside: Anti-wrinkles, lip care.* Retrieved Disember 12, 2015 from Indena.com: http://www.indena.com/pdf/asiaticoside_st_pc.pdf

Indianmirror. (2015). *Gotu Kola*. Retrieved Disember 12, 2015 from Indian Mirror: http://www.indianmirror.com/ayurveda/gotu-kola.html

Islam, A. K., Ismail, Z., Ahmad, M. N., Othman, A. R., Dharmaraj, S., & Shakaff, A. Y. (2003). Taste Profiling of *Centella Asiatica* by a Taste Sensor. *Sensors and Materia!s*, 209-218.

James, J., & Dubery, I. (2011). Identification and Quantification of Triterpenoid Centelloids. *Journal of Planar Chromatography*, 82–87.

Jamiepighin. (2003). *Your Guide to Plant Cell Culture*. Retrieved Disember 11, 2015, from The Science Creative Quarterly: http://www.scq.ubc.ca/your-guide-to-plant-cell-culture/

Jamil, S. S., Qudsia, N., & Salam, M. (2007). *Centella asiatica* (L.) Urban- A review. *Indian Journal of Natural Products and Resources*, 158-170.

Kabera, J. N., Semana, E., Mussa, A. R., & He, X. (2014). Plant Secondary Metabolites: Biosynthesis, Classification, Function and Pharmacological Properties. *Journal of Pharmacy and Pharmacology 2*, 377-392.

Kar, A. (2007). *Pharmacognosy and pharmacobiotechnology*. New Delhi: New Age International (P) Ltd.

Kasture, Veena S., Gosavi, Seema A., Ajage, Rohit K. Deshpande, Shraddha G., Inamke, Shekhar R., Kolpe Jyoti B. (2014). Comparative study of brahmi and brmhamanduki: A review. *World Journal of Pharmacy And pharmaceutical Sciences*, 2217-2230.

Kavitha, C., Agarwal, C., & Agarwal, R. (2011). Asiatic acid inhibits pro-angiogenic effects of VEGF and human gliomas in endothelial cell culture models. *PLoS One*.

Kazakevich, Y. (2010). *Diode-array detectors*. Retrieved from Analytical Chemistry: http://hplc.chem.shu.edu/NEW/HPLC_Book/Detectors/det_uvda.html

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., . . . Prithiviraj, B. (2009). Seaweed Extracts as Biostimulants of Plant Growth. *Journal Plant Growth Regulator*, 386–399.

Kim, W.-J., Kim, J., Veriansyah, B., Kim, J.-D., Lee, Y.-W., Oh, S.-G., & Tjandrawinatad, R. R. (2009). Extraction of bioactive components from Centella asiatica using subcritical water. *The Journal of Supercritical Fluids*, 211–216.

Kumar, K., & Rao, I. U. (2012). Morphophysiologicals Problems in Acclimatization of Micropropagated Plants in - Ex Vitro Conditions- A Review. *Journal of Ornamental and Horticultural Plants*, 271-283.

Kumar, V., Bharadwaj, R., Gupta, G., & Kumar, S. (2015). An Overview on HPLC Method Development, Optimization and Validation process for drug analysis. *The Pharmaceutical and Chemical Journal*, 30-40.

Kumar, V., Kumar, C. S., Hari, G., Venugopal, N. K., Vijendra, P. D., & Basappa, G. B. (2013). Homology modeling and docking studies onoxidosqualene cyclases associated with primary and secondary metabolism of Centella asiatica. *SpringerOpen Journal*, 1-12.

Kupiec, T. (2004). Quality-Control Analytical Methods: High-Performance Liquid Chromatography. *International Journal of Pharmaceutical Compounding*, 223-227.

Labster. (2014). *Plant Secondary Metabolites*. Retrieved November 27, 2015 from Labster: http://learn.labster.com/index.php/Plant_Secondary_Metabolites

Maffei, M. (2010). Sites of Synthesis, Biochemistry and Functional Role of Plant Volatiles. *South African Journal of Botany*, 612-31.

Mercier, L., Lafitte, C., Borderies, G., Briand, X., Esquerré-Tugayé, M.-T. and Fournier, J. (2001), The algal polysaccharide carrageenans can act as an elicitor of plant defence. *New Phytologist*, 149: 43–51

Merriam-Webster. (2015). *Partition Chromatography*. Retrieved November 30, 2015 from Merriam-Webster An Encyclopaedia Britannica Company : http://www.merriam-webster.com/medical/partition%20chromatography

Mithöfer A, Fliegmann J, Neuhaus-Url G, Schwarz H, Ebel J (2000) The hepta-beta-glucoside elicitor-binding proteins from legumes represent a putative receptor family. Biol Chem 381 705–713

Mohammadparast, B., Rasouli, M., Rustaiee, A., Zardari, S., & Agrawal, V. (2014). Quantification of asiatic acid from plant parts of Centella asiatica L. and enhancement of its synthesis through organic elicitors in in vitro. *Hortic. Environ. Biotechnol.*, *55*(6), 578-582. http://dx.doi.org/10.1007/s13580-014-0168-5

Mondal, D., Ghosh, A., Prasad, K., Singh, S., Bhatt, N., Zodape, S. T., Ghosh, P. (2015). Elimination of gibberellin from *Kappahycuz alvarezii* seaweed sap foliar spray enhances corn stover production without compromising the grain yield advantage. *Plant Growth Regul*, 657-666.

Nicolaou, K. C., Chen, J. S., & Elias, J. C. (2011). *Classics in Total Synthesis. Further Targets, Strategies, Methods III.* Wiley-VCH.

Ophardt, C. (2003). *Polarity of Organic Compounds*. Retrieved from Virtual Chembook: http://chemistry.elmhurst.edu/vchembook/213organicfcgp.html

Orhan, I. E. (2012). *Centella asiatica* (L.) Urban: From Traditional Medicine to. *Evidence-Based Complementary and Alternative Medicine*, 1-8.

Patel, H., & Krishnamurthy, R. (2013). Elicitors in Plant Tissue Culture. *Journal of Pharmacognosy and Phytochemistry*, 60-65.

Poulev, A., O'Neal, J., Logendra, S., Pouleva, R., Timeva, V., Garvey, A., . . . Raskin, I. (2003). Elicitation, a new window into plant chemodiversity and phytochemical drug discovery. *Journal Med Chem*, 7-2542.

PubChem. (2015). *Asiatic Acid, Madecassic Acid, Asiaticoside, Madecassoside*. Retrieved December 11, 2015 from PubChem Open Chemistry Database: https://pubchem.ncbi.nlm.nih.gov/compound/24825670

Qadry, J. S. (2010). Plant Tissue Culture Techniques. In *Pharmacognosy* (pp. 40-51). Prof. J. S. Qadry.

Rafamantana, M., Rozet, E., Raoelison, G., SU, R., Hubert, P., & Quetin-Leclercq, J. (2009). An improved HPLC-UV method for the simultaneous quantification of triterpenic glycosides and aglycones in leaves of Centella asiatica (Apiaceae). *Journal of Chromatography B*, 2396-2402.

Rao, K., Rao, S., & Sadanandam, M. (1999). Tissue culture studies. *Indian J. of Pharm. Sci.*,, 392-395.

Robards, K., Jackson, P. E., & Haddad, P. A. (2012). Chromatographic Separation Simply Explained. In *Principles and Practice of Modern Chromatographic Methods* (p. 8). Elsevier.

Schlaq, E., & McIntosh, M. (2006). Ginsenoside content and variation among and within American ginseng (Panax quinquefolius L.) populations. *Phytochemistry*, 9- 1510.

Schultz, J. (2015). *Secondary Metabolites in Plants*. Retrieved 28 November from Biology Reference: http://www.biologyreference.com/Re-Se/Secondary-Metabolites-in-Plants.html

Seevaratnam, V., anumathi, P.B., Premalatha, M. R., Sundaram, S. P. & Arumugam, T. (2012). Functional properties Of *Centella Asiatica (*L.): A Review. *International Journal of Pharmacy and Pharmaceutical Sciences*, 1-7.

Selleckchem.com. (2013) *Asiatic acid*. Retrieved Disember 11, 2015 from Selleckchem.com: http://www.selleckchem.com/products/Asiatic-acid.html

Shah, S. A. A. (2015). *Terpenoids*. Presentation, Faculty of Pharmacy, UiTM Puncak Alam.

Sharma, N., Chauhan, S. R., & Sood, H. (2015). Seaweed extract as a novel elicitor and medium for mass propagation and picroside-I production in an endangered medicinal herb Picrorhiza curroa. *Plant Cell Tissue Organ Culture*, 57-65.

Sharma, S., & Vimala, Y. (2010). Adenine sulphate enhanced in vitro shoot regeneration in centella asiatica (l.) Urban. *Indian Botanical Society*, 30-33.

Shimadzu. (2016). *Principles and Practical Applications of Shimadzu's ELSD-LT 2 Evaporative Light Scattering Detector*. Retrieved from http://www.shimadzu.com/: http://www.ssi.shimadzu.com/products/literature/lc/c190e108.pdf

Shimadzu. (2015). *Preparing Buffer Solutions*. Retrieved Disember 11, 2015 from Shimadzu Excellence in Science: http://www.shimadzu.com/an/hplc/support/lib/lctalk/38/38lab.html

Sigma-Aldrich. (2015). *Madecassoside from Centella asiatica*. Retrieved Disember 12, 2015 from Sigma-Aldrich: http://www.sigmaaldrich.com/catalog/product/sigma/m6949?lang=en&region=MY

Singh P, Singh JS. (2002) Recruitment and competitive interaction between ramets and seedlings in a perennial medicinal herb *Centella asiatica*. Basic Appl Ecol.; 3:65–76.

Singh, S., Gautam, A., Sharma, A., & Batra, A. (2010). Centella asiatica (L.): a Plant with Immense Medicinal Potential But Threatened. *International Journal of Pharmaceutical Sciences Review and Research*, 8-17.

Stadnik, M. J., & Freitas, M. B. (2014). Algal polysaccharides as source of plant resistance inducers. *Tropical Plant Pathology*, vol. 39(2):111-118.

Stamp, N. (2003). Out of the Quagmire of Plant Defense Hypotheses. *The Quarterly Review of Biology 78*, 23-55.

Stiff, C. (1998). *Plant Responses to Different Growth Regulators (Hormones) in Tissue Culture*. Retrieved from Austin Community College: http://www.austincc.edu/cstiff/CIS/page57.pdf

Stough, C., & Scholey, A. (2013). *Advances in natural medicines, nutraceuticals and neurocognition*. Boca Raton, Fla.: CRC Street, H. E. (1973). *Plant Tissue and Cell Culture*. University of California Press.

Takita, K., Herlenius, E., Yamamoto, Y., & Lindahl, S. (2000). Effects of Neuroactive Substances on the Morphine-Induced Respiratory Depression; an in vitro Study. *Brain Research 884*, 5-201.

Thangapandian, R., Suganya, D., & Theresa. (2012). Rapid Micro Propagation Techniques For Conserving *Centella asiatica* a valuable medicinal herb. *Journal of Pharmacognosy*, 104-107.

Thongnopnua, P. (2008). High-performance liquid chromatographic determination of asiatic acid in human plasma. *Thai J. Pharm. Sci.*, 10-16.

Tiwari, S., Gehlot, S., & Gambhir, I. (2011). Centella asiatica: A concise drug review with probable clinical uses. *Journal of Stress Physiology & Biochemistry*, 38-44.

TreatingGlioblastoma.com. (2008). *Asiatic acid*. Retrieved December 11, 2015 from Treating Glioblastoma a patient advocacy site: http://www.treatingglioblastoma.com/supplements/asiatic_acid.htm#references

Trigiano, R. N., & Gray, J. D. (2005). Valuable Secondary Products from In Vitro Culture. In M. A. Lila, *Plant Development and Biotechnology* (p. 288). CRC Press.

Vince, Ö., Zoltán, M., Egyetem, D., Egyetem, N.-M., & Egyetem, P. (2011). *Plant Physiology: Secondary metabolites in plant defences*. Retrieved November 26, 2015 from Digitalis Tankonyvtar: http://www.tankonyvtar.hu/en/tartalom/tamop425/0010_1A_Book_angol_01_novenyelettan/ch03s05.html

Vohra, K., Pal, G., Gupta, V. K., Singh, S. & Bansal, Y. (2011). An insight on *Centella Asiatica* Linn.:A review on recent research. *Pharmacologyonline2*, 440-462.

Wagner, H., & Bladt, S. (2001). A Thin Layer Chromatography Atlas. In *Plant Drug Analysis* (pp. 324-325). Springer.

Watson, G. D. (2005). *Pharmaceutical Analysis: A Textbook for Pharmacy Students and Pharmaceutical Chemists.* Harcourt Publishers.

Weber, J. (2015). *Generalities of Pharmacognosy & Adsorption and Partition of Chromatography*. Presentation, Faculty of Pharmacy, UiTM Puncak Alam.

Zodape, S. T., Gupta, A., Bhandari, S. C., Rawat, U. S., Chaudhary, D. R., Eswaran, K., & Chikara, J. (2011). Foliar application of seaweed sap as biostimulant for enhancement of yieldand quality of tomato (Lycopersicon esculentum Mill.). *Journal of Scientific & Industrial Research*, 215-219.

APPENDICES

Appendix I : Murashige and Skoog (MS) Stock Preparation

Stock	Compounds	Concentration (g/L)
MACRO (10X)	NH_4NO_3	16.5
	KNO_3	19.0
	$CaCl_2.2H_2O$	4.4
	$KHPO_4$	1.7
	$MgSO_4.7H_2O$	3.7
MICRO (100X)	$MnSO_4.4H_2O$	2.2
	$ZnSO_4.7H_2O$	0.86
	H_3BO_3	0.62
	KI	0.083
	$Na_2MoO_4.2H_2O$	0.025
	$CuSO_4.5H_2O$	0.025
	$COCl_2.6H_2O$	0.0025
FERUM (100X)	Na_2 EDTA	373
	$FeSO_4.7H_2O$	2.7
VITAMIN (100X)	Glycine	0.2
	nicotinic acid	0.05
	pyridine HCl	0.05
	Thiamine HCl	0.01