TISSUE CULTURE STUDIES, SECONDARY METABOLITES AND PIGMENT EXTRACTION FROM *Allamanda cathartica* L.

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FACULTY OF SCIENCE
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ABSTRACT

Allamanda cathartica L. is a potential medicinal plant which starts to achieve public awareness due to its nutritional value that able to treat various types of diseases, especially in the treatment of jaundice, malaria and cancer. Tissue culture studies were undertaken to examine the effects of different plant growth regulators (PGRs) on the callus induction from leaf and stem explants of this species. Surface sterilization by mercuric (II) chloride ($HgCl_2$) was utilized in order to surface sterilize the leaf (0.1%) and stem (0.2%) explants. The leaf and stem explants were cultured on full-strength Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), Naphthaleneacetic acid (NAA) or 4-amino-3,5,6-trichloropicolinic acid (picloram) alone (0.5 and 1.0 mg/L) or in combinations of 2,4-D (0.5, 1.0 and 1.5 mg/L) and BAP (0.5, 1.0 and 1.5 mg/L) or NAA (0.5 and 1.0 mg/L) and BAP (0.5 mg/L). MS basal medium was used as a control medium. From the results obtained, the leaf and stem explants cultured on 1.0 mg/L 2,4-D and 1.0 mg/L BAP gave the best callus response (100%) with yellow-white, greenish friable callus (0.3857 ± 0.0939 g and 0.0707 ± 0.0549 g for callus fresh and dry weights) and brown-white, greenish friable callus (0.4177 ± 0.0108 g and 0.0207 ± 0.0009 g for callus fresh and dry weights), respectively.

In vitro plant regeneration from the nodal explants of *A. cathartica* L. was also achieved. Surface sterilization of the nodal explants by 0.2% $HgCl_2$ was tested and the explants were cultured on MS medium supplemented with BAP at 1.0, 3.0 and 5.0 mg/L for shoot multiplication. MS basal medium was used as a control and also for shoot elongation. Microscopic studies on the surfaces of the leaf and stem specimens from *in vitro* and *in vivo*

shoots were also examined using scanning electron microscope (SEM). For shoot multiplication, MS supplemented with 5 mg/L BAP gave the best response (100%) with formation of multiple shoots (6.0 ± 0.6 shoots per explant) from the nodal explants. In the PGR-free medium, the elongated shoots were developed (1.01 ± 0.07 cm) with white hairy roots. SEM micrographs showed the *in vitro* specimens to be cleaner in term of surfaces compared to *in vivo* specimens. Abundant stomata were observed on the leaf abaxial surface.

The ethanolic dried and fresh extracts of the leaves, stems, petals and roots of *A. cathartica* L. were used and the extracts were screened for the presence of phytochemicals and their effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH) was used to determine the free radical scavenging activity. Phytochemical screening of all the extracts showed the presence of reducing sugars, anthraquinones, terpenoids and steroids, glycosides and essential oils. Saponins and flavonoids were absence in the fresh and dried leaf extracts. Tannins were similarly absent from the root (dried material) and stem (fresh material) extracts. Concentrations of the plant extracts required for 50% inhibition of DPPH radical scavenging effect (IC_{50}) were recorded as 2.40 μg/ml, 2.59 μg/ml, 4.05 μg/ml and 4.58 μg/ml for ethanolic dried extracts of roots, petals, stems and leaves, respectively. As for the ethanolic fresh extracts of roots, stems, petals and leaves, the IC_{50} values were recorded as 3.49 μg/ml, 4.14 μg/ml, 4.83 μg/ml and 5.04 μg/ml, respectively. Meanwhile, the positive control of ascorbic acid showed IC_{50} value of 3.28 μg/ml.

Studies of pigment extraction and coloured coating development were also carried out for the detection of pigments and the coloured coating was developed from the natural

pigments of *A. cathartica* L. Three extracting solvents, 0.15% HCl in 99.9% methanol, 0.01% HCl in 70% acetone and 95% ethanol in different volumes (5.0, 12.5, 20.0 and 25.0 ml) were utilized for each 1 g of plant material (leaves, stems, petals and calli) and compared. The coloured coating development was performed using the mixture of polyvinyl alcohol (PVA) solution and plant extracts (ethanol and methanol extracts) in the mixing ratio of 1:1 painted on the glass slides and cotton wools, which then proceeded with the salt and heat tests. UV-VIS spectrophotometer was used to measure the absorbance values. The pigments, chlorophyll *a* and chlorophyll *b* showed the maximum absorption in 20 ml methanol leaf extract, ethanol stem extract and acetone callus extract, whereas the carotenoid pigments present in the petals exhibited intense absorption in 5.0 ml of ethanol. The natural colours of the pigments were found to be potential new alternative sources of synthetic colourants for the paint and textile industries.

ABSTRAK

Allamanda cathartica L. merupakan salah satu tumbuhan perubatan yang amat berpotensi disebabkan nilai perubatannya yang dapat mengubati pelbagai jenis penyakit, terutamanya dalam rawatan jaundis, malaria and kanser. Kajian kultur tisu untuk melihat kesan pelbagai hormon untuk induksi kalus telah dijalankan ke atas eksplan daun dan batang. Merkurik (II) klorida ($HgCl_2$) digunakan untuk pensterilan permukaan eksplan daun (0.1%) dan batang (0.2%). Exsplan daun and batang kemudian dikultur atas medium Murashige and Skoog (MS) ditambah dengan asid 2,4-dichlorophenoxyacetic (2,4-D), 6-benzylaminopurine (BAP), asid naphthaleneacetic (NAA) atau asid 4-amino-3,5,6- trichloropicolinic (picloram) sahaja (0.5 dan 1.0 mg/L) atau dalam kombinasi hormon 2,4-D (0.5, 1.0 dan 1.5 mg/L) dan BAP (0.5, 1.0 dan 1.5 mg/L) atau NAA (0.5 dan 1.0 mg/L) dan BAP (0.5 mg/L). Medium MS tanpa hormon digunakan sebagai medium kawalan. Daripada keputusan yang didapati, penginduksian kalus optima (100%) telah diperolehi apabila eksplan daun dan batang dikultur dalam medium MS dengan 1.0 mg/L 2,4-D dan 1.0 mg/L BAP. Kalus yang dihasilkan oleh eksplan daun dan batang adalah berwarna kuning-putih kehijauan dengan struktur rapuh (0.3857 ± 0.0939 g dan 0.0707 ± 0.0549 g untuk kalus berat basah dan kering) dan perang-putih kehijauan dengan struktur rapuh (0.4177 ± 0.0108 g and 0.0207 ± 0.0009 g untuk kalus berat basah dan kering), masing-masing.

Regenerasi tumbuhan secara *in vitro* daripada eksplan nod *A. cathartica* L. juga telah berjaya didapati. Kepekatan 0.2% $HgCl_2$ telah digunakan untuk pensterilan permukaan eksplan nod dan dikultur atas medium MS ditambah dengan BAP pada 1.0, 3.0 dan 5.0 mg/L bagi penggandaan pucuk. Medium MS basal bertindak sebagai kawalan dan juga

digunakan untuk pemanjangan pucuk. Kajian mikroskopik pada permukaan spesimen daun dan batang dari pucuk *in vitro* dan *in vivo* juga diperiksa dengan menggunakan mikroskop imbasan electron (SEM). Bagi penggandaan pucuk, medium MS yang ditambah dengan 5 mg/L BAP memberikan respon yang terbaik (100%) dengan penghasilan pucuk berganda (6.0 ± 0.6 pucuk bagi setiap eksplan) dari eksplan nod. Dalam medium MS tanpa hormon, pemanjangan pucuk telah dihasilkan (1.01 ± 0.07 cm) dengan akar berbulu putih. Mikrograf SEM menunjukkan bahawa spesimen *in vitro* didapati lebih bersih dari segi permukaan berbanding dengan spesimen *in vivo*. Banyak stomata dapat diperhatikan di permukaan abaxial daun.

Ekstrak etanol daun, batang, kelopak bunga dan akar *A. cathartica* L. dari sampel kering dan segar digunakan dan diskrinkan untuk kehadiran bahan fitokimia serta kesan ekstrak tumbuhan pada 1,1-difenil-2-picrylhydrazyl (DPPH) bagi menentukan aktiviti radikalnya. Kehadiran gula ringkas, anthraquinone, terpenoid dan steroid, glikosida dan minyak pati telah ditunjukkan di semua ekstrak tumbuhan dalam kajian penskrinan fitokimia. Ekstrak daun untuk sampel kering dan segar tidak mengandungi saponin dan flavonoid. Ekstrak akar (sampel kering) dan batang (sampel segar) juga tidak mengandungi tanin. Kepekatan ekstrak tumbuhan untuk merencatkan DPPH radikal aktiviti sebanyak 50% (IC_{50}) telah direkodkan pada 2.40 μg/ml, 2.59 μg/ml, 4.05 μg/ml dan 4.58 μg/ml untuk ekstrak etanol akar, kelopak bunga, batang dan daun (sampel kering), masing-masing. Nilai IC_{50} sebanyak 3.49 μg/ml, 4.14 μg/ml, 4.83 μg/ml and 5.04 μg/ml telah direkodkan bagi ekstrak etanol akar, batang, kelopak bunga dan daun (sampel segar), masing-masing. Sementara itu, asid askorbik yang merupakan kawalan positif menunjukkan nilai IC_{50} pada 3.28 μg/ml.

Kajian pengekstrakan pigmen dan penghasilan salutan berwarna turut dijalankan untuk mengesan kehadiran pigmen dan lapisan berwarna telah dihasilkan daripada pigmen semula jadi *A. cathartica* L. Tiga pelarut, iaitu 0.15% HCl dalam metanol 99.9%, 0.01% HCl dalam aseton 70% dan etanol 95% dalam isipadu yang berlainan (5.0, 12.5, 20.0 dan 25.0 ml) telah digunakan untuk setiap 1 g bahan tumbuhan (daun, batang, kelopak bunga dan kalus) dan dibandingkan. Penghasilan salutan berwarna dilakukan dengan menggunakan campuran daripada larutan alkohol polivinil (PVA) dan ekstrak tumbuhan (ekstrak etanol dan metanol) dalam nisbah campuran 1:1 dan ia dicatkan ke atas slaid kaca dan kapas kaca, kemudian diteruskan dengan ujian garam dan haba. UV-VIS spektrofotometer digunakan untuk mengukur nilai daya serap. Pigmen klorofil *a* dan klorofil *b* telah menunjukkan daya serap maksimum dalam 20 ml ekstrak metanol daun, ekstrak etanol batang dan ekstrak aseton kalus, manakala pigmen karotenoid dari kelopak bunga mempunyai daya serap maksimum dalam 5 ml etanol. Warna semulajadi dari pigmen tumbuhan merupakan alternatif baru yang berpotensi untuk menggantikan warna sintetik dalam industri cat dan tekstil.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
ºC	Degree Celsius
µg/ml	Microgram per millilitre
2,4-D	2,4-dichlorophenoxyacetic acid
2iP	Isopentenyladenine
ABA	Abscisic acid
BAP	6-benzylaminopurine
cm	Centimetre
Dicamba	3,6-dichloro-2-methoxybenzoic acid
DNA	Deoxyribonucleic acid
et al.	Latin: at alii or English: and others
etc	Latin: et cetera or English: and other thins/so on
EtOAc	Ethyl acetate
$FeCl_3$	Iron (III) chloride
FeNaEDTA	Ethylenediaminetetraacetic acid
g	Gram
GA_3	Gibberellic acid
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HEPA	Hight Efficiency Particulate Air

$HgCl_2$	Mercuric (II) chloride
HIV	Human Immunodeficiency virus
HPLC	High Performance Liquid Chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid or indolebutyric acid
IC_{50}	Half maximal inhibitory concentration
Kinetin	Furfiryalamino purine
L	Litre
mg/L	Milligram per litre
mm	Millimetre
MS medium	Murashige and Skoog's medium (1962)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAA	Naphthaleneacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometre
PGR	Plant growth regulator
Picloram	4-amino-3,5,6-trichloropicolinic acid
psi	Pound per square inch
ROS	Reactive oxygen species
SEM	Scanning Electron Microscope
TDZ	1-phenyl-3-(1,2,3 thia-diazol-5-yl)urea

TEM	Transmission Electron Microscope
THE	Theanine
Tween 20	Polyoxyethylene sorbitan monolaurate
UV-VIS	Ultraviolet-Visible
v/v	Volume per volume
w/v	Weight per volume
Zeatin	4-hydroxy-3-methyl-trans-2-butenylaminopurine

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION TO MEDICINAL PLANTS

Agriculture is known to have flourished nearly 10,000 years ago and there were records on medicinal plants even in ancient time (Muhamad and Mustafa, 1994). Tropical forests contain more than half of the world's estimated 500,000 plant species and less than 1% of these plants have been researched for medicinal property. Tropical plant species also proved to contain three to four times of active chemical constituents compared to the temperate counter-parts (Stasi *et al*., 2002). Malaysia is rich in natural resources basic to traditional medicine. In Peninsula Malaysia, there are 550 genera containing 1300 species of plants with some of them are medicinal plants which are good for the human body and health (Muhamad and Mustafa, 1994).

Medicinal plants have always been a basic resource for human health. Plants, their parts and products of all descriptions, particularly those with medicinal properties, have been used since time immemorial as principle ingredients of various traditional medicines. Their low cost and wide accessibility in addition to their preventive and therapeutic value often remain as a stronghold in many traditional cultures. Herbal medicine has its origins in ancient cultures including those of the Egyptians, American Indians and Chinese. It involves the medicinal use of plants to treat diseases and enhance general health and human well being. In recent years, interest in herbal medicine has increased globally,

leading to a greater scientific interest in the medicinal use of plants. Many international studies have shown that plants are capable of treating diseases and improving health, often without any significant side effects (Bihani, 2005).

Information about the biologically active chemical components that are responsible for the claimed medicinal effects has been generated on the research of medicinal plants. Medicinal substances found in the medicinal plants are the products of natural metabolic processes. On the other hand, each plant species has its own genetic structure that governs the presence of chemical components and it is also influenced by the environment and differences among cultivars within each species. Therefore, some of the plants can produce medicinally useful compounds; some do not or produce in very small quantities (Li, 2000).

Currently, interest in the medicinal use of plants has increased dramatically. The World Health Organization (WHO) reported that there were approximately 75-80% of the world's population uses plant medicines either in part or entirely (Blythe, 1999). In Malaysia, traditional medicine is accepted as one of the various treatment systems and is being practiced widely in the society (Muhamad and Mustafa, 1994). Medicinal plants are serving as the target for cosmetic and international drug industries due to two important reasons. The first reason is due to increasing cases of drug allergies in western societies indicating intolerance to chemically derived medicines and appearance of side effects after prolonged drug use in chronic illnesses. The second reason is due the importance of finding an indigenous curing tradition as drug sources or new drug by international pharmaceutical industry (Sahai, 2000). Thus, many researches on medicinal plants have been carried out in

order to discover the new sources of alternative medicine in the areas of pharmaceuticals, healthcare products and cosmeceuticals (Wakdikar, 2004).

1.2 PLANT PIGMENTS AS NATURAL COLOURANTS

Since old times, human being has been attracted by wonderful colours produced by nature and started applying them as dyeing materials in textiles (example: *Juglans nigra* and *Rubia tinctorium* for red, *Crocus sativa* and *Reseda luteola* for yellow and *Isatis tinctoria* for blue), printing (example: flowers of *Delphinium zalil* along with roots of *Datisca cannabina* for yellow in calico priting), painting (example: heartwood of *Caesalpinia sappan*, *Pterocarpus santalinus* and rhizomes of *Alpinia galangal* for red, seeds of *Bixa orellana* and rhizomes of *Curcuma longa* for yellow and leaves of *Indigofera tinctoria* for blue) and colouring food stuffs (example: *Bixa orellana*, *Carthamus tinctorius*, *Curcuma longa*) (Kumar and Sinha, 2004; Nayar *et al*., 1999). Besides, history reveals that the art of vegetable dyeing has been in practice since time immemorial. This can be proved from the wall paintings (mural art) of Ajanta, Ellora, Sithannavasal, Mithila (about 40 plants were used for making colours) and Egyptian pyramids which had been exclusively done with natural colourants (Jha, 2002).

However, after the synthesis of ‘Mauveine’ by William Henry Perkin in 1856, synthetic dyes began to be used in place of the natural dyes (Brill, 1980). Due to a wide range of applications in various fields such as food, cosmetic, photodynamic therapy, non-linear optical activity and textile, the acceptability of synthetic dyes became faster in the later period. But too bad, due to an increased environmental awareness, harmful effects

because of either toxic degraded products or non-biodegradable nature from the synthetic dyes and contain serious health hazards like allergenicity and carcinogenicity, the use of synthetic dyes is gradually receding during the last few decades (Kumar and Sinha, 2004).

Natural dyes can be defined as those colourants (dyes and pigments) obtained from plant or animal without processing. Most of the historical important colourants are members of the anthraquinones, naphthoquinone, indigoid and carotenoid groups (Hancock, 1997). The advantages of using natural colourants are multifaceted as they are ecofriendly, safe for body contact, unsophisticated, harmonized with nature, obtained from renewable sources, do not cause health hazards, no disposal problem and also their preparations involves a minimum possibility of chemical reactions. However, the natural dyes have their own limitations like availability, colour yield, stability, complexity of dyeing process and reproduction of shades. Altogether, natural dyes comprise a separate section in colour index, where they are arranged according to hue and their applications (Kumar and Sinha, 2004).

Natural colourants are classified in miscellaneous ways markedly based on structure and their application and the most commonly available natural colourants include chlorophylls, carotenoids, flavonoids, anthocyanins and etc which can be obtained from the plants. Chlorophyll is a mixture of four different pigments, i.e. chlorophyll *a* (blue black), chlorophyll *b* (dark greem), carotene (orange red) and xanthophylls (yellow) which indeed a dyestuff for colouring edible fats, chewing gum, confectionary, oils, cosmetics, liniments, lotions, mouthwashes, soaps, inks, waxes and etc. Carotenes from the group of carotenoids occur in three isomeric forms, i.e. α - (violet), β - (red) and γ - (dark red) of which β -

form is common; it is widely used in colouring foods, drinks, confectionary, cosmetics and animal feed. Moreover, among flavonoids, flavones and flavonols are more widely distributed and are mostly responsible for yellow colours in flowers. Anthocyanins are the best known natural food colourants since they are widely distributed in plant kingdom and their colour range from red of apples, to blue of grapes and to purple of brinjals, also responsible for a vast array of beautiful colours in flowers (Kumar and Sinha, 2004).

1.3 BIOTECHNOLOGICAL TECHNIQUES FOR PLANT CONSERVATION

Plants have played a major role in affecting man and his thoughts. Equally, forms and characteristics of plants also influenced by man in helping them cope with man's progress (Muhamad and Mustafa, 1994). In recent years, biotechnology is changing the agricultural and plant scene in three main areas, i.e. (1) control of growth and development in vegetative, generation and reproduction/propagation, (2) protecting plants against the threats of biotic and abiotic stress and (3) producing specialty foods, biochemicals and pharmaceuticals for expanding the horizons (Altman, 1999).

An advanced biotechnological method of culturing plant cells and tissues in plant tissue culture can provide new means of conserving and rapidly propagating value, rare and endangered plants (Nalawade *et al*., 2003). Plant tissue culture can also provide potential impact on production and improvement of medical plants, as well as *in vitro* manufacturing of crude drugs and active compounds (Yaniv and Bachrach, 2005). In Germany, due to the reasons of plant species protection and interested in a stable, long-term supply of raw material of high and standardized quality, production of phytopharmaceuticals are initiated

in developing field culture procedures for plants that are collected from the wild (Yaniv and Bachrach, 2005).

Furthermore, the increasing public interest in plant-based medicine coupled with rapid expansion of pharmaceutical industries have lead to over-exploitation that harm the survival of many rare species leading to extinction at an alarming rate due to rapid agriculture, urban development, uncontrolled deforestation and indiscriminate collection (Nalawade *et al*., 2003). To stop the violence against nature, efforts should be directed to both, preservation of the plant populations and elevating the knowledge level for sustainable utilization of these plants in medicine – traditional, alternative and allopattic (Tasheva and Kosturkova, 2011). With the combinations of *in vitro* propagation techniques (Fay, 1992) and cryopreservation (Mikulik, 1999), it may help in the conservation of biodiversity of locally used medicinal plants.

In vitro cell and tissue culture techniques are of great interest for the collection, multiplication and storage for plant germplasm. Tissue culture systems allow propagating plant material with high multiplication rates in an aseptic environment. Besides, virus-free plants can be produced through meristem culture in combination with thermotherapy, thus ensuring the production of disease-free stocks and simplifying quarantine procedures for the international exchange of germplasm (Engelmann, 2011). Cryopreservation of *in vitro* cultures of medicinal plants is a useful technique for plant conservation. Cryopreservation is long-term conservation method in liquid nitrogen (-196ºC) in which cell division, metabolic and biochemical processes are stopped. A large number of cultured materials can be stored in liquid nitrogen without alteration or modification for a theoretically unlimited

period of time. Since whole plants can regenerate from frozen culture, cryopreservation provides an opportunity for conservation of endangered medicinal plants. Moreover, cryopreservation requires limited space, protects material from contamination, involves very little maintenance and is considered to be a cost-effective option for storage a range of tissue types, including meristems, anthers/pollens, embryos, calli and even protoplast (Tripathi and Tripathi, 2003; Rao, 2004).

Additionally, slow growth procedures are biotechnological method that provides short- and medium-term storage options for plant conservation. In this method, the germplasm accessions are kept as sterile plant tissues or plantlets on nutrient gels and allow the plant material to be held for 1-15 years under tissue culture conditions with periodic subculturing. Generally, a low temperature range of 0-5ºC are employed with cold tolerant species, but a temperatures between 15ºC and 20ºC are used for tropical species in order to limit growth. It is also possible to limit growth by modifying the culture medium, i.e. by reducing the sugar and/or mineral elements concentration and reduction of oxygen level available to cultures by covering explants with a layer of liquid medium or mineral oil (Rao, 2004).

1.4 RESEARCH OBJECTIVES

Allamanda cathartica L. is one of the medicinal plants in Malaysia which attracts great interest to researchers due to its medicinal properties. However, scientific investigation and plant tissue culture studies are limited. This study therefore was

conducted to determine the potential of *A. cathartica* L. for tissue culture studies and do an assessment of its therapeutic potential and medicinal properties.

The specific objectives include:

1. To establish a suitable protocol of surface sterilization for the leaf, stem and nodal explants of *A. cathartica* L.;

2. To examine the effect of various concentrations of PGRs on the callus induction from the leaf and stem explants;

3. To establish an efficient regeneration protocol using nodal explants;

4. To investigate some medicinal properties of *A. cathartica* L. through phytochemical screening and antioxidant activity;

5. To study the pigments (chlorophylls and carotenoids) that are present in by using the typical absorption spectrum as a reference; and

6. To develop coloured coatings from the natural pigments (chlorophylls and carotenoids) that are present in the leaf and petal.

CHAPTER 2

LITERATURE REVIEW

2.1 *Allamanda cathartica* L.

The family of Apocynaceae consists of approximately 424 genera with about 3500 species (Endress and Bruyns, 2000). One of the genera of this family is *Allamanda* which is named to honor Frederic Louis Allamand (c. 1736-c. 1803), a Swiss botanist and physician and a contemporary of Linnaeus. Allamand had been in correspondence with Linnaeus from 1770 (Mitchell, 2007). In 1771, Allamand had collected the seeds of this plant in Surinam and had suggested the name *Gararips*, but instead Linnaeus honoured him with *Allamanda* in his *Mantissa Plantarum Altera* (Mitchell, 2007; Nellis, 1997). The species epithet for this plant, "*cathartica*" in the Latin word is given the meaning of "cathartic" (Nellis, 1997) because the leaves, roots and flowers may be used in the preparation of a powerful cathartic (PrettyFlowerz, 2012). Moreover, due to the huge amount of *Allamanda cathartica* thru the entire city of Canovanas in the Caribbean Island of Puerto Rico and its rivers, the city hence named this plant as its official flower (PrettyFlowerz, 2012).

Allamanda species are apparently native to northern Brazil, Guyana, Surinam and probably French Guiana (Tropilab Inc., 2011). In the aspect of ecology, the wild and naturalized *Allamanda* can be found on riverbanks in Suriname, on disturbed sites in Florida, along the roads and on forsaken house places, farms and around clandestine dumps

in Puerto Rico (Francis, 2002). The species of *Allamanda* can be grown best in the place of full sun, well drained, moist, sandy soils rich in organic matter but does not tolerate shade, salty soils, highly alkaline conditions and is killed by temperatures of -1ºC (Tropilab Inc., 2011). Besides, *Allamanda* can also grow below brush and a thin forest canopy (Francis, 2002).

A. cathartica L. commonly known as 'Akar Chempaka Hutan' or 'Bunga Akar Kuning' in Malaysia (University of Connecticut, 2012). The common names of *A. cathartica* are Golden trumpet, Yellow bell, Buttercup flower, Angel's trumpet, Ilamarada, Wilkens-bita or Yellow allamanda, is from the family of Apocynaceae (Plants of Southeast Asia, 2009). Its synonymous names are *Echites verticillatus*, *Echites salicifolius*, *Orelia grandiflora*, *A. grandiflora*, *A. hendersonii*, *A. aubletii*, *A. cathartica* forma *salicifolia*, *A.cathartica* var. *grandiflora*, *A. cathartica* var. *hendersonii*, *A. cathartica* var. *nobilis*, *A. cathartica* var. *schottii*, *A. cathartica* var. *williamsii*, *A. latifolia*, *A. linnaei*, *A. nobilis*, *A. salicifolia*, *A. schottii*, *A. wardleyana* and *A. williamsii* (Plants of Southeast Asia, 2009). Table 2.1 showed the scientific classification of *A. cathartica* L.

2.1.1 Characteristics of *Allamanda cathartica* L.

Allamanda (Apocynaceae) is a genus of climbing shrubs (Schmidt *et al*., 2006). The distribution of this species is global but is mainly present in subtropical to tropical, rarely temperate regions with non-succulent or succulent leaves (Vinckier and Smets, 2002). *A. cathartica* L. is a scrambling, perennial shrub or vine which can grow up to15

Table 2.1: The scientific classification of *Allamanda cathartica* L.

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Asteridae
Order:	Gentianales
Family:	Apocynaceae
Genus:	*Allamanda*
Species epithet:	*cathartica*
Botanical name:	*Allamanda cathartica* L.

(Source: United States Department of Agriculture, 2012).

feet tall (Tropilab Inc., 2011). The species can climbs a few meters into the crowns of tall brush and low trees (Francis, 2002). Figure 2.1(A) showed the leathery, yellow-green leaves which have a very variable size from 6 to 16 cm long by 1 to 6 cm wide, arise in rosettes of 4 or 5, obovate to oblong-lanceolate, pointed at both ends and have entire margins and short petioles (5 mm) (Francis, 2002; Burkhill, 1985; Raidah, 2009). Furthermore, the older plants of *A. cathartica* L. often have multiple stems from the root crown and long stems with relatively few branches. Bark of lower stems is brown and furrowed and twigs are green or yellow green in colour. The stems and twigs of this plant would exclude a white milky sap when wounded and is considered poisonous. This plant contains 2n = 18 chromosomes (Floridata, 1999; Francis, 2002).

A. cathartica L. produces flowers all year round in most habitats (Raidah, 2009). The bright yellow bell-like flowers (Figure 2.1(B)) are arising on hairy pedicels with 5 to 7.5 cm across (Francis, 2002; Burkhill, 1985). Flowers of cultivated varieties are often larger and may be coloured white, cream, pink or orange. The seed capsules are oval and prickly with winged seed (Figure 2.1(C)) that fly about when the pod dries and breaks

open (Plants of Southeast Asia, 2009). Seed capsules are rarely produced by cultivated varieties; therefore, the propagation of this plant is usually by cuttings (Nelson *et al*., 2007).

Figure 2.1: Characteristics of *Allamanda cathartica* L. plant. (A)Yellow-green leaves; (B) Bright yellow bell-like flowers; (C) Oval winged seeds; (Source: Tropilab Inc., 2011).

2.1.2 Chemical Constituents

A. cathartica L. has long been used in traditional medicine for different purposes and chemical investigation on this plant with respect to its chemical composition and pharmacological properties have been analyzed (Schmidt *et al*., 2006). In 1974, a continuing search of tumor inhibitors from the plant origin has been done by Kupchan *et al*., which reported that a new antileukemic iridoid lactone, allamandin (Figure 2.2(A)) and the new companion iridoids, allamandicin (Figure 2.2(B)) and allamdin (Figure 2.2(C)) were found in the ethanolic root extract of *A.cathartica* L. Besides, two weakly cytotoxic plumericin (Figure 2.2(D)) and isoplumericin (Figure 2.2(E)) were also found in the EtOAc leaf extract of *A. cathartica* L., which known to have the properties of antifungal, algicidal and barnicidal activities (Abdel-Kader *et al*., 1997). In term of structural elucidation, molecular formula of $C_{15}H_{16}O_7$ for allamandin was established using elemental analysis

and mass spectrometry. This corresponded to the addition of the elements of water to either plumericin or isoplumericin with the infrared spectrum showed the hydroxyl absorption at 2.98 μ (Kupchan *et al*., 1974).

Apart from the chemical constituents stated above, the leaves of *A. cathartica* L. were also found to contain triterpene esters, plumieride (Figure 2.3 (F)), ursolic acid (Figure 2.3 (G)), β-amyrin (Figure 2.3 (H)) and β-sitosterol (Figure 2.4 (I)) (Daniel, 2006). Plumieride is a strong fungitoxicity against some dermatophytes that caused dermatomycosis to animals and human beings (Tiwari *et al*., 2002). The stem contains most of the chemical compounds than present in the leaves, glabridin (Figure 2.3(Q)) and

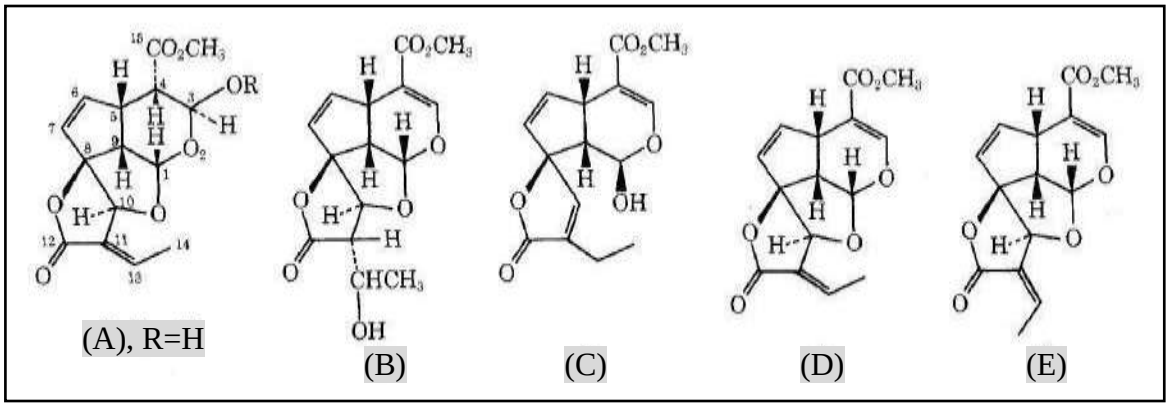

Figure 2.2: Structures of chemical constituents that isolated from *Allamanda cathartica* L. (A) Allamandin; (B) Allamandicin; (C) Allamdin; (D) Plumericin and (E) Isoplumericin (Source: Kupchan *et al*., 1974).

naringenin (Figure 2.3 (R)) were found the first time in the stem of this plant (Yamauchi *et al*., 2011). Other than allamandin, allamandicin and allamdin, the roots of *A. cathartica* L. also contain fluvoplumeirin (Figure 2.3 (J)), plumericin, isoplumericin and plumieride as well as lupeol (Figure 2.3 (K)), fatty alcohols and fatty acids. Fluvoplumeirin is triterpene

lactone that demonstrated the properties of antifungal, antileukemic, antimicribiological and anti-HIV (Kardono *et al*., 1990; Tan *et al*., 1991).

Flavonoids, quercetin (Figure 2.3 (L)), kaempferol (Figure 2.3 (M)) (Daniel, 2006) and 17-methyl-5,9-octadecadienoic acid (fatty acid) (Figure 2.3 (P)), which was the first report of iso-anteiso branched Δ5,9 nonadecadienoic acids (Carballeira and Cruz, 1998), were also produced in the petals of this plant. Moreover, two new iridoid glycosides of plumieride coumarate (Figure 2.3 (N)) and plumieride coumarate glucoside (Figure 2.3 (O)) have also been isolated from *A. cathartica* L. (Coppen, 2001).

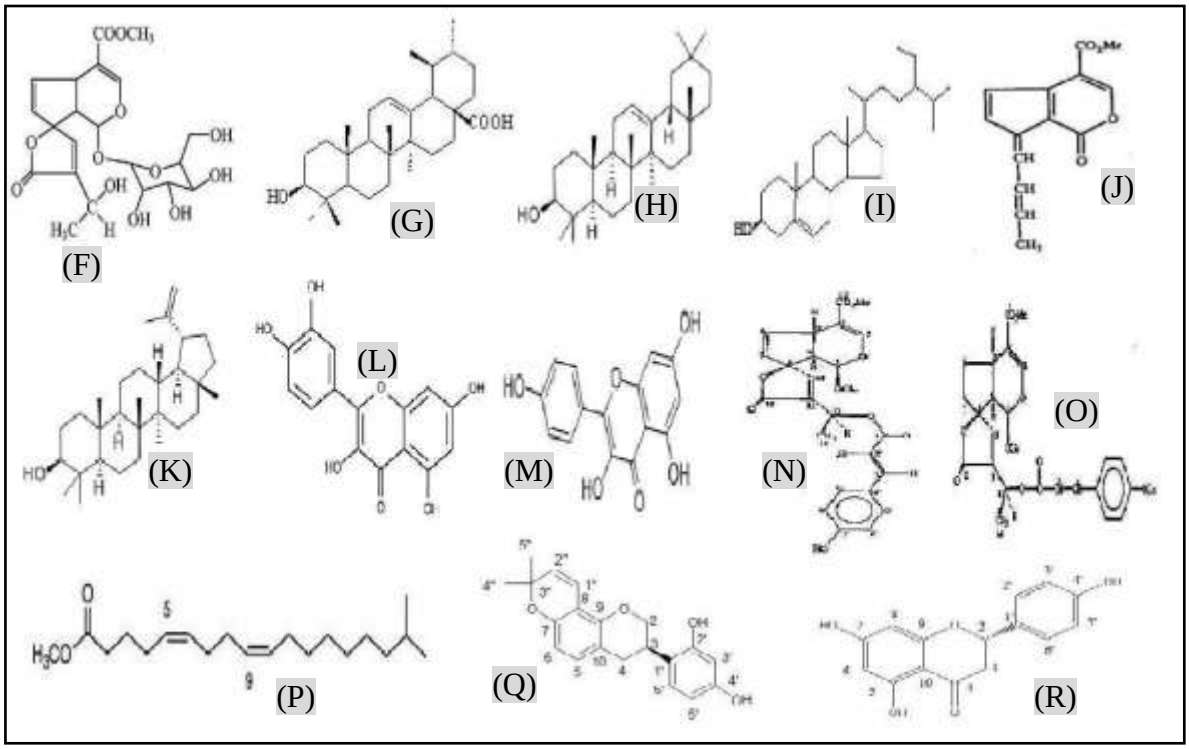

Figure 2.3: Structures of other chemical constituents that isolated from *Allamanda cathartica* L. (F) Plumieride; (G) Ursolic acid; (H) β-amyrin; (I) β-sitosterol; (J) Fluvoplumeirin; (K) Lupeol; (L) Quercetin; (M) Kaempferol; (N) Plumieride coumarate; (O) Plumieride coumarate glucoside; (P) 17-methyl-5,9-octadecadienoic acid; (Q) Glabridin and (R) Naringenin (Source: Schmidt *et al*., 2006; Begum *et al*., 1994; Devprakash *et al*., 2012; Carballeira and Cruz, 1998;Yamauchi *et al*., 2011).

2.1.3 Medicinal Properties

Every part of the plant pictures the usage of the herb in treating diseases. *A. cathartica* L. is starting to achieve more attention especially in the field of traditional medicine due to its nutritional value that were able to treat various types of diseases and it is good for healthcare maintenance.

In general, *A. cathartica* L. can be used to relieve coughs in which the leaves are boiled and the vapor is inhaled to clear the nasal passages. Besides, the leaves of this medicinal plant can also be made into decoctions for use as a purgative (Morton, 1962). *A. cathartica* L. believe to have the properties of antibacterial, antifungal, anticancerous (Nassr-Allah *et al*., 2009), the root tea has also been used in various formulations to treat jaundice and malaria symptoms (Nellis, 1997) as well as wound healing (Nayak *et al*., 2006). The sap was used as a purgative and to eliminate intestinal worms, but the practice was unsafe and mostly abandoned (Nellis, 1997). Since the bark, leaves, fruit, seeds and sap of this plant are poisonous, processing this plant as an herbal remedy is not recommended to be done by people who have little or no experience in alternative medicine (Nelson *et al*., 2007).

2.1.3.1 Antimicrobial Activity of *Allamanda cathartica* L.

To test the antibacterial activity of *A. cathartica* L., disc diffusion method was the first choice, possibly due to its simplicity and capability to analyze a huge number of samples (Van, 2008). According to Mohammad *et al*. (2010), the antibacterial activities of different fractions of *A. cathartica* L.'s leaf extracts were evaluated using Gram-positive and Gram-negative bacteria. From the results, it showed mild to moderate antibacterial

activity with the choloroform solvent extract of *A. cathartica* L. gave the highest diameter of zone of inhibition against *Shigella dysenteriae* (13 mm). Moderate activity was shown against *Bacillus subtilis* and *Psedomonas aeruginosa* (Mohammad *et al*., 2010). Furthermore, the acetone, ethanol and methanol extracts of *A. cathartica* L. showed less inhibition zones against *Escherichia coli* (7 mm). 12 mm of inhibition zone against *Serratia marcescens* and 6-9 mm inhibition zone against *Proteus mirabilis* was showed on the acetone stem extracts and methanol leaf & stem extracts of *A. cathartica* L., respectively (Jeyachandran *et al*., 2010).

The methanolic crude leaf extracts and its different fractions, i.e. petroleum ether, carbon tetrachloride, chloroform and aqueous soluble fractions of *A. cathartica* L. were tested against different fungi (*Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae*) to find out their antifungal activity using food poison method (Mohammad *et al*., 2010). Mohammad *et al*. (2010) reported that the chloroform fraction of *A. cathartica* L. showed the largest inhibition zone against *A. niger* (12 mm) compared to the other fractions. Moreover, the chloroform fraction of *A. cathartica* L. also gave the moderate activity against *C. albicans* and *S.cerevisiae* with 10 mm and 9 mm diameter of inhibition zone, respectively.

2.1.3.2 Anticancer Activity of *Allamanda cathartica* L.

Cancer is a malignant growth, also known as neoplasm in the medical area, which means a relatively autonomous growth of tissues and commonly defined as an uncontrolled growth of cells, with loss of differentiation and commonly with metastasis, spread of the cancer to other tissues and organs (Rajwar *et al*., 2011). The search for an effective

anticancer drug to treat cancer has prompted researchers to investigate the efficacy of natural products in the treatment of cancer (Patel *et al*., 2010).

An investigation using hexane extract of *A. cathartica* L. was performed by standard MTT assay, in order to test its cytotoxicity in three cancer cell types, which were colorectal carcinoma (COLO-205), cervical carcinoma (Hela) and hepatoma (HepG2). The results showed that the leaves of *A. cathartica* L. had highest cytotoxic efficiency with IC_{50} at 41.3,227.6 and 600 μg/ml in COLO-205, Hela and HepG2, respectively and the stalk of this plant has less activity (Uthaisang and Khawsak, 2005). Furthermore, a previous report also showed that the allamandin derivatives extracted by water and/or ethanol from *A. cathartica* L. had significant activity *in vivo* against the p-388 leukemia in the mouse (Kupchan *et al*., 1976). Isoplumericin and plumericin present in *A. cathartica* L. also showed IC_{50} values of 25 and 100 μg/ml, respectively in a cytotoxicity assay with the Madison lung carcinoma (M109) cell line (Abdel-Kader *et al*., 1997).

2.1.3.3 Wound Healing Activity of *Allamanda cathartica* L.

Wound healing involves a complex series of interactions between different cell types, cytokine mediators and the extracellular matrix. Hemostasis, inflammation, proliferation and remodeling are the phases of normal wound healing (MacKay and Miller, 2003). According to Nayak *et al*. (2006), the excision and incision wound models were used to evaluate the wound-healing activity of *A. cathartica* L. i.e. by creating the wound of 2.5 cm in width (circular area = 4.90 cm^2) and 0.2 cm depth on the back of rats for excision wound. For the incision wound, 6 cm long of a longitudinal paravertebral incision was made through the skin and cutaneous tissue on the back of the rats, part of the skin was

sutured 1 cm apart using a surgical thread and curved needle after the incision. These models were then applied with the leaf extract of *A. cathartica* L. in order to evaluate the wound-healing activity.

From the results, it showed the leaf extract of *A. cathartica* L. promoted wound healing activity significantly in both excision and incision wound models studied. High rate of wound contraction ($P < .001$), decrease in the period of epithelialisation (10.2 ±0.13), high skin breaking strength (440.0 ± 4.53), significant increase in the weight of the granulation tissue ($P <.001$) and hydroxyproline ($P < .001$) content were observed in rats treated with the leaf extract of *A. cathartica* L. Histological studies of the granulation tissue from the treated group showed the presence of a lesser number of inflammatory cells, and increased collagen formation than the rats in control group. With this studied, it can be used to cure different types of wounds in human beings too (Nayak *et al*., 2006).

2.1.3.4 Tyrosinase Inhibitory Activity of *Allamanda cathartica* L.

Melanin is a pigment that is widely distributed on body surface, retina, nigra of brain, adrenal medullae and so on. It is biosynthesized in cells called melanocyte from tyrosine through oxidation process that is catalyzed by tyrosinase. In the area of cosmetic, melanin is a reason of sunburn and mottle, hence, compounds inhibiting melanin production, i.e. tyrosinase inhibitors played an important role to the application of cosmetic as whitening agent (Yamauchi *et al*., 2011). From the study of Yamauchi *et al*. (2011), glabridin isolated from the stem extract of *A. cathartica* L. showed the highest tryosinase inhibitory activity (IC_{50} = 2.93 μM) which is 15 times stronger than that of kojic acid used

as a positive control (IC_{50} = 43.7 μM). Kojic acid is one of the compounds included in whitening agent of cosmetic products (Yamauchi *et al*., 2011).

2.2 CONTAMINATIONS

The tissue culture techniques usually involves growing stock plants in ways that will minimize infection such as treating the plant material with disinfecting chemicals to kill superficial microbes and sterilizing of tools used for dissection, the vessels and media in which cultures are grown (Odutayo *et al*., 2004). However, contamination has been reported as a constant problem, which often compromise development of all *in vitro* techniques (Enjalric *et al*., 1988).

Contaminants of plant tissue culture are bacteria that reside on dust particles and fungi, including spores (Stephens, 1995). Tissue culture contamination frequently originates with the introduction into culture of explants contaminated with endophytic microorganisms or surface sterilization-resistant microorganisms (Cassells, 2001). The nutrient media in which the plant tissue is cultivated is a good source of nutrient that can support the rapid growth of bacteria and fungi (Odutayo *et al*., 2004). Once these contaminants are established in culture, they usually grow quickly competing adversely with plant tissue culture for nutrient, depleting the medium of nutrients and producing toxins that can affect the growth of the cultured plant tissue. The presence of these microbes in these plant cultures usually results in increased culture mortality as the cultivable contaminations may over-run the cultures killing the explants (Moore, 2007).

Some microorganisms may positively influence plant growth and development by acidification of the medium or by release of plant growth regulators. The presence of latent infections can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting. Conversely, some microorganisms are inhibited by acidification of culture media and by exudates from the plant tissue and may be weakly expressed or latent as endophytes in the tissues (Cassells, 2001).

Contamination by bacteria and fungi is an insidious process that continually threatens plant tissue cultures throughout the duration of the culture period. Despite the fact that plant tissue cultures may be sterile when initiated, microorganisms can often contaminate cultures at any point during subsequent tissue culture manipulations. Thus, it would be useful to provide a chemical agent that reduces or prevents the microbial contamination of plant tissue culture media and maintains the sterility of the media for the duration of the culture period (Strosse *et al*., 2004).

Contamination that results from improperly sterilized tissue will generally arise from the explant and be located in the medium adjacent to the explant. If contamination is due to poor techniques, contaminated transfer hood filters or improperly sterilized media, it will be scattered on or in the medium. Contamination of cultures by fungi appears as a fuzzy growth while bacterial contamination appears as smooth pink, white or yellow colonies. Contamination from insects will generally appear as tracks across the medium, which are visible due to bacterial or fungal growth on the insect tracks (Smith, 2000). Smith (2000) also explained that contamination from air vents and high foot traffic can be a problem. Foot traffic scuffs up the wax on floors as well as dust which help spread

contaminants. It is best to keep traffic around the transfer area to a minimum (Stephens, 1995).

2.3 STERILIZATION

One of the most important factors of plant tissue culture practice is establishing and maintaining aseptic conditions. Therefore, to avoid contamination and to preserve sterile conditions, tools, containers, media, explants and the working environment are sterilized and aseptic techniques are practiced (Dodds and Roberts, 1995).

Glasswares, filters, cotton wool plugs, plastic cap and instruments can be sterilized by autoclaving. Autoclaving is treatment by water vapour at high temperature and high pressure. The standard conditions used for sterilization are 121ºC at 15 psi for 15-20 minutes. Vessels should be closed with a cap or with aluminium foil and instruments should be wrapped in foil and placed in an autoclave bag. It is important that the steam can penetrate the items for sterilization (Dodds and Roberts, 1995). Plant tissue culture media are also generally sterilized by autoclaving 121ºC and 15 psi (Phyto Technology Laboratories Inc., 2007).

The laminar flow chamber provides a constant flow of filter-sterilized air over the work surface (Evans *et al*., 2003). The laminar flow hoods operate under positive pressure and are equipped with HEPA filters, which are approximately 3 inches thick. The HEPA filters are 99.99% efficient at removing particulate matter 0.3 microns or larger. With this, bacteria and fungal spores are trapped by the filter (Purdue University, 2007). The airflow

is directed either downward (a vertical flow system) or outward (a horizontal flow system) over a non-porous work surface. The constant flow of filter- sterilized air prevents non-sterilized air from entering the working area and thus creates a barrier against airborne contaminants. If proper techniques were used while working inside these hoods, contamination of plant tissue culture can be virtually eliminated.

The laminar flow chamber can be sterilized by exposure to UV light. The time of exposure depends on the size of hood. Sterilization should only be done when the hood is not in use, as UV is harmful to plant material and damaging to eyes. Laminar flow hoods are sterilized by wiping the work surface with 70% ethanol and the airflow should remain on continuously for at least 30 minutes before use. Laminar flow hoods should also be sterilized by periodic (twice a month) spraying or wiping with commercial brands of bactericides and fungicides (Dodds and Roberts, 1995).

Frequently, instruments used for aseptic manipulations, such as forceps, scalpel, holders, needles and spatula, previously sterilized by dry heat or autoclaving are removed from their wrapping and re-sterilized just prior to use by dipping in 95% ethanol and placing in the flame of a Bunsen burner. After use, instruments can be re-flamed before reusing (IAEA, 2004). Safety is a major concern with this technique because of high flammability of ethanol and the risk of a flash fire (Evans *et al*., 2003). For flame sterilization of instruments, 95% alcohol has been found entirely satisfactory. The alcohol should be regularly changed as *Bacillus circulans* strains persist in alcohol for more than one week (Bhojwani and Razdan, 1996).

As *in vitro* cultures are normally axenic, it is necessary to eliminate possible contaminants from the surface of the explants (Fay, 1992). Surfaces of plant parts carry a wide range of microbial contaminants such as fungal, bacterial and algal spores. To avoid this source of infection, the tissue must be thoroughly surface sterilized before planting it on the nutrient medium. To disinfect plant tissues, various sterilizing agents have been used. Hypochlorite solution (sodium or calcium) have proved to be effective in most cases (Bhojwani and Razdan, 1996). Other sterilants used include mercuric (II) chloride and hydrogen peroxide.

A wetting agent such as Tween 20 is often included to improve contact between the sterilant and the plant tissue (Fay, 1992). It is important to realize that a surface sterilant is also toxic to the plant tissue. Therefore, the concentration of the sterilizing agent and the duration of treatment should be chosen to minimize tissue death (Bhojwani and Razdan, 1996).

2.4 PLANT TISSUE CULTURE

Plant tissue culture is a technique of *in vitro* cultivation of plant cells and organs, which divide and regenerate into callus or particular plant organs. The technique relies on the explants which is a small tissue excised from any part of the plant, aseptic environment to avoid contamination from microorganisms and nutrient media that strongly govern the growth and morphogehesis of plant tissues (Rai, 2007).

Plants cultured *in vitro* yield thousands of genetically identical plants (clones) from a single plant. Through the use of biotechnology, tissue-cultured plants have been genetically engineered to provide insight into plant molecular biology and gene regulation (Mineo, 1990), so that the desirable genetic traits can be transferred from one organism to another by transfer of DNA. Many more plants with the desirable DNA can be regenerated from small pieces of the transformed plant tissue over a very short period of time (Montgomery College Biotech Institute, 2009).

Furthermore, in order to understand plant tissue culture, plasticity and totipotency have to be known. Plasticity is the ability of the plants to adapt to the environmental conditions by altering their metabolism, growth and development. Meanwhile, totipotency is the maintenance of genetic potential whereby plant cells have the ability to express the total genetic potential of the parent plant when correct stimuli is given (Slater *et al*., 2003). Thus, the cells have all the genetic information necessary to regenerate into a complete plant.

2.4.1 Callus Culture

Callus is a tissue consisting of dedifferentiated cells generally produced as a result of wounding or of culturing tissues in the presence of plant growth regulators (Bhojwani, 1996). Callus generally formed at the cut end of a stem or root and consists of amorphous mass of loosely arranged thin-walled parenchyma cells arising from the cultured explants (Dodds and Roberts, 1995). Callus that formed from the explants is thought to be a protective response generated by the plant to seal off the damaged tissue (Smith, 2000).

In the modes of a culture, the plant cells if cultured on a solid surface will grow as unorganized mass of cells called callus. For growth, the cells need to undergo division, whereas, the cells breaking up from explants are mature and non-dividing. Therefore, the differentiated tissue will undergo modifications, in both morphology and metabolism to become non-differentiated (Rai, 2007). This phenomenon of a mature cell to revert back to non-differentiated callus tissue is called dedifferentiation and its consequence is that most plant cultures will lose the ability to photosynthesize due to unmatchable metabolic profile with the donor plant (Gamborg, 2002).

Callus culture is the culture of non-differentiated mass of cell on agar media produced from explants of a seedling or other plant part. For callus formation, auxins and cytokinins are required. Callus can be subcultured periodically by transferring to fresh medium. Subculturing is required to consider the cell growth, nutrient depletion and medium drying (Rai, 2007).

The rate of growth of callus on solid agar medium is relatively slow. The new cells are formed on the periphery of existing callus mass. Consequently, callus consists of cells which vary considerably in age. Since nutrients are gradually depleted from the agar, a vertical nutrient gradient is formed. The usefulness of callus in experimental system is limited, due to low degree of uniformity among cells in callus, slower growth rate and development of nutrient gradients. Thus, callus culture is mainly use for maintaining of cell lines and morphogenesis (Rai, 2007).

2.4.2 Micropropagation

Micropropagation or clonal propagation is referred to as *in vitro* propagation of plants vegetatively by tissue culture to produce genetically identical copies of a cultivar (Rai, 2007). Use of tissue culture for micropropagation was initiated in 1962 and found this as the only commercially viable approach for orchid propagation (Razdan, 2003). Since then several crop species have been micropropagated and recipes are now available which can be adopted by growers trained in aseptic manipulations and plant husbandry (Behera *et al*., 2009).

Furthermore, micropropagation has proved to be useful for propagation of various plant species including medicinal plants, propagation of sexually sterile plant species like triploids, aneuploids which cannot be perpetuated by seeds, seedless plants like banana, cross bred perennials where heterozygosity is to be maintained, mutant lines like auxotrophs which cannot be propagated *in vivo* and disease free planting material of fruit trees and ornamentals (Rai, 2007).

Micropropagation is a complicated process requiring many stages in order to simplify the daily operation, accounting, and product cost. Murashige proposed four distinct stages that can be adopted for overall production of clones commercially (Figure 2.4). Stages I-III are under *in vitro* condition, whereas Stage IV is accomplished in a greenhouse environment. An additional Stage 0 (Figure 2.4) for various micropropagating systems was suggested due to the treatment and preparation of stock plants should be regarded as a separately numbered stage or stages (George *et al*., 2007).

Stage 0 (3 months)	Selection and maintenance of stock plants for culture initiation.
Stage I (3-24 months)	Preparation and establishment of explants on suitable culture medium (usually shoot tips and axillary buds used).
Stage II (10-36 months)	Regeneration: multiplication of shoots or somatic embryos on defined culture medium.
Stage III (1-6 weeks)	Rooting of regenerated shoots/ or somatic embryo *in vitro.*
Stage IV	Transfer of plantlets to sterilized soil for hardening under greenhouse environment.

Figure 2.4: Major stages that involves in micropropagation (Source: Dixon and Gonzales, 1994).

2.4.3 Organogenesis

Organogenesis is the formation of organs, such as leaves, shoots and roots on a plant organ, either directly from explants or indirectly from a callus culture (Chawla, 2002). Organogenesis generally occurs *de novo* or "from new" indicating that no previous meristems that were developmentally determined existed beforehand. The concept of organogenesis is composed of three phases, i.e. 'competence acquisition phase', 'induction phase' and 'morphological differentiation phase' which may be applicable to organogenesis in various tissue culture systems, but experimental identification of each phase is not easy in all cases (Ozawa *et al*., 1998).

Organogenesis depends on the inherent plasticity of plant tissues that is regulated by altering the components of the medium (Slater *et al*., 2003). One of the remarkable feats of physiological research on organogenesis *in vitro* was the identification of a predominant role of cytokinin and auxin as chemical determinants in plant development (Skoog and Miller, 1957). The classical studies of Skoog and Miller (1957) demonstrated that the relative ratio of cytokinin and auxin is important in determining the nature of organogenesis in tobacco pith tissue as high level of cytokinin initiates bud formation while high level of auxin promotes rooting. But some other plant species do not follow the concept of auxin/cytokinin ratio because the requirement of exogenous PGRs depends on their endogenous levels which might differ in different plant species and also in different plant materials (Rai, 2007).

Some other factors also would affect organogenesis. The larger the explants, more is the likelihood of shoot bud formation. Genotype of explants affects shoot regeneration as explants taken from different plant varieties of same species show different frequencies of shoot bud differentiation (Rai, 2007). Light has been shown to have inhibitory effect. Even Skoog and Miller (1957) reported that blue light shown to induce shoot formation and root by red light in tobacco. The optimum temperature required may vary with plant species.

2.4.4 Somatic Embryogenesis

The initial and development of embryos from somatic tissues in plant culture was first recognized in the cultures of *Daucus carota* (Slater *et al*., 2003). In somatic embryogenesis, the embryos regenerate from somatic cells, tissues or organs either *de novo* or directly from the tissue (adventive origin), which is opposite of zygotic or sexual

embryogenesis (Chawla, 2002). In scientific way, it is a process in which a bipolar structure resembling a zygotic embryo develops from a non-zygotic cell without vascular connection with the original tissue (Bhojwani, 1996).

Under natural conditions, this pathway is not normally followed, but from tissue culture somatic embryogenesis occurs most frequently and as an alternative to organogenesis for regeneration of whole plants (Razdan, 2003). Furthermore, induction of somatic embryogenesis requires a single hormonal signal to induce a bipolar structure capable of forming a complete plant, while in organogenesis it requires two different hormonal signals to induce first shoot organ, then a root organ (Chawla, 2002).

Somatic embryogenesis can be obtained via either direct or indirect method (Dodds and Robert, 1995). In direct somatic embryogenesis, the embryo is initiated directly from the explants tissue in the absence of callus proliferation which occurs through Pre-embryogenic determined cells (PEDC) where the cells are committed to embryogenic development and need only to be released. In indirect somatic embryogenesis, cell proliferation takes place, i.e. proliferation of callus from explants takes place from which embryos are developed and the cells from which embryos arise are called Induced embryogenic determined cells (IEDC) (Chawla, 2002). In majority cases, direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis.

In addition, floral and reproductive tissue has proven to be an excellent source of embryogenic material in somatic embryogenesis. Somatic embryogenesis encompasses various stages from callus initiation to embryo development and maturation and

subsequently plantlet formation. For many species, one medium is used for initial callusing and for the maintenance of callus, second medium is used for somatic embryo maturation and third medium is used to allow growing into plant (Evans *et al*., 2003). An elaborate sequence of media is required because induction of somatic embryogenesis is difficult.

Plantlets produced from somatic embryogenesis are called as true type. Somatic embryogenesis is an important prerequisite for the usage of biotechnological tools for genetic improvement (Ruvalcaba *et al*., 1998). Somatic embryogenesis has also been carried out on huge number of medicinal plants.

2.4.5 Shoot Multiplication

Shoot multiplication involves the process of micropropagation which is a true-to-type propagation (Trigiano and Gray, 2004). Multiplication can be divided into three approaches which included (1) multiplication through the growth and proliferations of meristems excised from apical and axillary shoots of the parent plant (Example: shoot culture), (2) induction and multiplication of adventitious meristems through processes of organogenesis or somatic embryogenesis directly on explants (Example: node culture) and (3) multiplication of calli derived from organs, tissues, cells or protoplast and their subsequent expression of either organogenesis or somatic embryogenesis in series subcultures. Shoots obtained from these calli can be multiplied further (Razdan, 2003).

The simplest method of shoot multiplication in *in vitro* propagation is via node culture which is of value for propagating species that produce elongated shoots in culture. This type of shoot multiplication is called adventitious shoot multiplication and dependent

on the presence of organized explants tissue such as stems, nodes, leaf blades and so on (Razdan, 2003). Hassan and Debergh (1987) have reported that, even in potato, node culture was preferable. Currently, this method becomes very famous in commercial micropropagation due to its clonal stability. Although the rate of shoot multiplication is slower than that of the shoot culture, there is less likelihood of associated callus development and the formation of adventitious shoots and thus reduced the genetic irregularity (George *et al*., 2007).

Moreover, node culture is a simplified form of shoot culture (Trigiano and Gray, 2004). The primary explants for node culture is a shoot apex, a lateral bud or a piece of shoot bearing one or more buds, i.e. having one or more nodes. Typically, a single elongated unbranched shoots consisted of multiple nodes and these shoots can either rooted or acclimatized to *ex vivo* conditions or repeatedly subdivided into nodal cuttings to initial additional cultures (George *et al*., 2007).

Nevertheless, although shoot culture has been proved to be a widely applicable method of micropropagation, the appreciation of its potential value developed only slowly and utilisation largely depended on improvements in tissue culture technology (George *et al*., 2007). The two major developments which made shoot culture feasible were the development of improved media for plant tissue culture and the discovery of the cytokinins as a class of PGRs with an ability to release lateral buds from dormancy, but it is not immediately applied to the shoot culture.

The most suitable PGRs in shoot multiplication belongs to the class of cytokinins because they are effective in removing the apical dominance of shoots. Their use can be combined with pinching the apex of shoots or placing explants in horizontal position. A cytokinin treatment in tissue culture can not only promote the formation of multiple shoots, either axillary or adventitious, but also cause the shoots formed to be too short for rooting and transfer if the compound used is unsuitable or the concentration used is too high (George *et al*., 2007).

2.5 FACTORS AFFECTING PLANT TISSUE CULTURE

2.5.1 Genotypes

Plant tissue culture are influenced by many factors, among them the genotype are regarded to be the major source of variation in *in vitro* culture. In plant tissue culture, it is well known that no two genotypes give similar response under a given set of culture conditions (Nehra *et al*., 1989; Nehra *et al*., 1990). Although all plant cells contain the entire set of genetic information necessary to form a whole plant if proper induction conditions are provided, their *in vitro* response varies tremendously. Gurel and Kazan (1998) have reported that genotype played an important role for callus induction like shoot regeneration in tissue culture studies.

Also, in the study of Ozyigit *et al*. (2007), the same media, hormones and culture conditions were used and it was seen that genotype is an important factor for callus induction with five commercially important cultivars of sunflower with different genotypes were used. Ceriani *et al*. (1992) reported that especially in sunflower tissue cultures,

hypocotyls and cotyledon explants are good regeneration materials that show different regenerative behavior when kept in a culture, depending on their genotype.

2.5.2 Explants

Tissue cultures are started from piece of whole plants. A plant organ or piece of tissue used to initiate a culture is called explant. To obtain an explant, it is depends on the kind of culture to be initiated, the purpose of the proposed culture and the plant species to be used. Virtually, any plant part can serve as explant for organized development (Thorpe, 1982). These include stem segments and apices, root sections, leaf and petioles, flower petals, ovular tissue, seedling parts and seed embryos (Vasil and Thorpe, 1994).

Particularly in difficult-to-work-with species, the explant which contains mitotically active cells and immature often gives much better results in callus induction and subsequent plantlet regeneration (Vasil and Thorpe, 1994). The mature and well-differentiated tissues with abundant storage reserves, typically give a poor embryogenic response (Trigiano and Gray, 2004). Thus, the correct choice of explant material can have an important effect on the success of tissue culture (George *et al*., 2007).

Chen and Chang (2002) reported the regeneration of *Oncidium* through direct somatic embryogenesis is an effective method and has been achieved using young leaf explant due to young leaves explants have a high regeneration capacity and may provide large numbers of embryos and plantlets in a short period of time. Besides, the position of explant was also significantly affected the embryo formation. In the study of Chen and Chang (2002), leaf tip segments had a higher embryogenic response than other segments of

leaves and adaxial-side-up orientation significantly promoted embryogenesis as compared to abaxial-side-up orientation.

In addition, the result of present study demonstrated that the induction of direct embryogenesis is also highly dependent on type, size and orientation of cultured explants. Among the explants, the immature cotyledons showed high frequency of embryo induction (Naz *et al*., 2008; Adkins and Adkins, 1995; Ramnaua *et al*., 1996; Ozais, 1989).

2.5.3 Culture Media

Plant tissues and organs are grown *in vitro* on artificial media, which supply the nutrients necessary for growth. The success of plant tissue culture as a means of plant propagation is greatly influenced by the nature of the culture medium used. In addition to the chemical composition of culture medium, its physical form can also influence the growth and multiplication rate of a culture (Mellor and Stace-Smith, 1969; Bhagyalakshmi and Singh, 1988).

In earlier plant tissue culture experiments, the media formulated by White were employed which consisted of the nutrients normally required by plant cells and widely used for root cultures. However, inadequate of particular nitrogen and potassium in this medium caused it cannot sustain the maximum growth of callus and cell suspension cultures. Therefore, richer mineral salt mixture was compensated for by adding the 'undefined' components such as yeast extract, protein hydrolysates, amino acids, coconut milk or other organic supplements. In order to replace White's medium, other media have been formulated and are adequate for cell culture (Gamborg, 2002).

The most commonly and frequently used medium is the formulation from MS. The purpose of this medium was used for optimal growth of tobacco callus and the development involved a large number of dose-response curves for the various essential minerals. In this medium, the relatively low levels of phosphorus (P), calcium (Ca) and magnesium (Mg) are evident. The most striking differences for this medium are the high levels of molybdenum (Mo) and chlorine (Cl) and low level of copper (Cu) (George *et al*., 2007). Each plant species has its own characteristic elementary composition in order to adapt the formulation of medium.

The principal and basic components of most plant tissue culture media are generally made up of inorganic nutrients (macronutrients and micronutrients), carbon source, vitamins, amino acids or other nitrogen supplements, gelling agents and PGRs (Phyto Technology Laboratories Inc, 2003). Complex nutritive substances that are added to the medium and composed of 'chemically defined' compounds are referred as a 'synthetic medium' (Razdan, 2003). Moreover, it is very important that the composition of a medium should be the same each time it is prepared.

Inorganic nutrients are divided into macronutrients and micronutrients. Elements required in concentrations greater than $0.5mmol^{-1}$ are referred to as macronutrients and those less than $0.05mmol^{-1}$ as micronutrients. Both macronutrients and micronutrients that exist as salts are mix together providing continuous source of nutrition, in addition to carbon, hydrogen and oxygen. Macronutrients in tissue culture consist of six major elements, i.e. nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), Magnesium (Mg) and sulphur (S). Micronutrients such as iron (Fe), manganese (Mn), zinc (Zn), boron (B),

copper (Cu) and molybdenum (Mo) are required in trace quantity but essential for plant cell and tissue growth (Razdan, 2003).

The organic nutrients in culture media usually included carbohydrates and vitamins. Carbohydrate such as sucrose serves as a carbon source in culture medium and usually added in concentrations of 1-5%. Plant cells and tissues in the culture medium lack of autotrophic ability and thus need external carbon source for energy that can enhance proliferation of cells and regeneration of green shoots (Razdan, 2003). Besides, the carbohydrate requirements have been established to depend upon the stage of culture and may show differences depending on the plant species (Baskaran and Jayabalan, 2005). Baskaran and Jayabalan (2005) also reported that among the carbon sources used, sucrose proved to be better for shoot regeneration than fructose or glucose.

Vitamins are usually required to achieve the best growth of plant tissues because some essential vitamins are synthesised only in suboptimal quantities when plants are grown *in vitro* (Razdan, 2003). Agar is a high molecular weight polysaccharide that can bind water and is derived from seaweed. Agar is a gelling agent or solidifying agent used to solidify culture media into a gel. Gelling agent permit the explant to be placed in precise and proper contact with the medium, but remain aerated (Trigiano and Gray, 1999). Agar is added to the culture medium ranging from 0.5-1.0% (w/v) in concentrations. Plant growth can be influenced by the consistency of the agar. If the concentration of agar is too high, the medium becomes harder which cause reduce plant growth. If agar concentration is lower or the pH is low, the medium will become very soft and hyperhydric plants may result (Trigiano and Gray, 1999).

2.5.4 Plant Growth Regulators (PGRs)

According to the Environmental Protection Agency, a PGR or plant hormone is defined as “any substance or mixture of substances intended, through physiological action, to accelerate or retard the rate of growth or maturation, or otherwise modify the characteristic of plants or their produce. In addition, PGRs are characterized by their low rates of applications; high rates of application of the same compounds often are considered herbicidal” (Lemaux, 1999). Besides, PGRs are produced by plants that modify or control some specific physiological processes in a plant. PGRs can be either natural or synthetic and divided into two main classes: auxin and cytokinin. Other PGRs are gibberellins, abscisic acid and ethylene.

Auxin is a group of hormones involved with growth responses of plants. The term “auxin” is derived from Greek word which means “to increase or grow” and was the first plant hormone group discovered (Saupe, 2009). In tissue culture, auxins cause cell division, cell elongation, elongation of stem, internodes, tropism, apical dominance, abscission, swelling of tissues and the formation of adventitious roots. Auxins have also been reported to induce callus formation and maximum callus growth was found in the culture (Gopi and Vatsala, 2006). Khan *et al*. (2000) reported the frequency of callus induction was affected by various concentrations of auxin, such as 2,4-D. The commonly used auxins in tissue culture are IBA, NAA, 2,4-D, IAA, dicamba and picloram. IAA is naturally occurring in plant tissues and others are synthetic auxins (Razdan, 2003). Auxins are generated in apical meristems and other immature part of plants.

Cytokinins are derivatives of adenine compounds that promote cell division and differentiation of adventitious shoots from callus and organs. Cytokinins also promote axillary shoot formation by decreasing apical dominance (Arteca, 1996). According to Arteca (1996), kinetin was the first cytokinin isolated from autoclaved herring sperm DNA due to its ability to promote cell division or cytokinesis in tobacco pith tissue. The members of cytokinin include BAP, kinetin, zeatin and 2iP. Zeatin and 2iP are naturally occurring in plant. Cytokinins are produced in meristematic regions and areas of continued growth potential such as roots, young leaves, developing fruits and seeds.

The combination between auxins and cytokinins is often use in plant tissue culture and their ratio is very important with regards to morphogenesis in the culture system. A high cytokinin to auxin ratio generally leads to the shoot formation, whereas a high auxin to cytokinin ratio is leads to the formation of root and an intermediate ratio between auxin and cytokinin favours callus formation (Razdan, 2003). In the study of Thomas and Puthur (2004), shoot regeneration in combination of TDZ with NAA has been established from morphogenic callus generated from the nodal segments and reported that the highest number of shoot buds per explants was recorded on MS medium supplemented with 3 μM TDZ and 0.5 μM NAA. Moreover, the combination of different concentrations of BAP with 2,4-D were also reported to behave in different fashion in callus initiation (Khan *et al*., 2000).

Gibberellins are a class of plant growth regulators having an ent-gibberel-lane skeleton and stimulating cell division and/or cell elongation (Arteca, 1996). One of the most common gibberellins used is GA_3 which promotes the growth of cell cultures at low

density, enhances the growth of callus and induces elongation of dwarf or stunted plantlets (Razdan, 2003). GA_3 can be found in roots and shoot tips, young leaves and seeds.

ABA is a 15-carbon sesquiterpenoid which either induces or inhibits the callus growth depending on the species. ABA is reported useful in embryo culture due to it is heat stable but light sensitive (Smith, 2000). ABA is produced in the leaves, fruits, root caps and seeds. Ethylene is another type of plant hormone that exists as a form of gas. Ethylene is used to stimulate ripening, release of dormancy, shoot and root growth and differentiation, ripening of climacteric fruits, leaf and fruit abscission, stimulates flower opening and stimulates flower and leaf senescence (Sengbusch, 2003). It is produced in the roots, shoot apical meristems, leaf nodes, aging flowers and ripening fruits.

2.5.5 Media pH

In tissue culture, pH of particular medium is extremely important, because it influences the uptake of nutrients and PGRs by regulating the solubility of culture media. Media pH also has been proven to influence many of plant developmental processes *in vitro*, such as shoot regeneration (Bhatia and Ashwath, 2005), micropropagation rate, organogenesis, secondary product formation, cell division, adventitious rooting, somatic embryogenesis and etc (Owen *et al*., 1991).

According to Bhatia and Ashwath (2005), a pH higher than 6 gives a fairly and hard medium and below 5 caused unsatisfactory gelling. If the pH is lower than 4.5 and higher than 7, the growth and development of *in vitro* processes will generally stop. Thus, the effective range of pH for tissue culture medium is limited and its optimal pH must not

disrupt the functioning of cell membranes or the cytoplasm's buffered pH. Normally, the pH of a culture medium will be adjusted between 5.2 to 5.8 prior to autoclaving (Skirvin *et al*., 1986) and a pH of 5.7 ± 0.1 is the most optimum.

Additionally, Mellor and Stace-Smith (1969) observed that the pH of the medium dropped within a week from 5.7 to 5.4. The changes of pH during culture period is due to the uptake of compounds required as essential nutrients or as buffer components such as ammonium (NH_4^+), nitrate (NO_3^-) and phosphorus (PO_4^{3+}) (Ramage and Williams, 2002), secretion of acids such as malate, succinate, lactate which is particularly pronounced during the stationary growth stage and an efflux of protons or hydroxyl ions (Bhatia and Ashwath, 2005). Besides, the pH of the medium decreased during ammonia assimilation and increased during nitrate uptake as reported by Ramage and Williams (2002).

In the study of Baksha *et al*. (2003), the effect of different pH level on induction and growth of adventitious roots was determined. Among the pH levels used, the highest percentage of explants showing rooting was recorded on the medium that has pH adjusted to 5.5 to 5.7 and the lowest percentage of rooting was observed at the pH of 4.0 to 5.0. From the study, it indicated that the level of media pH greatly influenced the plant developmental processes *in vitro*.

2.5.6 Incubation Conditions

Environmental factors greatly affect the process of growth and differentiation of tissues in cultures (Razdan, 2003). These incubation conditions include temperature, photoperiod, illumination and relative humidity.

Temperature in a culture room is controlled by the air-conditioning equipment with average temperature of $25 \pm 2\,°C$. The temperature in culture room may be a few degrees higher than the room temperature due to the greenhouse effect. It is recommended that the room should be prepared with an alarm device in order to indicate when the temperature has reached pre-set maximum or minimum limits (Evans *et al*., 2003). Besides, the airflow must also be uniform to ensure the same temperature in the whole environment within the culture room. Kozai *et al*. (1996) reported that the fresh weight of sweet potato plantlets (*Ipomoea batatas* L. Lam) increased at air temperature of $28\,°C$ to $30\,°C$ and the length of stem was longer with higher temperatures.

Photoperiod is the duration of a plant's daily exposure to light, considered especially with regard to the effect of the exposure on growth and development (George *et al*., 2007). Plant growth and developments as well as physiological and biochemical pattern of the cultures may be affected by light and dark conditions. Most plant tissue cultures are grown under lower irradiance level with broad spectral quality. Photoperiod usually is 16 hours light, 8 hours dark, some are in continuous light condition and others are in dark condition (Artanti and McFarlane, 1996). As shown in the study of Senapati and Rout (2008), the frequency of shoot multiplication was higher under 16 hours photoperiod as

compared to continuous light. Artanti and McFarlane (1996) reported that callus cultures of *Coronilla rostrata* had slightly higher fresh weight under high continuous light.

Illumination in the culture room is provided by the fluorescent lamps in the shelves that are made of open mesh with the advantage that light tend to be shared between different levels on the rack. Most of the fluorescent lamps are emitting light deficient in the far-red wavelength, thus suitable spectral balance must be taken into consideration for growth of cultures (Biswas *et al*., 2007). Generally, most of the cultures require an illumination that varies between 500 to 3000 lux. Matsuura and Kakuda (1990) have reported the effect of illumination on THE accumulation in tea callus and was better in dark condition rather than in the illuminated condition.

Relative humidity (RH) is indirectly controlled by the air-conditioning equipment and the RH of the culture environment is usually close to 100% (~20-98%). Generally, low RH causes medium drying and high RH increases chances of contamination. The pattern of organized development can also be influenced by RH surrounding the cultures. In addition, the regulation of RH in certain situations may be necessary for a specific form of differentiation (Vasil and Thorpe, 1994). Elgimabis (2008) reported that plastic tunnels with water mist (higher RH) gave the best rooting and vegetative growth followed by the cutting under plastic tunnels without mist (lower RH), irrespective of season.

2.6 SCANNING ELECTRON MICROSCOPY (SEM)

Electron microscopy is defined as a technology for examining the extremely fine detail or ultrastructure of biological specimens. It is routinely used as a tool in many areas such as anatomy, anthropology, biochemistry, cell biology, forensic medicine, microbiology, immunology, pathology, physiology, plant biology, toxicology and zoology There are two basic types of electron microscopy, namely SEM and TEM (Bozzola and Russell, 1999). As the name implies, SEM generates an image by scanning the specimen's surface with a beam of electrons, while TEM forms an image from electrons that are transmitted through the specimen being examined (Becker *et al*., 2006).

For the present study, SEM was preferred for viewing the surfaces of bulk specimens of *A. cathartica* L. In the SEM, the area to be examined or analyzed is irradiated with a finely focused electron beam, which may be swept in a grating across the surface of the specimen to form images which are recorded either on photographic films or captured digitally using computer interfaces. The interaction between the electron beam and the specimen produced different types of signals which are included secondary electrons, backscattered electrons, characteristic x-rays and other photons of various energies. These signals can be used to examine many features of the specimen such as crystallography, surface topography, composition and etc (Goldstein *et al*., 2003).

There are seven primary operational systems in SEM which function together in order to determine the results and qualities of a micrograph , i.e. vacuum system, electron beam generation system (electron gun), electron beam manipulation system, beam

specimen interaction system, detection system, signal processing system, display and recording system. Firstly, a vacuum system is required when using electron beam due to the quickly motions, either disperse or scatter of electrons which collide with other molecules. Electron beam generation system is located at the top of the microscope column (Figure 2.5) and is known to generate the primary (1 º) electron beam. The function of the electron beam manipulation system is to control the size, shape and position of the electron beam on the specimen surface and it is consists of electromagnetic lenses and coils located in the microscope column (Figure 2.5). Beam specimen interaction system involves the interaction of the electron beam with the specimen and the types of signals that can be detected. The detection system consists of several different detectors which are sensitive to different energy or particle emissions that occur on the specimen. The signal generated by the detection system will be processed by the signal processing system and allows extra electronic manipulation of the image. Lastly, the display and recording systems will allow visualization of an electronic signal by a cathode ray tube and enables recording of the results using photographic or magnetic media (Dunlap and Adaskaveg, 1997).

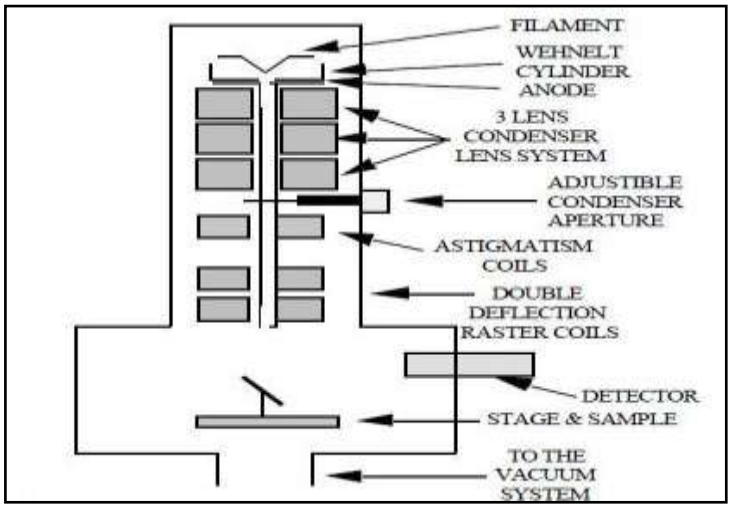

Figure 2.5: Diagram of major components (part of seven primary operational systems) of a SEM (Source: Dunlap and Adaskaveg, 1997).

2.6.1 Specimen Preparation Techniques

The methods that were used in the preparation of specimens for electron microscope are more complicated, time-consuming and costly compared with methods used for the light microscopy. Besides, specimens to be examined by electron microscopy can be prepared in several different ways, depending on the type of microscope and the kind of information the microscopist wants to obtain (Becker *et al*., 2006).

The goal when preparing a specimen for SEM is to preserve the structural features of the cell surface and to treat the tissue in a way that minimizes damage by the electron beam. Initially, the specimen is fixed in glutaraldehyde and Sorensen's phosphate buffer, postfixed in osmium tetroxide and dehydrated by processing through a series of alcohol solution. The specimen is then put in a fluid such as liquid carbon dioxide in a heavy metal canister called a Critical Point Dryer (CPD), in order to dry the specimen under controlled temperature and pressure. CPD helps to maintain the structures on the surfaces of the specimens' tissue in almost the same condition as they were before dehydration. Next, the specimens are mounted on stubs with carbon adhesive tape and coated with a layer of gold or a mixture of gold and palladium, using a modified form of vacuum evaporation called sputter coating. After mounting and coating, the specimens are ready to be examined in the SEM (Becker *et al*., 2006).

2.7 THE NATURAL PRODUCTS FROM PLANTS

Photosynthesis (the metabolism of carbon assimilation) and respiration (the metabolism of energy conversions) are the major processes that occurred in all plants.

Primary plant metabolites such as sugar phosphates, amino acids, lipids, proteins, carbohydrates and nucleic acids are the molecules required by all the plants for proper cellular processes. Secondary plant metabolites or plant natural products are the molecules that may have no obvious role in growth and development of cells. They generally occurred in relatively low quantities and their production may be widespread or restricted to particular families, genera or even species. Furthermore, the natural products produced in the plants have significant economic and medicinal value. The secondary plant metabolites or natural products are divided into four main classes, i.e. terpenes, phenolic compounds, glycosides and alkaloids (Hopkins and Huner, 2004).

Terpenoids are probably the largest and most diverse class of organic compounds found in plants (Zwenger and Basu, 2008) with molecular structures containing carbon backbones made up of isoprene (2-methyl-1,3-butadiene) units, in which the isoprene contains five carbon atoms (Sell and Royal Society of Chemistry, 2003). The terpenoids play diverse functional roles in plants as hormones (gibberellins and abscisic acid), photosynthetic pigments (phytol and carotenoids), electron carriers (ubiquinone and plastoquinone), mediators of polysaccharide assembly (polyprenyl phosphates), latex (the basic for natural rubber), pharmaceuticals (artemisinin and taxol), agrochemicals (pyrethrins and azadirachtin) and many of the essential oils that provide plants their attractive odors and flavors. Moreover, many specific compounds of terpenoid also serve in communication and defense, i.e. as attractants for pollinators and seed dispersers, competitive phytoxins, antibiotics and herbivore repellents and toxins (McGarvey and Croteau, 1995).

Generally, phenolic compounds are the compounds that have one or more hydroxyl group attached directly to an aromatic ring (Vermerris and Nicholson, 2008) and well known as radical scavengers, metal chelators, reducing agents, hydrogen donors and singlet oxygen quenchers (Proestos *et al*., 2006). It is ranging from simple phenolic acids to very large and complex polymers such as tannins, lignin, coumarins and flavonoids. Tannins are divided into two categories, i.e. condensed tannins (also called proanthocyanidins or procyanidins) and hydrolyzed tannins. The astringent property of tannins is a component in the flavor of many fruits as well as drinks such as coffee, tea and red wine (Dykes and Rooney, 2007). Tannins also tend to reduce digestibility of dietary protein by binding with protein in the gut. Lignin is a very large polymer that can be found in cell walls, especially the secondary walls of tracheary elements in the xylem. Moreover, lignin is insoluble in water and most of the organic solvents, not readily digested by herbivores and can slow the rate of cell wall degradation at the site of fungal penetration. Coumarins are widespread family of lactones called benzopyranones which have the roles of antimicrobial agents, feeding deterrents and germination inhibitors (Hopkins and Huner, 2004). Flavonoids such as anthocyanins, flavanols, flavones, flavanones and flavonols are compounds with a C_6-C_3-C_6 skeleton that consists of two aromatic rings joined by a three-carbon link (Dykes and Rooney, 2007). Flavonoids have been show to possess the properties of hepatoprotective, anti-thrambotic, anti-inflammatory, antioxidant, anticarcinogenic, anticancer and antiviral activities (Saija *et al*., 1995).

Glycoside is a molecule in which a sugar group is bonded via its anomeric carbon to another group through a glycosidic bond. Many plants store chemicals in the form of inactive glycosides and can be activated by enzyme hydrolysis (Arias, 2007), which causes

the sugar part to be broken off, making the chemical available for use. Saponins, cardiac glycosides (cardenolides) and cyanogenic glycosides are the secondary metabolites that occur as glycosides. Saponins contain the properties of a surfactant or detergent due to the combination of a relatively hydrophobic triterpene with a hydrophilic sugar. As in plant, saponins appear to preform defence against attack by fungi. Cardiac glycosides are structurally similar to the steroid saponins with similar detergent properties; it can be distinguished by the extra lactone ring (attached at C17) and the rare sugars. Most of the cyanogenic glycosides appear to be derived from one of four amino acids (phenylalanine, tyrosine, valine and isoleucine) or from nicotinic acid and the plants with this type of glycosides would release a toxic HCN when synthesize particular chemicals. Low concentration of cyanogenic glycosides in soy, apples, apricots, peaches and plums are not normally a health hazard but large amount of cyanogenic glycosides in cassava, for example, is poisonous (Hopkins and Huner, 2004).

Alkaloids are low molecular weight nitrogen-containing compounds found mainly in plants with one or more nitrogen atoms, typically as primary, secondary or tertiary amines and this usually confers basicity on the alkaloid (Dewick, 2011). Alkaloids exhibit high biological activity and about 1200 of them have been found to occur in approximately 20 percent of the species of flowering plants (mostly herbaceous dicots). In humans, alkaloids generate varying degrees of physiology and psychological response by interfering with neurotransmitters. Furthermore, alkaloids or alkaloid-rich extracts have been used for a variety of pharmacological purposes, such as tranquilizers, muscle relaxants, antitussives, pain killers, poisons and mind-altering drugs but large doses of alkaloids are highly toxic and may have therapeutic value in smaller doses (Hopkins and Huner, 2004).

2.8 ANTIOXIDANT PROPERTIES OF NATURAL COMPOUNDS IN PLANTS

Recently, there is an increasing interest in antioxidants. The main feature of an antioxidant is its ability to trap the free radicals which may oxidize nucleic acid, proteins, lipids or DNA and can initiate degenerative disease (Prakash *et al*., 2001). In the living systems, there are various metabolic processes and environmental stresses which generate various reactive species of free radicals, i.e. reactive oxygen species (ROS). Structure of biomolecules and their functions can be damaged or modified by increasing level of ROS which may lead to cellular dysfunction and even cell death. The cumulative effect of increased ROS can further increase the oxidative stress in systemic level and it is manifested in the form of a variety of health problems such as cancer, age related disease and cardiovascular diseases (Grune *et al*., 2001; Noguchi and Niki, 2000).

Nature has bestowed living systems with numerous antioxidant molecules. For the prevention of presumed adverse effects of free radicals in the human body and the deterioration of fats and other constituents of foodstuffs, antioxidants from the natural sources (plants) are preferable compared to the synthetic sources (Abdalla and Roozen, 1999). Phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl that are present in the plants are the antioxidant compounds that can inhibit the oxidative mechanisms that lead to degenerative diseases (Prakash *et al*., 2001). Therefore, it is very essential to isolate the naturally occurring antioxidants and use various types of method to estimate the efficiency of these antioxidants presence in the plants.

2.8.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) Assay

One of the most famous methods for monitoring the antioxidant activities from the plant extracts is by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, which is a rapid, simple and inexpensive method. DPPH assay is routinely practiced for assessment of free radical scavenging potential of an antioxidant molecule and considered as one of the standard and easy colorimetric methods for the evaluation of antioxidant properties of pure compound (Mishra *et al*., 2012). DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, to evaluate antioxidant activity of plant's compounds and to quantify antioxidants in complex biological systems. The DPPH method can be used for solid or liquid samples and is not specific to any particular antioxidant component. It can be used to determine the overall antioxidant capacity of the sample and understand the functional properties of the plants (Prakash *et al*., 2001). Compared with other methods, the DPPH assay has many benefits, such as good stability, credible sensitivity, simplicity and feasibility (Deng *et al*., 2011). Moreover, the antioxidant activity of the plant extracts depends on the type and polarity of extraction solvent, the isolation procedures, purity and identify of antioxidant active components from the raw materials (Abdalla and Roozen, 1999).

The molecule of DPPH (molecular formula $C_{18}H_{12}N_5O_6$) is used to assess the free radical scavenging property of natural products (Sarker *et al*., 2005) and is characterized as a stable free radical by the virtue of the delocalization of the spare electron over the molecule as a whole (Molyneux, 2004). The odd electron in the DPPH free radical gives a strong adsorption at 517 nm and is purple in colour. When the odd electron of the nitrogen in the DPPH radical becomes paired with a hydrogen from a free radical scavenging

antioxidant, the purple colour (free radical form) of DPPH turns to yellow colour (non-radical form) as the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 to form the reduced DPPH-H (Figure 2.6). The resulting decolorization is stoichiometric with respect to the number of electrons captured (Prakash *et al*., 2001) and the colour change can be tested spectrophotometrically and utilized for the determination of parameters for antioxidant properties (Mishra *et al*., 2012). In addition, the suitable standard reference or positive control that are widely used in DPPH assay are ascorbic acid and α - tocopherol (Molyneux, 2004).

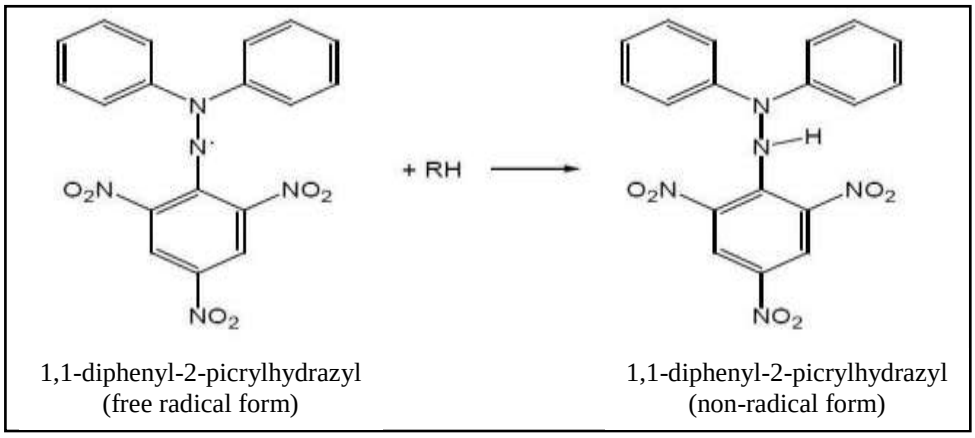

Figure 2.6: Structure of DPPH and its reduction form by the antioxidant RH (Source: Rohman *et al*., 2010).

2.9 PHOTORECEPTORS IN PLANTS

Photoreceptors are defined as pigment molecules that process the energy and informational content of light into a form that can be used by the plant. The photoreceptors present in plants such as chlorophylls, phycobilins and carotenoids will be discussed as follows:

2.9.1 Chlorophylls

Chlorophyll is a pigment primarily responsible for harvesting light energy used in photosynthesis. Its name is derived from the Greek words *chloros* meaning green and *phyllon* meaning leaf (Sur, 2011). The molecule of chlorophyll consists of a porphyrin head and a long hydrocarbon or phytol tail (Figure 2.7). A porphyrin is a cyclic tetrapyrrole that is made up of four nitrogen-containing pyrrole rings arranged in a cyclic fashion. The long and lipid soluble phytol tail is a derivative of the 5-carbon isoprene which is the precursor to a variety of important molecules, such as other pigments (carotenes), hormones (gibberellins) and steroids. The chemical structure of chlorophyll contains a magnesium ion (Mg^{2+}) chelated to the four nitrogen atoms in the center of the ring (Figure 2.7). A non-green product, pheophytin which serves as an early electron acceptor in the chloroplast will be formed when the Mg^{2+} is lost from chlorophyll (Hopkins and Huner, 2004).

There are four types of chlorophyll, i.e. cholorophyll *a*, *b*, *c* and *d*. Chlorophyll *a* is the primary photosynthetic pigment present in all higher plants, algae and cyanobacteria. Chlorophyll *b* is found in virtually all higher plants and green algae and its chemical structure is similar with that of chlorophyll *a*, except a formyl group (-CHO) is substituted by a methyl group on ring II (Figure 2.7). Chlorophyll *c* lacks a phytol tail and can be found in the diatoms, dinoflagellates and brown algae. Chlorophyll *d* is only present in the red algae and a (-O-CHO) group replaces the ($-CH=CH_2$) group on ring I in the chemical structure of chlorophyll *a* (Figure 2.8) (Hopkins and Huner, 2004).

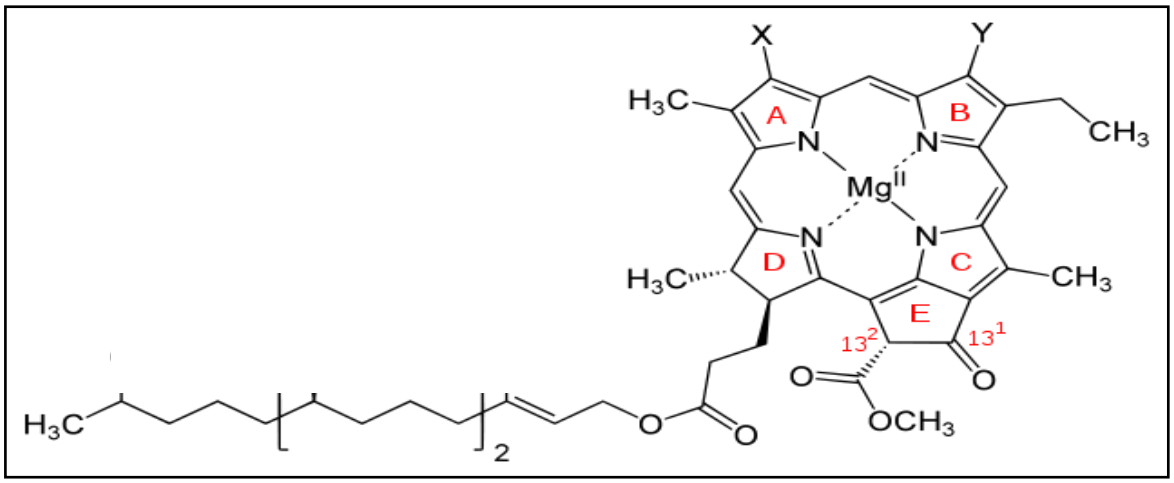

Figure 2.7: Chemical structure of chlorophylls. Chlorophyll *a* - X: $CH=CH_2$, Y: CH_3; Chlorophyll *b* – X: $CH=CH_2$, Y: CHO; Chlorophyll *c* – similar to chlorophyll *a* except that it lacks the long hydrocarbon tail and Chlorophyll *d* – X: -O-CHO, Y: CH_3 (Source: Open WetWare, 2010).

In the organic solvents, all the chlorophylls generally show the similar absorption spectra but with absorption maxima at distinctly different wavelengths in both blue and red regions of the spectrum. Chlorophylls absorbed strongly in the blue and red regions of the spectrum and transmittance in the green is what gives chlorophyll its characteristic green colour (Hopkins and Huner, 2004). An absorption spectrum is a graph that indicates which wavelengths are most strongly absorbed by a pigment molecule. Chlorophyll *a* (absorption peak at 660 and 420 nm) and chlorophyll *b* (absorption peak at 643 and 453 nm) absorb light in the red and blue portions of the visible spectrum (Figure 2.10) and the presence of both types of chlorophylls ensure that more wavelengths of the visible spectrum are absorbed than would be the case with either one individually, this leads to the efficiency of photosynthesis to be enhanced. Furthermore, chlorophyll *c* contains the absorption peak at 625 and 445 nm, whereas chlorophyll *d* indicates the absorption peak at 690 and 450 nm in an absorption spectrum (Campbell and Farrell, 2007).

2.9.2 Phycobilins

From the word of "phycobilin", the prefix *phyco*- designated pigments that are originally from algal. Four phycobilins are known with three of them are involved in the process of photosynthesis, i.e. phycperythrin (also known as phycoerythrobilin), phycocyanin (phycocyanobilin), allophycocyanin (allophycocynobilin) and the fourth, phytochromobilin, is an important photoreceptor that regulates various aspects of growth and development. In term of chemical structure, phycobilin is differs from chlorophyll for the open-chain tetrapyrrole group (chromophore) (Figure 2.8) is covalently linked with a protein that forms a part of the molecule (chromoprotein). A chromophore is the part of the molecule responsible for absorbing light and hence, colour. Except phytochromobilin, the other three phycobilin pigments are only found in the cyanobacteria and red algae (Rhodophyta) where they assume a light-harvesting function in photosynthesis and absorb light energy in the green region of the visible spectrum where chlorophyll does not absorb. The fourth phytochromobilin, i.e. phytochrome is significant to higher plants which plays an important role in many photomorphogenic phenomena. In the absorption spectrum, phycobilins absorb strongly in the range of 520-670 nm (Figure 2.10) where the absorption of chlorophyll is minimal (Nobel, 2009; Hopkins and Huner, 2004).

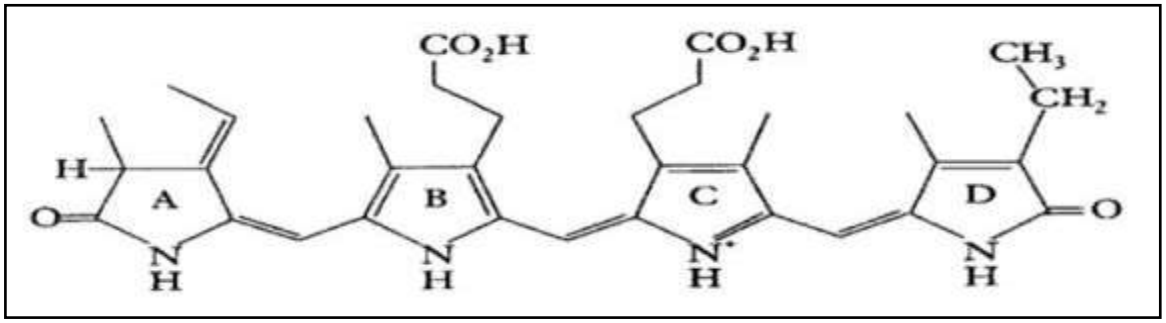

Figure 2.8: The open-chain tetrapyrrole chromophore of phycocyanin (Source: McDonald, 2003).

2.9.3 Carotenoids

Carotenoid pigments are C_{40} terpenoids derived from the isoprenoid pathway which comprise a family of orange and yellow pigments, i.e. carotenes and xanthophylls. Carotenoids are lipid soluble and found either in the chloroplast membranes or in specialized plastids called chromoplasts in most photosynthetic organisms. Carotenes are predominantly orange or red-orange pigments with α-carotene is the major carotenoid found in higher plants and algae and γ-carotene is found in the green photosynthetic bacteria. In term of the chemical structure, both ends of α-carotene and β-carotene molecules are cyclized (Figure 2.9), whereas γ-carotene has only one end cyclized and lycopene has both ends open (Figure 2.9). Xanthophylls (yellow carotenoids) such as lutein, zeaxanthin and violaxanthin are oxygenated carotenes. Carotenoid pigments absorb strongly in the blue region of the visible spectrum (435-480 nm) (Figure 2.10) (Hopkins and Huner, 2004).

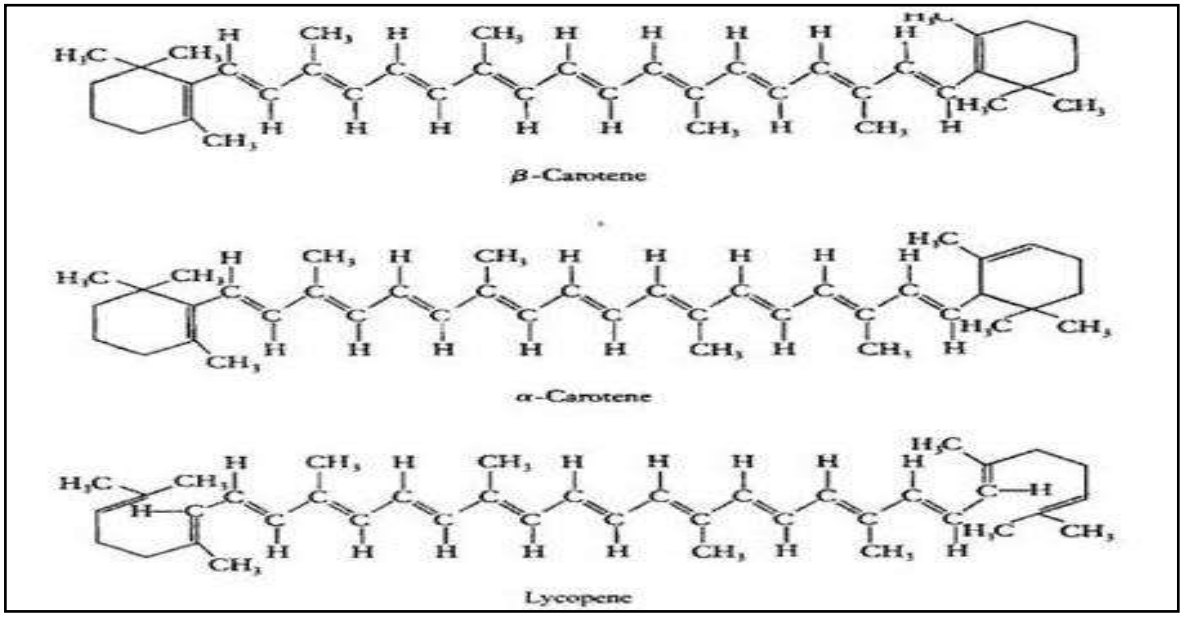

Figure 2.9: The chemical structures of representative carotenes (Source: McDonald, 2003).

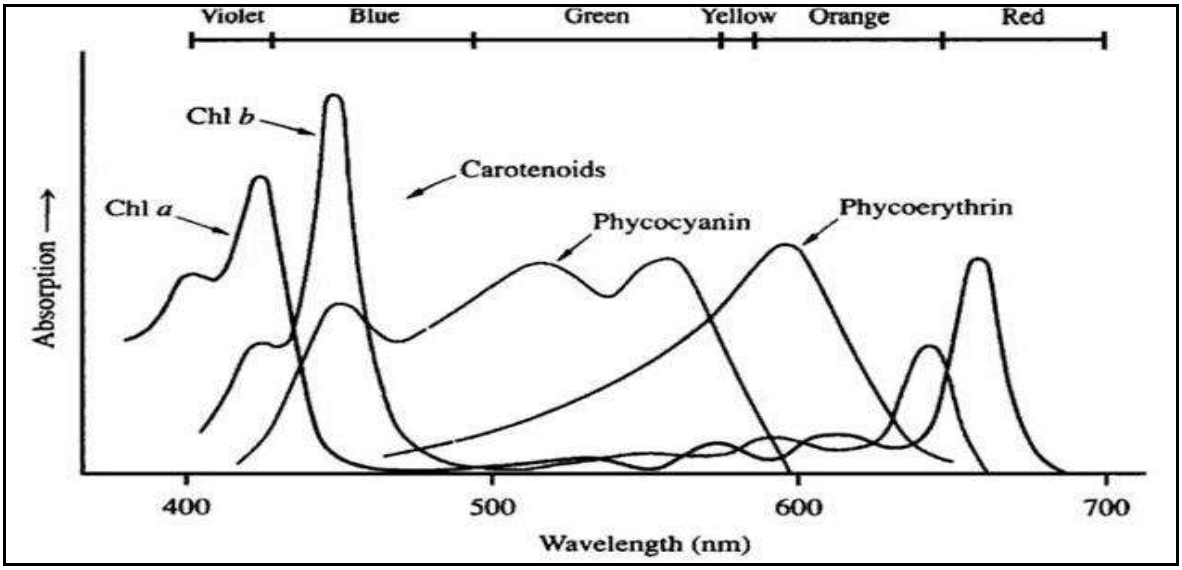

Figure 2.10: The absorption spectra of the major photosynthetic pigments (Source: McDonald, 2003).

CHAPTER 3

METHODOLOGY

3.1 TISSUE CULTURE AND MICROSCOPIC STUDIES

3.1.1 Plant Materials

The intact plant of *A. cathartica* L. (2-month-old) (Figure 3.1) was purchased from Bukit Jalil Nursery Sdn Bhd, Malaysia.

Figure 3.1: Intact plant of *Allamanda cathartica* L.

3.1.2 Surface Sterilization

Before surface sterilization, all the required glassware and apparatus were autoclaved at 121ºC and 15 psi in an autoclave machine (TOMY, ES-315). The healthy shoots were collected and the plant parts were separated into leaves, stems and nodes for surface sterilization. Throughout the process, the explants were sterilized separately. For surface sterilization using the Clorox solution, the explants were first washed with Dettol for 3 minutes. Then, the explants were washed under running tap water for 2 hours. After 2 hours, the explants were transferred into the laminar flow chamber (MDH, MICROFLOW) to be surface-sterilized. Different concentrations of Clorox solution, i.e. (A) 70%, 50%, 30%, 10%; (B) 80%, 60%, 40%, 20%; (C) 90%, 70%, 50%, 30% and (D) 100%, 80%, 60%, 40% were used to establish the suitable surface sterilization protocol for the leaf, stem and nodal explants. The explants were continuously shaken in Clorox solution containing two drops of Tween 20 for 3 minutes for each of the Clorox solution's concentration. The explants were then rinsed with 70% ethanol for 1 minute and rinsed 3 times with sterile distilled water for 2 minutes.

For surface sterilization using the $HgCl_2$ solution, the explants were first washed thoroughly under running tap water for 2 hours with few drops of Tween 20, then, the leaves, stems and nodes were washed with Dettol for 3 minutes and 6 minutes, respectively. In the laminar flow chamber (MDH, MICROFLOW), the explants were rinsed with sterile distilled water for 5 times. The leaf, stem and nodal explants were soaked in 0.1% and 0.2% $HgCl_2$ for 1 minute and 2 minutes, respectively. Finally, the explants were rinsed with sterile distilled water for 5 times.

The leaf explants were then cut into squares with approximate size of 0.5 cm X 0.5 cm, stem and nodal explants were cut into approximately 1-2 cm in length. All the explants were placed in the sterile culture tubes containing MS medium without any supplementation of PGR for two weeks observation.

3.1.3 Preparation of MS Basal Medium

The medium used in this study was Murashige and Skoog (MS) (1962). This medium was chosen based on previous studies by other researchers and the formulation for MS medium is given in Appendix I. However, MS medium used in this study was the ready-to-use powdered form purchased from SIGMA Chemical Company, Sigma-aldrich, St. Louis, USA. Full strength of MS medium was utilized throughout this study for callus induction and plant regeneration (shoot multiplication and shoot elongation). The MS basal medium (1L) was composed of 4.4 g MS's powder (including all the macronutrients, micronutrients, FeNaEDTA and vitamins) or PGR (if needed). Sucrose at 30 g (w/v) was added as the carbon source and then dissolved completely with distilled water. The pH of the medium was adjusted to 5.7±0.1 by using NaOH or HCl with a pH meter (METTLER TOLEDO, 320 pH Meter). Agar Technical at 8 g (w/v) was added to solidify the medium. The medium was then autoclaved at 121ºC and 15 psi for 21 minutes. After autoclaving, the autoclaved medium was let cooled before dispensing into the sterile culture tubes in the laminar flow hood.

3.1.4 Callus Induction from the Leaf and Stem Explants

After 2 weeks from the day of surface sterilization, the uncontaminated leaf and stem explants were transferred into MS medium supplemented with different concentrations of 2,4-D, BAP, NAA or picloram alone (0.5 and 1.0 mg/L) or in combination of 2,4-D (0.5, 1.0 and 1.5 mg/L) and BAP (0.5, 1.0 and 1.5 mg/L) or NAA (0.5 and 1.0 mg/L) and BAP (0.5 mg/L) . MS medium without any supplementation of PGR was used as the control. The mouth of sterile culture tube was sealed with parafilm. After 4 weeks of culture, the callus formed was excised from the explants in order to determine the callus fresh weight and then dried in the oven at 34ºC for 1 week for callus dry weight determination.

3.1.5 Plant Regeneration (Shoot Multiplication and Shoot Elongation)

After 2 weeks from culture initiation, the uncontaminated nodal explants were cultured vertically by inserting about 0.4 cm of the basal end into semi-solid MS medium containing 1.0, 3.0 and 5.0 mg/L BAP. MS medium without any supplementation of PGR was used as the control. The mouth of sterile culture tube was sealed with parafilm. After 4 weeks of culture, the new shoots formed from the nodal part were transferred to a new sterile culture tube containing MS medium without any supplementation of PGR for shoot elongation.

3.1.6 Culture Conditions

All apparatus such as forceps, scapel, petri dishes, conical flasks and etc need to be cleaned and sterilized before being utilized. These culture apparatus were wrapped in aluminium foil and autoclaved for 30 minutes. Forceps and scalpel were dipped in 99.9%

alcohol, sterile distilled water, dried and dipped into hot bead sterilizer to ensure the sterility of culture apparatus. Hot bead sterilizer reached a temperature of 250ºC. Forceps and scalpel need to be cooled by dipping them into sterile distilled water before being used to excise plant tissues.

All the cultures were labeled and incubated in the culture room under a photoperiod of 16 hours light and 8 hours darkness at 25±1ºC with a light intensity of 1000 lux provided by cool white fluorescent tubes. This was suitable to allow cultures to respond and grow.

3.1.7 Microscopic Studies (Scanning Electron Microscopy - SEM)

In this study, the same developmental stages (2-month-old) of *in vitro* and *in vivo* (intact) shoots were used. The leaf and stem specimens of *A. cathartica* L. were treated with different chemical materials separately. Firstly, the specimens were fixed in 4% glutaraldehyde and Sorensen's phosphate buffer with the ratio of 1:1 and incubated at room temperature for 1 hour. After 1 hour, the specimens were rinsed with Sorensen's phosphate buffer and distilled water with the ratio of 1:1. Next, the specimens were soaked in 1% osmium and distilled water with the ratio of 1:1 and incubated at room temperature for 1 hour in the fume hood. The solution of osmium was pipette out and the specimens were rinsed with distilled water. The fixations of specimens were then carried out using the ethanol series (10% to 100%) for 15 minutes each step. The specimens were soaked in the mixture of 100% ethanol and 100% acetone for 20 minutes in the ratios of 3:1, 1:1 and 1:3, respectively. Then, the specimens were soaked in 100% acetone for 20 minutes and this step was repeated for 3 times. Replacement of acetone with carbon dioxide for several

times was carried out using the CPD equipment (BAL-TEC, CPD 030). Then the specimens were kept inside the CPD chamber for 1 hour. After the process of CPD, the specimens were mounted on stubs with carbon adhesive tape. The specimens were then placed inside of SPI-Module Sputter Coater chamber for covering the specimen surfaces with gold. In this step, the air of the chamber was replaced with argon gas using a pump, which had been attached to the gold coater equipment. Coating the specimens with gold was accomplished for 60 seconds. Finally, the specimens were observed using a scanning electron microscope (JEOL, JSM-6400).

3.2 INVESTIGATION ON SECONDARY METABOLITES OF *Allamanda cathartica* L.

3.2.1 Plant Material Extraction

For the preparation of ethanol extracts using the dried plant materials, the fresh leaves, stems, petals and roots of *A. cathartica* L. were air-dried at room temperature (26ºC) for 1 week, after which it was grinded to a uniform powder. The ethanol extracts were prepared by soaking 5 g each of the dried powdered plant materials in 50 ml of ethanol (99.9%) at room temperature for 4 days. After 4 days, the extract was filtered through a Whatmann filter paper No. 42 and then through cotton wool. The extracts were concentrated using rotary evaporator (Bochi Rotavapor, R-200/205) with the water bath set at 40ºC. For the preparation of ethanol extracts using the fresh plant materials, the fresh leaves, stems, petals and roots of *A. cathartica* L. were grinded into small pieces directly without air-dried at room temperature and the ethanol extracts were prepared by soaking the plant materials in 99.9% ethanol followed by the steps that mentioned above. Both

ethanol extracts of dry and fresh plant materials were used for phytochemical screening and antioxidant activity determination.

3.2.2 Phytochemical Screening

3.2.2.1 Test for Reducing Sugars (Fehling's Test)

The aqueous ethanol extract (0.5 g in 1 ml of distilled water) with 5-8 drops of Fehling's solution (A and B) were boiled in a test tube. Formation of a brick red precipitate was observed for the presence of reducing sugar (Ayoola *et al*., 2008).

3.2.2.2 Test for Anthraquinones (Bontrager's Test)

The extract (0.5 g) was boiled with 10 ml of concentrated H_2SO_4 for 5 minutes and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer (lower layer) was pipetted into another test tube and 1 ml of dilute ammonia (10%) was added. A rose pink to red colouration in the ammonical layer indicates the presence of anthraquinones (Ayoola *et al*., 2008).

3.2.2.3 Test for Terpenoids and Steroids (Salkowski Test)

Two ml of chloroform was added to 0.5 g of extract. Three ml of concentrated H_2SO_4 was carefully added to form a layer. A red violet colouration of the interface indicates the presence of terpenoids and green bluish colour for steroids (Chhetri *et al*., 2008).

3.2.2.4 Test for Saponins

Distilled water (5 ml) was added to 0.5 g of extract in a test tube. The solution was shaken vigourously and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigourously after which it was observed for the formation of an emulsion (Ayoola *et al*., 2008).

3.2.2.5 Test for Tannins

The extract (0.5 g) was boiled in 1 ml of distilled water in a test tube and 1-2 drops of 0.1% $FeCl_3$ was added. Blue colour was observed for gallic tannins and green black for catecholic tannins (Chhetri *et al*., 2008).

3.2.2.6 Test for Glycosides

The extract (0.5 g) was hydrolyzed with 1 ml of HCl and neutralized with 1 ml of NaOH solution. A few drops of Fehling's solution (A and B) were added. Red precipitate indicates the presence of glycosides (Uddin *et al*., 2011a).

3.2.2.7 Test for Essential Oils

To 2 ml of the extract, 0.1 ml of 2M NaOH was added, followed by a small quantity of 2M HCl and shaken. White precipitate indicates the presence of essential oils (Ahumuza and Kirimuhuzya, 2011).

3.2.2.8 Test for Flavonoids

Two methods were used to test for flavonoids. First, 5 ml of dilute ammonia (10%) was added to portion of an aqeous filtrate of the extract (0.5 g). Concentrated H_2SO_4 (1 ml) was then added. A yellow colouration that disappears on standing indicates the presence of flavonoids (Ayoola *et al*., 2008). Second, 1 ml of dilute NaOH (5%) was added to 1 ml of extract. Golden reddish precipitate indicates the presence of flavonoids (Kadiri and Ajayi, 2009).

3.2.3 Determination of Antioxidant Activities

For both dried and fresh plant materials, the antioxidant activities of leaf, stem, petal and root extracts of *A. cathartica* L. were determined using the DPPH free radical scavenging assay. Initially, 0.004% (w/v) DPPH solution was prepared in 95% methanol. The crude leaf, stem, petal and root's ethanol extracts of *A. cathartica* L. was mixed with 95% methanol to prepare the stock solution (100 μg/ml). From the stock solution, 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of this solution were taken into five centrifuge tubes and by serial dilution with 95% methanol and was made the final volume of each centrifuge tube up to 10 ml whose concentration was then became 20 μg/ml, 40 μg/ml, 60 μg/ml, 80 μg/ml and 100 μg/ml, respectively. Next, 0.5 ml of freshly prepared DPPH solution was added in each of these centrifuge tubes containing *A. cathartica* L. extract. After 2 hours, the absorbance was taken at 517 nm using UV-VIS spectrophotometer (MFLAB, NV203). Ascorbic acid was used as a reference standard and dissolved in distilled water to make the stock solution with the same concentration (100 μg/ml) of extract of *A. cathartica*. 95% methanol was used as blank and control.

3.3 PIGMENT EXTRACTION AND COLOURED COATING DEVELOPMENT FROM NATURAL PIGMENTS (CHLOROPHYLLS AND CAROTENOIDS)

3.3.1 Pigment Extraction

For pigment extraction, the fresh leaves, stems, petals and calli of *A. cathartica* L. were dried in an oven at 41ºC for 5 minutes. The plant materials (1 g each) were ground separately in the ice bath under dim light condition with different extracting solvents, i.e. 0.15% HCl in 99.9% methanol, 0.01% HCl in 70% acetone and 95% ethanol in different volumes (5.0, 12.5, 20.0 and 25.0 ml). The ground plant material was then centrifuged at 5000 rpm for 5 minutes and the supernatant (plant sample) was collected. Lastly, the sample was measured using UV-VIS spectrophotometer (SHIMADZU, UV-1650) in order to identify the spectral characteristics of pigments and compared.

3.3.2 Preparation of Plant Materials for Coloured Coating Development from Natural Pigments (Chlorophylls and Carotenoids)

In order to achieve the great colouration from the natural pigments, the fresh leaves and petals of *A. cathartica* L. were chosen and air-dried at room temperature (26ºC) for 1 week, after which it was ground to a uniform powder. The methanol and ethanol extracts were prepared by soaking 5 g each of the dried powdered plant materials in 50 ml of ethanol (95%) and methanol (0.15% HCl in 99.9% methanol) at room temperature for 5 days. After 5 days, the extract was centrifuged at 5000 rpm for 5 minutes and the supernatant (plant sample) was used as the source for the development of coloured coating.

3.3.3 Preparation of Polyvinyl Alcohol (PVA) Solution

In order to prepare 200 ml of 15% polyvinyl alcohol (PVA) solution, 170 ml of distilled water and 30 g of PVA powder were measured. The PVA powder was added to the distilled water with stirring. The mixture was heated and stirred occasionally, until a clear solution was obtained. The solution was covered with aluminum foil and allowed to cool.

3.3.4 Coloured Coating Development from Natural Pigments (Chlorophylls and Carotenoids)

The coloured coating development from natural pigments was performed on the glass slides and cotton wools. The PVA solution and the plant extract (supernatant) with mixing ratio of 1:1 were mixed and painted on a glass class and a cotton wool by a brush. For the natural coloured coating on the glass slide, it was preceded with the salt test (0.5% NaCl, 1.0% NaCl and 1.5 NaCl) and heat test (temperature of the oven set at 40ºC) with different parameter of times, i.e. 0, 15, 30, 45 and 60 minutes for the salt test and 24, 48, 72, 96, 120, 144 and 168 hours for the heat test. After each of the test, the absorbance was taken at 662 nm and 430 nm for the leaf sample and 450 nm for the petal sample by UV-VIS spectrophotometer (MFLAB, NV203). The mixture of the PVA solution and the extracting solvent (1:1 ratio) without any plant sample was used as the control. For the natural coloured coating on the cotton wool, the degradation of colour was observed over 1 week interval for 8 weeks.

3.4 EXPERIMENTAL DESIGN

3.4.1 Experimental Replication

For surface sterilization, a total of four leaves, two stem and two nodal explants were used in each sterile culture tube. Each explant would have 10 replicates and the whole experiment was repeated for three times. For callus induction from the leaf and stem explants, a total of two leaves and two stem explants were used for each treatment. Each treatment would have five replicates and the experiment was repeated for three times. For shoot multiplication, a total of two nodal explants were cultured in each sterile culture tube with five replicates for each treatment and the experiment was repeated for three times. After shoot multiplication, a total of 15 selected explants were transferred into MS medium without any supplementation of PGRs for the shoot elongation studies. For microscopic studies (SEM) from the leaf and stem (*in vitro* and *in vivo*) specimens, a total of two leaves and two stems specimens were used for specimen preparation and viewing under the microscope once. For phytochemical screening, determination of antioxidant activity, pigment extraction and coloured coating development, the whole experiment was repeated for three times for reliable results.

3.4.2 Data Collection

3.4.2.1 Contamination Frequency

Data collected for contamination frequency were the type and morphology of the contaminant over 2 weeks of culture. The data calculated were the total percentage of

contamination and the percentage of contamination for each contaminant with the following formula:

$$\text{Total percentage of contamination (\%)} = \text{Sum of contamination percentage for each contaminant}$$

$$\text{Percentage of contamination for each contaminant (\%)} = \frac{\text{Number of contaminated explants}}{\text{Total number of explants cultured}} \text{ X } 100\%$$

3.4.2.2 Callus Induction

For callus induction, data collected were the day of initial callus formation, callus morphology, callus fresh and dried weights over 4 weeks of culture. The data calculated were the percentage of callus formation. The formula to calculate the percentage of callus formation was shown as follow (Chowdhury *et al.*, 2011):

$$\text{Percentage of callus formation (\%)} = \frac{\text{Number of explants that formed callus}}{\text{Total number of explants cultured}} \text{ X } 100\%$$

3.4.2.3 Plant Regeneration

For shoot multiplication, data collected were the number of shoots formed from the nodal part and shoot morphology. The data calculated were the percentage of shoot formation and average number of shoots formed over 4 weeks of culture. The formulas were shown as below (Burbulis *et al.*, 2010):

$$\text{Percentage of shoot formation (\%)} = \frac{\text{Number of explants that formed shoot}}{\text{Total number of explants cultured}} \text{ X } 100\%$$

$$\text{Number of shoots formation per explant} = \frac{\text{Total number of shoot formed per treatment}}{\text{Total number of explants cultured}}$$

The length of elongated shoot, shoot morphology and root formation were observed for 4 weeks intervals after the multiplied shoots were transferred into MS medium without any supplementation of PGR.

3.4.2.4 Microscopic Studies (Scanning Electron Microscopy - SEM)

Data collected for microscopic studies (SEM) was the micrographs of leaf and stem (*in vitro* and *in vivo*) specimens of *A. cathartica* L. which generated by SEM. Observations and comparisons were made on the differences of abaxial and adaxial surfaces and the presence of stomata and trichomes on the specimens of leaf and stem (*in vitro* and *in vivo*).

3.4.2.5 Investigation on Secondary Metabolites of *Allamanda cathartica* L.

Data collected for phytochemical screening were the presence of phytochemical constituents for each of the phytochemical test.

Data collected for determination of antioxidant activity was the absorbance value taken at 517 nm. The data calculated were the percentage yield of extract, IC_{50} from the graph of percentage of scavenging of DPPH against respective concentrations used and percentage of scavenging of the DPPH free radical with the following formula (Chhetri *et al*., 2008; Ripa *et al*., 2009; Ayoola *et al*., 2008):

$$\text{Percentage yield of extract (\%)} = \frac{\text{Weight of the extract}}{\text{Weight of raw material}} \text{ X } 100\%$$

$$\text{Percentage scavenging activity (\%)} = \frac{\text{Absorbance of the control} - \text{Absorbance of the test sample}}{\text{Absorbance of the control}} \times 100\%$$

3.4.2.6 Pigment Extraction and Coloured Coating Development from Natural Pigments (Chlorophylls and Carotenoids)

For pigment extraction, data collected was the graph of absorbance versus wavelength which measured under UV-VIS spectrophotometer for all the methanol, acetone and ethanol extracts of leaves, stems, petals and calli of *A. cathartica* L. For coloured coating development from the natural pigments on the glass slide, data collected was the absorbance value at 662 nm and 430 nm (leaf sample), 450 nm (petal sample) at different parameter of times for both the salt and heat tests. For the natural coloured coating on the cotton wool, data collected was the observation for colour degradation over 1 week interval for 8 weeks.

3.4.3 Data Analysis

SPSS version 15.0 for Window® was used to perform the overall statistical analysis. One way analysis of variance (One-Way ANOVA) was used to analyse the result of each experiment. Post hoc multiple comparisons Tukey's Honestly Significant Difference (HSD) test was used to analyse the homogeneous subsets of the collected data. The statistic significance of differences between means was predicted at 95% of confidence interval ($p<0.05$).

CHAPTER 4

RESULTS

4.1 TISSUE CULTURE AND MICROSCOPIC STUDIES

4.1.1 Contamination Frequency

To establish a suitable surface sterilization protocols for leaf, stem and nodal explants of *A. cathartica* L., different concentrations of Clorox solution were used, i.e. (A) 70%, 50%, 30%, 10%; (B) 80%, 60%, 40%, 20%; (C) 90%, 70%, 50%, 30% and (D) 100%, 80%, 60%, 40%. $HgCl_2$ at the concentrations of (E) 0.1% and (F) 0.2% were also used to surface sterilize the leaf, stem and nodal explants. All the explants were continuously shaken in each Clorox solution for 3 minutes, 0.1% $HgCl_2$ for 1 minute and 0.2% $HgCl_2$ for 2 minutes.

Contamination was observed on the leaf, stem and nodal explants after two weeks of culture for all the treatments of Clorox solution. Based on Figure 4.1, the total percentage of contamination using Clorox solution at the concentrations of 70%, 50%, 30%, 10% was 99.2 ± 0.8% for the leaf explants, 98.3 ± 1.7% for the stem and nodal explants. For Clorox solution at the concentrations of 80%, 60%, 40%, 20%, leaf explants had 97.5 ± 1.4% contamination while stem and nodal explants had 93.3 ± 6.7% and 95.0 ± 2.9% contamination, respectively (Figure 4.1). For Clorox solution at the concentrations of 90%, 70%, 50%, 30%, leaf, stem and nodal explants showed a total of 95.8 ± 2.2%, 91.7 ± 4.4% and 88.3 ± 6.0% contamination, respectively (Figure 4.1). While, the total percentage of

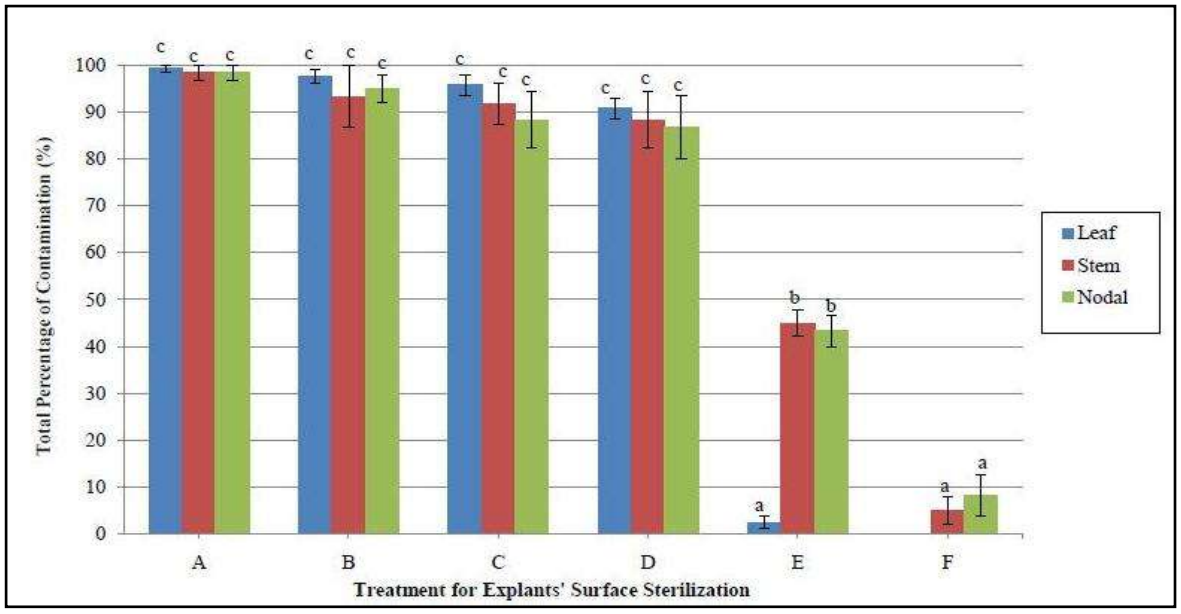

Figure 4.1: Total percentage of contamination on leaf, stem and nodal explants of *Allamanda cathartica* L. treated with different concentrations of Clorox solution and mercuric (II) chloride ($HgCl_2$). (A) 70%, 50%, 30%, 10%; (B) 80%, 60%, 40%, 20%; (C) 90%, 70%, 50%, 30%; (D) 100%, 80%, 60%, 40%; (E) 0.1% $HgCl_2$ and (F) 0.2% $HgCl_2$. The error bars indicate the standard error. Same alphabet on top of the bars indicate that the means of percentage of contamination do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

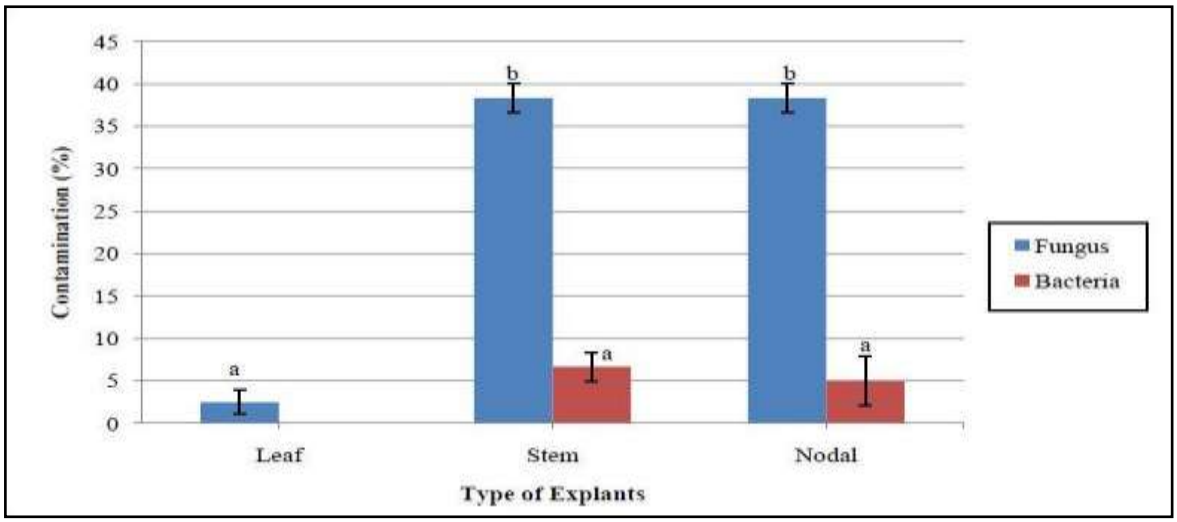

Figure 4.2: Percentages of fungus and bacteria contaminations on leaf, stem and nodal explants of *Allamanda cathartica* L. treated with 0.1% $HgCl_2$ for 1 minute after 2 weeks of culture. The error bars indicate the standard error. Same alphabet on top of the bars indicate that the means of percentage of contamination do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

contamination in the Clorox treatment of 100%, 80%, 60%, 40% gave 90.8 ± 2.2%, 88.3 ± 6.0% and 86.7 ± 6.7% contamination on leaf, stem and nodal explants, respectively (Figure 4.1). Moreover, surface sterilization using 0.1% and 0.2% $HgCl_2$ was also carried out in order to sterilize the explants. For 0.1% $HgCl_2$, the leaf, stem and nodal explants were showed to have 2.5 ± 1.4%, 45.0 ± 2.9% and 43.3 ± 3.3% of contamination, respectively (Figure 4.1). The explants that surface sterilized using 0.2% $HgCl_2$ were gave 0%, 5.0 ± 2.9% and 8.3 ± 4.4% contamination for leaf, stem and nodal explants, respectively according to Figure 4.1.

Statistical analysis by One-Way analysis of variance (ANOVA) using Tukey's HSD test indicated that there were no significant difference on the total percentage of contamination between leaf, stem and nodal explants treated in all concentrations of Clorox solution. For the treatment of $HgCl_2$, there were no significant difference on the total percentage of contamination between leaf explants treated with 0.1% $HgCl_2$, stem and nodal explants treated with 0.2% $HgCl_2$ but a significant difference was observed on the stem and nodal explants treated with 0.1% $HgCl_2$. However, a significant difference on the total percentage of contamination was also observed between the leaf, stem and nodal explants between the treatments of Clorox solution and $HgCl_2$ solution.

Among the different protocols of surface sterilization tested above, 0.1% of $HgCl_2$ was the most suitable agent which to sterilize the leaf explants with the total percentage of contamination of 2.5 ± 1.4%, whereas 0.2% $HgCl_2$ was the most suitable agent which to sterilize the stem (5.0 ± 2.9%) and nodal (8.3 ± 4.4%) explants (Figure 4.1). Moreover, high concentration of $HgCl_2$, i.e. 0.2% caused the leaf explants to die. By using 0.1%

$HgCl_2$, it was found that the percentage of fungus contamination was 2.5 ± 1.4% while no bacteria contamination was observed for leaf explants (Figure 4.2). In the case of stem explants, 38.3 ± 1.7% contamination was due to fungus contamination while the percentage of bacteria contamination was 6.7 ± 1.7% (Figure 4.2). The percentages of fungus and bacteria contamination for the nodal explants were 38.3 ± 1.7% and 5 ± 2.9%, respectively (Figure 4.2). There were no significant differences between the percentage of fungus and bacteria contaminations on stem and nodal explants but a significant difference was observed on the percentage of fungus contamination between the leaf, stem and nodal explants.

For 0.2% $HgCl_2$, no fungus and bacteria contamination were observed because leaf explants were dead after sterilization. In the case of stem explants, 5.0 ± 2.9% contamination was due to fungus contamination and no bacteria contamination was found (Figure 4.3). The percentages of fungus and bacteria contaminations for the nodal explants were 5.0 ± 2.9% and 3.3 ± 1.7%, respectively (Figure 4.3). There were no significant differences on the percentages of fungus and bacteria contaminations between the stem and nodal explants after statistical analysis were performed.

Throughout the study, only fungus and bacteria contaminations were observed for leaf, stem and nodal explants where fungus contamination occurred more frequently than bacteria contamination. Contamination of cultures by fungus could be recognized by their fuzzy growth, which was white or grey in colour (Figure 4.4). On the other hand, bacteria contamination could be characterized as smooth cream, pink or white colonies (Figure 4.4). Fungus contamination was observed after three or four days of culture, whereas bacteria

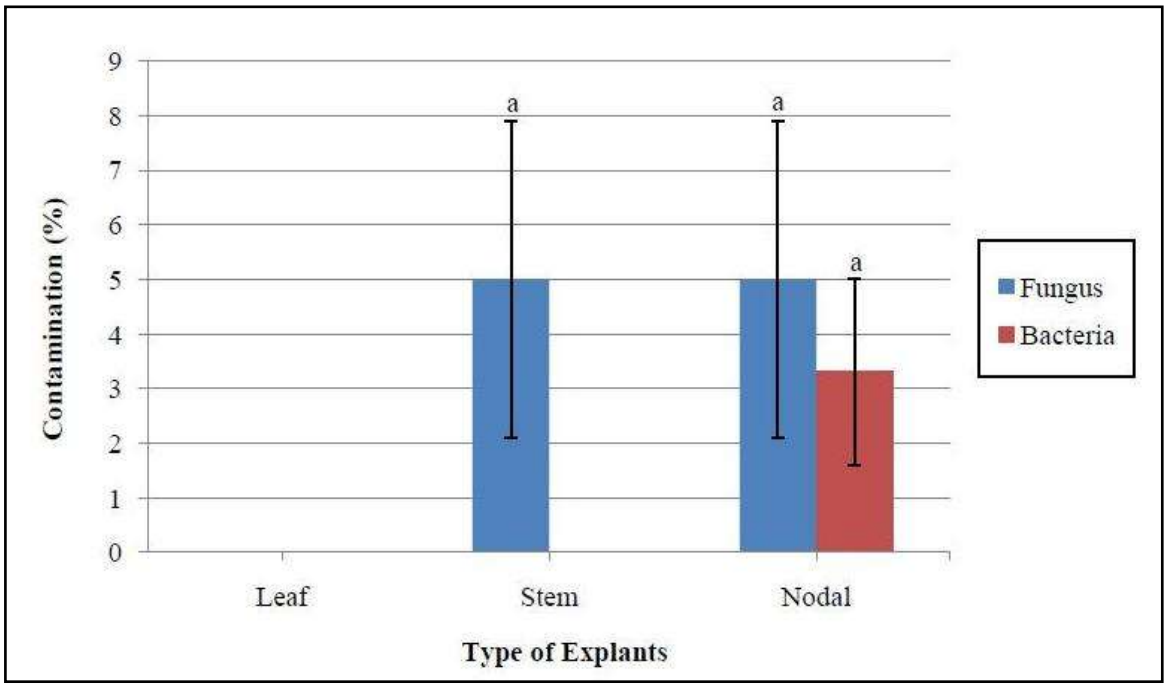

Figure 4.3: Percentages of fungus and bacteria contaminations on leaf, stem and nodal explants of *Allamanda cathartica* L. treated with 0.2% $HgCl_2$ for 2 minute after 2 weeks of culture. The error bars indicate the standard error. Same alphabet on top of the bars indicate that the means of percentage of contamination do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

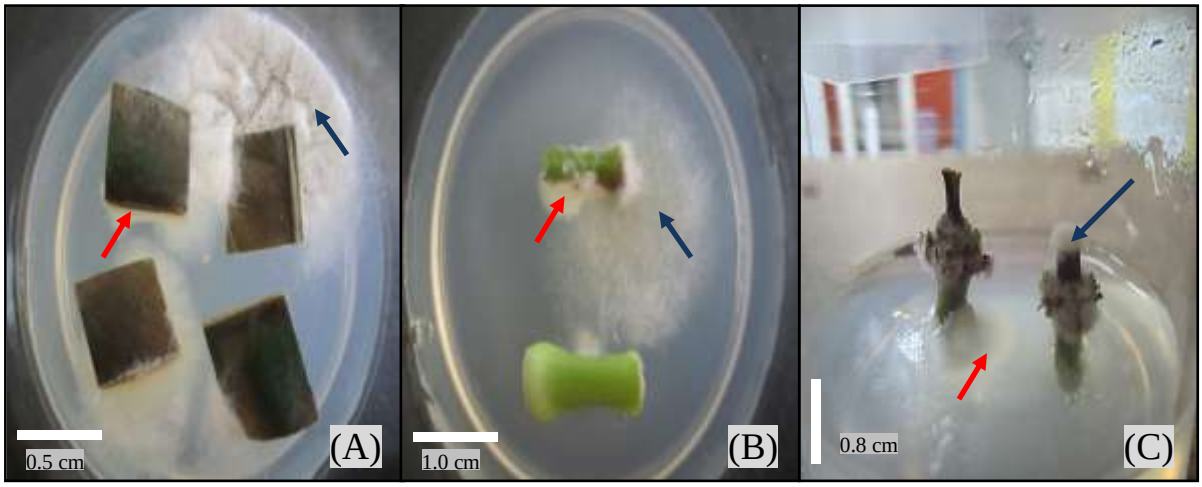

Figure 4.4: Fungus (blue arrow) and bacteria (red arrow) contaminations observed on the explants of *Allamanda cathartica* L. (A) Leaf explants; (B) Stem explants and (C) Nodal explants.

contamination was observed after 6 or 7 days of culture. Fungus was observed to grow much faster than bacteria. Fungus contaminated the whole surface of medium from a single site within five to six days while bacteria spread much slower than obvious growth could only be observed after a week.

4.1.2 Callus Induction from the Leaf Explants

The result on callus induction from leaf explants of *A. cathartica* L. revealed that there was no sign of callogenesis in the control medium under the photoperiod of 16 hours light and 8 hours darkness condition (Table 4.1). All the leaf explants cultured in the control medium were unhealthy, turned from green to brown in colour after 4 weeks and eventually died (Figure 4.5). There were no significant differences on the day of initial callus formation, percentage of callus formation, callus fresh and dry weights were observed for the control medium.

In the treatment of auxins alone, 2,4-D, NAA and picloram were used in this study. Callus formation was observed on MS medium supplemented with 2,4-D and explants cultured on MS medium fortified with either 0.5 or 1.0 mg/L of 2,4-D provided the same percentage of callus formation, i.e. 16.7 ± 12.0% and 16.7 ± 6.7%, respectively (Table 4.1) MS medium fortified with 0.5 mg/L 2,4-D required the shorter time (16.0 ± 1.9 days) to induce the callus formation as compared to MS medium fortified with 1.0 mg/L 2,4-D (17.0 ± 1.5 days). In term of the callus fresh and dry weights, 1.0 mg/L 2,4-D (0.0102 ± 0.0001 g and 0.0018 ± 0.0001 g) showed to produce higher weight of callus compared to 0.5 mg/L 2,4-D (0.0020 ± 0.0010 g and 0.0002 ± 0.0001 g). There were no significant differences observed on the percentage of callus formation, day of initial callus formation,

Table 4.1: Effects of MS medium supplemented with auxins (2,4-D, NAA and picloram) and cytokinin (BAP) at different concentrations on callus induction from leaf explants of *Allamanda cathartica* L. after 4 weeks of culture.

Concentration of auxin (mg/L)	Concentration of cytokinin (mg/L)	Day of initial callus formation (Day ± SE[a])	Percentage of callus formation (%) ± SE[a]	Callus fresh weight (g) ± SE[a]	Callus dry weight (g) ± SE[a]	Callus morphology
2,4-D	**BAP**					
0	0	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
0.5	0	16.0 ± 1.9^{d}	16.7 ± 12.0^{a}	0.0020 ± 0.0010^{a}	0.0002 ± 0.0001^{a}	Yellow-white, greenish friable callus
1.0	0	17.0 ± 1.5^{d}	16.7 ± 6.7^{a}	0.0102 ± 0.0001^{a}	0.0018 ± 0.0001^{a}	Yellow-white, greenish friable callus
0	0.5	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
0	1.0	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
0.5	0.5	8.0 ± 0.3^{b}	90.0 ± 10.0^{bcd}	0.2451 ± 0.0283^{bcd}	0.0325 ± 0.0141^{a}	Yellow-white, greenish friable callus
0.5	1.0	6.0 ± 0.3^{b}	93.3 ± 6.7^{cd}	0.1814 ± 0.0035^{bc}	0.0158 ± 0.0016^{a}	Yellow-white, greenish friable callus
0.5	1.5	9.0 ± 1.2^{bc}	86.7 ± 6.7^{bcd}	0.2590 ± 0.0467^{bcd}	0.0419 ± 0.0146^{a}	Yellow-white, greenish friable callus
1.0	0.5	10.0 ± 1.7^{bc}	80.0 ± 10.0^{bcd}	0.2169 ± 0.0318^{bc}	0.0251 ± 0.0049^{a}	Yellow-white, greenish friable callus
1.0	1.0	6.0 ± 0^{b}	100.0 ± 0^{d}	0.3857 ± 0.0939^{d}	0.0707 ± 0.0549^{a}	Yellow-white, greenish friable callus
1.0	1.5	9.0 ± 0.6^{bc}	93.3 ± 6.7^{cd}	0.1949 ± 0.0371^{bc}	0.0217 ± 0.0050^{a}	Yellow-white, greenish friable callus
1.5	0.5	9.0 ± 1.2^{bc}	96.7 ± 3.3^{d}	0.1368 ± 0.0053^{abc}	0.0147 ± 0.0026^{a}	Yellow-white, greenish friable callus
1.5	1.0	6.0 ± 0.3^{b}	96.7 ± 3.3^{d}	0.1914 ± 0.0244^{bc}	0.0176 ± 0.0017^{a}	Yellow-white, greenish friable callus
1.5	1.5	6.0 ± 0.3^{b}	96.7 ± 3.3^{d}	0.2850 ± 0.0100^{cd}	0.0523 ± 0.0206^{a}	Yellow-white, greenish friable callus
NAA	**BAP**					
0.5	0	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
1.0	0	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
0.5	0.5	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
1.0	0.5	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed
Picloram						
0.5	0	13.0 ± 1.5^{cd}	60.0 ± 5.8^{b}	0.1116 ± 0.0394^{ab}	0.0120 ± 0.0029^{a}	Yellow-white, greenish friable callus
1.0	0	8.0 ± 0.6^{b}	63.3 ± 6.7^{bc}	0.1526 ± 0.0202^{abc}	0.0124 ± 0.0016^{a}	Yellow-white, greenish friable callus

SE[a] = Standard Error. The value for the same alphabet is not significantly different (Tukey's multiple range test, $p<0.05$).

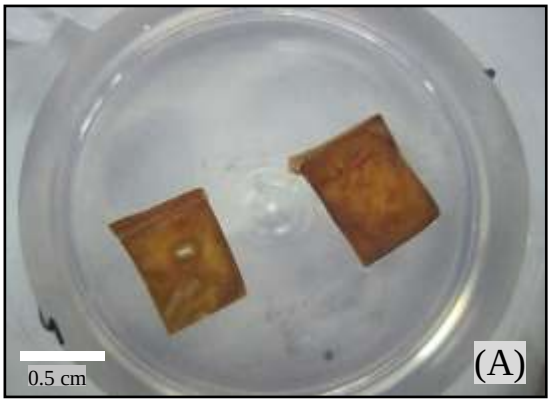

Figure 4.5: Induction of callus from leaf explants of *Allamanda cathartica* L. on MS medium without any supplementation of PGRs. (A) Control medium.

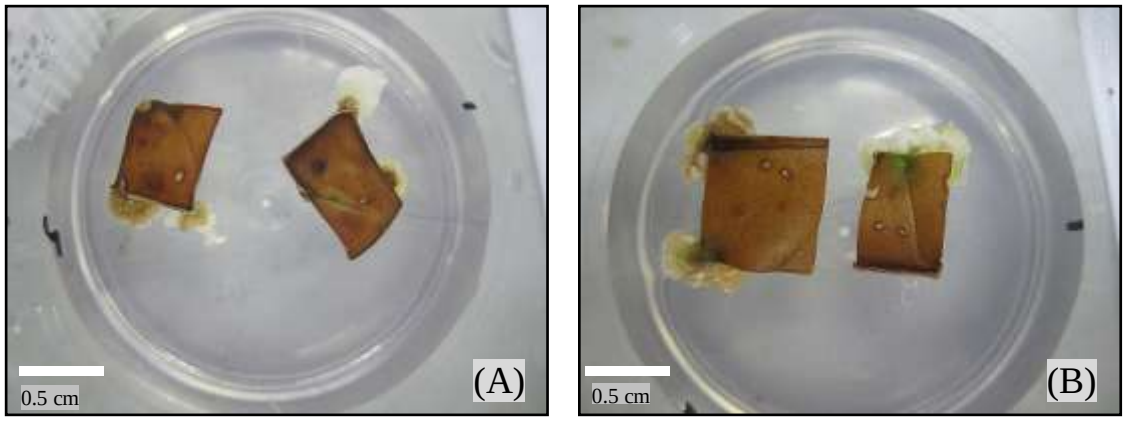

Figure 4.6: Induction of callus from leaf explants of *Allamanda cathartica* L. on MS medium supplemented with different concentrations of 2,4-D. The pictures were taken on the fourth week of culture.(A) 0.5 mg/L 2,4-D and (B) 1.0 mg/L 2,4-D.

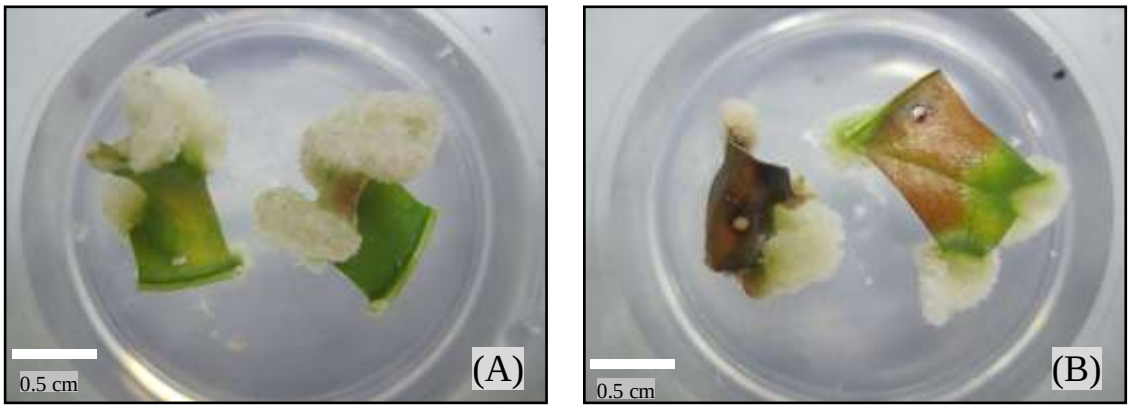

Figure 4.7: Induction of callus from leaf explants of *Allamanda cathartica* L. on MS medium supplemented with different concentrations of picloram.The pictures were taken on the fourth week of culture (A) 0.5 mg/L picloram and (B) 1.0 mg/L picloram.

callus fresh and dry weights among the two different concentrations of 2,4-D. The yellow-white, greenish friable calli were formed at the cutting edges of the leaf explants for all the concentrations of 2,4-D (Figure 4.6).

For the treatment of NAA, explants cultured in MS medium supplemented with either 0.5 or 1.0 mg/L of NAA showed no sign of callogenesis (Table 4.1) and the leaf explants were unhealthy, turned from green to brown in colour after 4 weeks of culture and eventually died. There were no significant differences on the day of initial callus formation, percentage of callus formation and callus fresh and dry weights were observed for all the concentrations of NAA tested. Callus formation was also observed in MS medium supplemented with picloram. Among the two concentrations used, 1.0 mg/L picloram was found to give the highest percentage (63.3 ± 6.7%) of callus formation. As for 0.5 mg/L picloram, only 60.0 ± 5.8% of callus was formed (Table 4.1). The explants cultured on 1.0 mg/L of picloram gave the earliest callus formation (8.0 ± 0.6 days) compared to the explants cultured on 0.5 mg/L of picloram (13.0 ± 1.5 days). A poor growth of calli was noted from the explants cultured on MS medium supplemented with 0.5 mg/L picloram (0.1116 ± 0.0394 g and 0.0120±0.0029 g) compared to 1.0 mg/L picloram (0.1526 ± 0.0202 g and 0.0124±0.0016 g). A significant difference on the percentage of callus formation, day of initial callus formation and callus fresh weight was observed between two concentrations of picloram treatments but no significant difference was observed on callus dry weight. Yellow-white, greenish and friable calli were formed around the edges of cutting sides of the leaf explants (Figure 4.7).

In the treatment of cytokinin, BAP was used in this study. No callus formation was observed in MS medium supplemented with either 0.5 or 1.0 mg/L of BAP. All the leaf explants cultured in all concentrations of BAP were unhealthy, turned from green to brown in colour after 4 weeks and eventually died. There were no significant difference on the day of initial callus formation, percentage of callus formation, callus fresh and dry weights were observed for all the concentrations of BAP tested.

Furthermore, the combination between auxins and cytokinins is often used in plant tissue culture. In the present study, the combinations of 2,4-D or NAA with BAP at various concentrations were utilized. MS medium supplemented with 1.0 mg/L 2,4-D + 1.0 mg/L BAP gave the best response on the formation of callus from the leaf explants among all the auxins, cytokinins and combinations tested, which gave the highest percentage (100.0 ± 0%) of callus formation. A total of 80.0 ± 10.0% and 93.3 ± 6.7% of callus formation were observed on explants cultured on both 1.0 mg/L 2,4-D with either 0.5 or 1.5 mg/L BAP, respectively (Table 4.1). A significant difference on the percentage of callus formation was observed between the three combinations of BAP with 1.0 mg/L 2,4-D. According to Table 4.1, it reveals that the explants cultured on 1.0 mg/L 2,4-D + 1.0 mg/L BAP showed the shortest time for initiation of callus formation (6.0 ± 0 days) with the heaviest fresh and dry weights (0.3857 ± 0.0939 g and 0.0707 ± 0.0549 g) compared to 1.0 mg/L 2,4-D + 0.5 mg/L BAP and 1.0 mg/L 2,4-D + 1.5 mg/L BAP. There were no significant differences on the day of initial callus formation and callus fresh weight between 1.0 mg/L 2,4-D + 0.5 mg/L BAP and 1.0 mg/L 2,4-D + 1.5 mg/L BAP but a significant difference was noticed between the two treatments stated above with 1.0 mg/L 2,4-D + 1.0 mg/L BAP. For callus

dry weight, no significant differences observed for all the treatments. All the calli formed were friable and yellow-white, greenish in colour (Figure 4.8).

Among the three different concentrations of BAP (0.5, 1.0 and 1.5 mg/L) with 0.5 mg/L of 2,4-D tested, MS supplemented with 0.5 mg/L 2,4-D + 1.0 mg/L BAP showed the highest percentage (93.3 ± 6.7%) of callus formation, followed by 0.5 mg/L 2,4-D + 0.5 mg/L BAP (90.0 ± 10.0%) and 0.5 mg/L 2,4-D + 1.5 mg/L BAP showed the lowest percentage (86.7 ± 6.7%) of callus induction. Even though 0.5 mg/L 2,4-D + 1.0 mg/L BAP gave the highest percentage of callus formation, but its callus fresh and dry weights (0.1814 ± 0.0035 g and 0.0158 ± 0.0016 g) was the lowest compared to the other two combinations. There were no significant differences observed on the percentage of callus formation and callus fresh weight between 0.5 mg/L 2,4-D + 0.5 mg/L BAP and 0.5 mg/L 2,4-D + 1.5 mg/L BAP but a significant difference was observed between two treatments mentioned above with 0.5 mg/L 2,4-D + 1.0 mg/L BAP. For callus dry weight, no significant differences were observed for all the treatments. For the day of initial callus, similar trend was observed compared to the percentage of callus formation, i.e. 0.5 mg/L 2,4-D + 1.0 mg/L BAP showed the shortest day of initial callus formation (6.0 ± 0.3 days) compared to the other two treatments. There were no significant differences observed on the day of initial callus formation between 0.5 mg/L 2,4-D + 0.5 mg/L BAP and 0.5 mg/L 2,4-D + 1.0 mg/L BAP but a significant difference was observed between two combinations mentioned above with 0.5 mg/L 2,4-D + 1.5 mg/L BAP. The yellow-white, greenish friable calli were formed at the cutting edges of the explants (Figure 4.9).

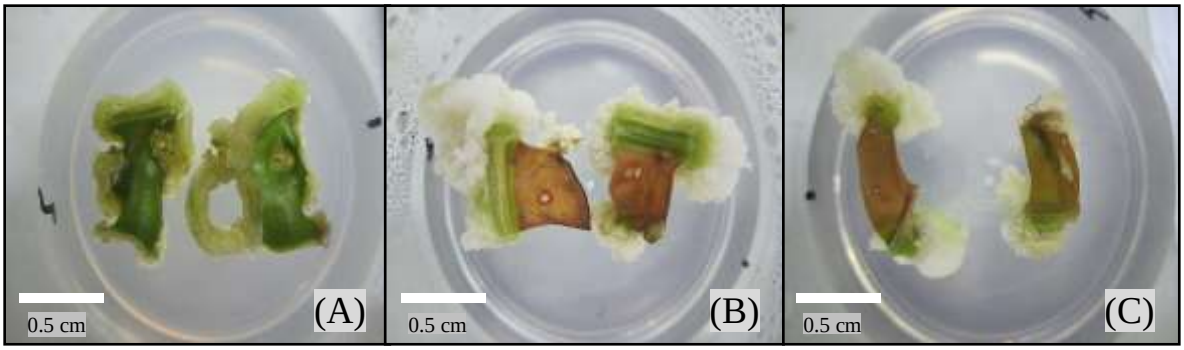

Figure 4.8: Induction of callus from leaf explants of *Allamanda cathartica* L. on MS medium supplemented with different combinations of 1.0 mg/L 2,4-D and BAP. The pictures were taken on the fourth week of culture. (A) 1.0 mg/L 2,4-D + 0.5 mg/L BAP; (B) 1.0 mg/L 2,4-D + 1.0 mg/L BAP and (C) 1.0 mg/L 2,4-D + 1.5 mg/L BAP.

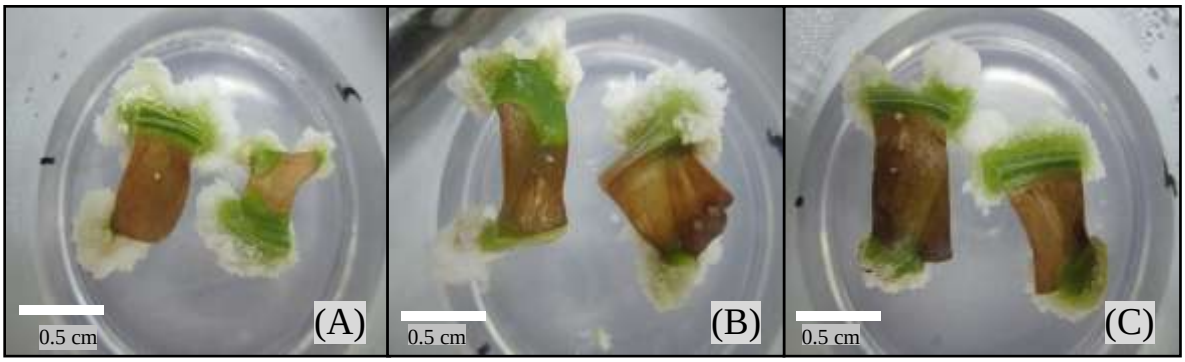

Figure 4.9: Induction of callus from leaf explants of *Allamanda cathartica* L. on MS medium supplemented with different combinations of 0.5 mg/L 2,4-D and BAP. The pictures were taken on the fourth week of culture. (A) 0.5 mg/L 2,4-D + 0.5 mg/L BAP; (B) 0.5 mg/L 2,4-D + 1.0 mg/L BAP and (C) 0.5 mg/L 2,4-D + 1.5 mg/L BAP.

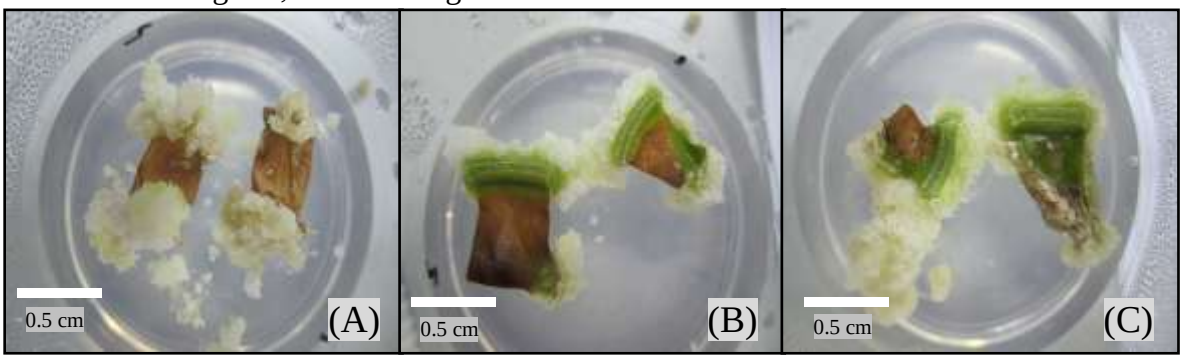

Figure 4.10: Induction of callus from leaf explants of *Allamanda cathartica* L. on MS medium supplemented with different combinations of 1.5 mg/L 2,4-D and BAP. The pictures were taken on the fourth week of culture. (A) 1.5 mg/L 2,4-D + 0.5 mg/L BAP; (B) 1.5 mg/L 2,4-D + 1.0 mg/L BAP and (C) 1.5 mg/L 2,4-D + 1.5 mg/L BAP.

Callus formation was also observed in the PGRs combinations of 1.5 mg/L 2,4-D with 0.5, 1.0 and 1.5 mg/L BAP. All the three combinations of 2,4-D and BAP were produced similar percentages of callus formation, i.e. 96.7 ± 3.3% (Table 4.1). There were no significant differences observed on the percentage of callus formation among the three different combinations of 2,4-D and BAP used. The explants cultured on 1.5 mg/L 2.4-D + 1.0 mg/L BAP and 1.5 mg/L 2,4-D + 1.5 mg/L BAP gave the earliest callus formation (6.0 ± 0.3 days) compared to the explants cultured on 1.5 mg/L 2,4-D + 0.5 mg/L BAP. There were no significant differences on the day of initial callus formation between 1.5 mg/L 2,4-D + 1.0 mg/L BAP and 1.5 mg/L 2,4-D + 1.5 mg/L BAP but a significant difference was observed between the other two combinations stated above with 1.5 mg/L 2,4-D + 0.5 mg/L BAP. MS medium supplemented with 1.5 mg/L 2,4-D + 1.5 mg/L BAP gave the highest fresh and dry weights of callus (0.2850 ± 0.0100 g and 0.0523 ± 0.0206 g), followed by 1.5 mg/L 2,4-D + 1.0 mg/L BAP (0.1914 ± 0.0244 g and 0.0176 ± 0.0017 g) and 1.5 mg/L 2,4-D + 0.5 mg/L BAP (0.1368 ± 0.0053 g and 0.0147 ± 0.0026 g). A significant difference on the callus fresh weight was observed but no significant difference on callus dry weight between three combinations of 2,4-D and BAP. All the calli formed from the combination of 1.5 mg/L 2,4-D with either 0.5, 1.0 or 1.5 mg/L BAP were friable and yellow-white, greenish in colour (Figure 4.10).

As for the combinations of NAA and BAP, there was no sign of callogenesis observed for the leaf explants cultured on MS medium fortified with 0.5 mg/L NAA + 0.5 mg/L BAP and 1.0 mg/L NAA + 0.5 mg/L BAP (Table 4.1). All the leaf explants cultured on these medium were unhealthy, turned from green to brown in colour after 4 weeks and eventually died. There were no significant differences on the day of initial callus formation,

percentage of callus formation, callus fresh and dry weights were observed for the combinations of NAA and BAP.

4.1.3 Callus Induction from the Stem Explants

As shown in Table 4.2, the results obtained for callus induction from stem explants of *A. cathartica* L. revealed that there were no signs of callogenesis in the PGR-free medium under photoperiod of 16 hours light and 8 hours darkness condition. All the stem explants cultured on the control medium were unhealthy, turned from green to brown in colour after 4 weeks and eventually died (Figure 4.11). There were no significant differences observed for the control medium based on the day of initial callus formation, percentage of callus formation, fresh and dry weights of callus.

In the treatment of auxins and cytokinin alone, 2,4-D, NAA, picloram and BAP were used in this study. Among the three auxins stated above, callus formation from the stem explants of *A. cathartica* L. was only noticed on MS medium fortified with picloram. A total of 63.3 ± 8.8% and 76.7 ± 3.3% (Table 4.2) of calli were formed using both 0.5 and 1.0 mg/L of picloram, respectively. The explants cultured on 1.0 mg/L picloram required the shortest period for callus formation, which was 7.0 ± 0.9 days. Meanwhile, MS medium supplemented with 0.5 mg/L picloram needed 12.0 ± 1.2 days for the formation of callus. For callus fresh and dry weights, 1.0 mg/L picloram showed to have more calli (0.1483 ± 0.0350 g and 0.0126 ± 0.0013 g) compared to 0.5 mg/L picloram (0.1127 ± 0.0310 g and 0.0103 ± 0.0010 g). Brown-white and greenish friable calli were noticed from all the explants cultured at different concentrations of picloram-supplemented MS media (Figure 4.12). However, there were significant differences observed on the day of initial callus

Table 4.2: Effects of MS medium supplemented with auxins (2,4-D, NAA and picloram) and cytokinin (BAP) at different concentrations on callus induction from stem explants of *Allamanda cathartica* L. after 4 weeks of culture.

Concentration of auxin (mg/L)	Concentration of cytokinin (mg/L)	Day of initial callus formation (Day ± SE[a])	Percentage of callus formation (%) ± SE[a]	Callus fresh weight (g) ± SE[a]	Callus dry weight (g) ± SE[a]	Callus morphology
2,4-D	**BAP**					
0	0	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
0.5	0	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
1.0	0	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
0	0.5	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
0	1.0	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
0.5	0.5	6.0 ± 0.3^{b}	80.0 ± 5.8^{bcd}	0.3283 ± 0.0640^{efg}	0.0194 ± 0.0001^{e}	Brown-white, greenish friable callus
0.5	1.0	8.0 ± 0.3^{b}	80.0 ± 5.8^{bcd}	0.1726 ± 0.0059^{bc}	0.0131 ± 0.0004^{bc}	Brown-white, greenish friable callus
0.5	1.5	7.0 ± 0.6^{b}	83.3 ± 3.3^{bcd}	0.1858 ± 0.0036^{bcd}	0.0134 ± 0.0002^{bc}	Brown-white, greenish friable callus
1.0	0.5	8.0 ± 0.9^{b}	93.3 ± 3.3^{cd}	0.2377 ± 0.0063^{cdf}	0.0175 ± 0.0015^{de}	Brown-white, greenish friable callus
1.0	1.0	5.0 ± 0.3^{b}	100.0 ± 0^{d}	0.4177 ± 0.0108^{g}	0.0207 ± 0.0009^{e}	Brown-white, greenish friable callus
1.0	1.5	8.0 ± 0.3^{b}	83.3 ± 8.8^{bcd}	0.2734 ± 0.0036^{def}	0.0181 ± 0.0010^{de}	Brown-white, greenish friable callus
1.5	0.5	7.0 ± 0.3^{b}	86.7 ± 6.7^{cd}	0.3477 ± 0.0029^{fg}	0.0194 ± 0.0001^{e}	Brown-white, greenish friable callus
1.5	1.0	8.0 ± 0.3^{b}	73.3 ± 3.3^{bc}	0.2220 ± 0.0029^{cd}	0.0153 ± 0.0005^{cd}	Brown-white, greenish friable callus
1.5	1.5	6.0 ± 0.3^{b}	93.3 ± 3.3^{cd}	0.3550 ± 0.0033^{fg}	0.0206 ± 0.0003^{e}	Brown-white, greenish friable callus
NAA	**BAP**					
0.5	0	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
1.0	0	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
0.5	0.5	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
1.0	0.5	0^{a}	0^{a}	0^{a}	0^{a}	Green stem turned brown. No callus was formed.
Picloram						
0.5	0	12.0 ± 1.2^{c}	63.3 ± 8.8^{b}	0.1127 ± 0.0310^{b}	0.0103 ± 0.0010^{b}	Brown-white, greenish friable callus
1.0	0	7.0 ± 0.9^{b}	76.7 ± 3.3^{bc}	0.1483 ± 0.0350^{bc}	0.0126 ± 0.0013^{bc}	Brown-white, greenish friable callus

SE[a] = Standard Error. The value for the same alphabet is not significantly different (Tukey's multiple range test, $p<0.05$).

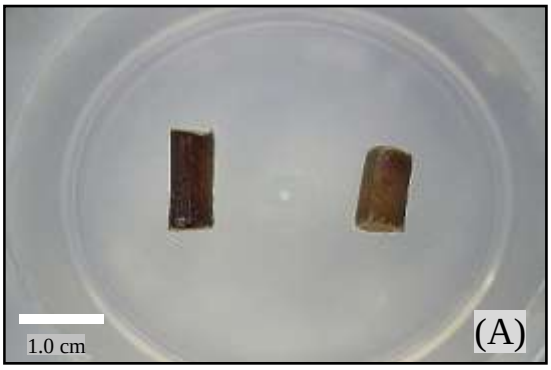

Figure 4.11: Induction of callus from stem explants of *Allamanda cathartica* L. on MS medium without any supplementation of PGRs. (A) Control medium.

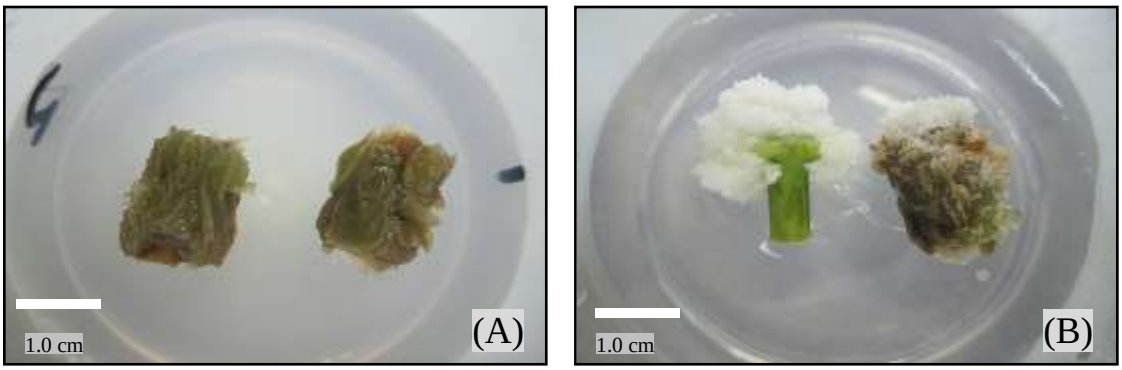

Figure 4.12: Induction of callus from stem explants of *Allamanda cathartica* L. on MS medium supplemented with different concentrations of picloram. The pictures were taken on the fourth week of culture. (A) 0.5 mg/L picloram and (B) 1.0 mg/L picloram.

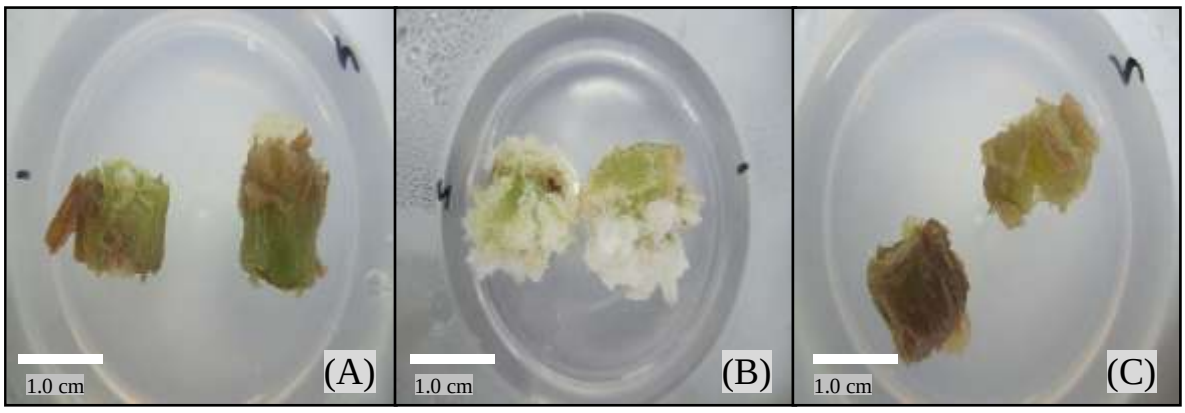

Figure 4.13: Induction of callus from stem explants of *Allamanda cathartica* L. on MS medium supplemented with different combinations of 1.0 mg/L 2,4-D and BAP. The pictures were taken on the fourth week of culture. (A) 1.0 mg/L 2,4-D + 0.5 mg/L BAP; (B) 1.0 mg/L 2,4-D + 1.0 mg/L BAP and (C) 1.0 mg/L 2,4-D + 1.5 mg/L BAP.

formation, percentage of callus formation, callus fresh and dry weights among the two different concentrations of picloram tested.

Table 4.2 reveals that MS media fortified with 2,4-D, NAA and BAP at the concentrations of 0.5 and 1.0 mg/L were not able to induce callus formation under the photoperiod of 16 hours light and 8 hours darkness condition. All the stem explants cultured on these media were unhealthy, turned from green to brown in colour after 4 weeks and eventually died. Meanwhile, there were no significant differences observed for MS medium fortified with 0.5 and 1.0 mg/L of 2,4-D, NAA and BAP based on the day of initial callus formation, percentage of callus formation, fresh and dry weights of callus.

As for the treatments of auxins and cytokinins' combinations, the combinations of 0.5 mg/L 2.4-D + 0.5 mg/L BAP, 0.5 mg/L 2,4-D + 1.0 mg/L BAP, 0.5 mg/L 2,4-D + 1.5 mg/L BAP, 1.0 mg/L 2.4-D + 0.5 mg/L BAP, 1.0 mg/L 2,4-D + 1.0 mg/L BAP, 1.0 mg/L 2,4-D + 1.5 mg/L BAP, 1.5 mg/L 2,4-D + 0.5 mg/L BAP, 1.5 mg/L 2,4-D + 1.0 mg/L BAP, 1.5 mg/L 2,4-D + 1.5 mg/L BAP, 0.5 mg/L NAA + 0.5 mg/L BAP and 1.0 mg/L NAA + 0.5 mg/L BAP were used in this study. MS medium supplemented with 0.5 mg/L NAA + 0.5 mg/L BAP and 1.0 mg/L NAA + 0.5 mg/L BAP showed no sign of callogenesis from the stem explants (Table 4.2). All the stem explants cultured on these medium were unhealthy, turned from green to brown in colour after 4 weeks and eventually died. There were no significant difference on the day of initial callus formation, percentage of callus formation, callus fresh and dry weights were observed for the combinations of NAA and BAP.

MS medium supplemented with 1.0 mg/L 2,4-D + 1.0 mg/L BAP showed the best response of callogenesis from stem explants regarding the day of initial callus formation (5.0 ± 0.3 days), percentage (100.0 ± 0%) of callus formation, callus fresh (0.4177 ± 0.0108 g) and dry (0.0207 ± 0.0009 g) weights as compared to the other combinations of 2,4-D or NAA with BAP, auxins (2,4-D, NAA and picloram) and cytokinin (BAP) alone (Table 4.2). MS media supplemented with 1.0 mg/L 2,4-D + 0.5 mg/L BAP amd 1.0 mg/L 2,4-D + 1.5 mg/L BAP showed the similar day of initial callus formation, i.e. 8.0 days with callus fresh weights of 0.2377 ± 0.0063 g and 0.2734 ± 0.0036 g, dry weights of 0.0175 ± 0.0015 g and 0.0181 ± 0.0010 g, respectively (Table 4.2). A significant difference was observed on the percentage of callus formation and callus fresh weight among the three combinations of 1.0 mg/L 2,4-D with 0.5, 1.0 or 1.5 mg/L BAP. Meanwhile, there were no significant differences observed for all the combinations of 1.0 mg/L 2,4-D on the day of initial callus formation but a significant difference was observed on the callus dry weight among other two combinations of 1.0 mg/L 2,4-D + BAP with 1.0 mg/L 2,4-D + 1.0 mg/L BAP. Brown-white and greenish friable calli were noticed from all the explants cultured at different combinations of 1.0 mg/L 2,4-D and BAP-supplemented MS media (Figure 4.13).

For the combinations of 0.5 mg/L 2,4-D with 0.5, 1.0 or 1.5 mg/L BAP, the percentage of callus formation increased as concentrations of BAP increased. MS medium supplemented with 0.5 mg/L 2,4-D + 1.5 mg/L BAP showed the highest percentage (83.3 ± 3.3%) of callus formation as compared to 0.5 mg/L 2,4-D + 0.5 mg/L BAP and 0.5 mg/L 2,4-D + 1.0 mg/L BAP. The explants cultured on 0.5 mg/L 2.4-D + 0.5 mg/L BAP required the shortest period for callus formation, which was 6.0 ± 0.3 days. Meanwhile, MS media

supplemented with 0.5 mg/L 2,4-D + 1.0 mg/L BAP and 0.5 mg/L 2,4-D + 1.5 mg/L BAP needed 8.0 ± 0.3 days and 7.0 ± 0.6 days, respectively for formation of callus. For callus fresh and dry weights, 0.5 mg/L 2,4-D + 0.5 mg/L BAP showed to have more calli (0.3283 ± 0.0640 g and 0.0194 ± 0.0001 g) compared to the other two combinations. There were no significant differences observed on the day of initial callus formation and percentage of callus formation but a significant difference was observed on the callus fresh weight among the three combinations of 0.5 mg/L 2,4-D. For callus dry weight, a significant difference was observed among other two combinations of 0.5 mg/L 2,4-D + BAP with 0.5 mg/L 2,4-D + 0.5 mg/L BAP. Brown-white and greenish friable calli were observed from all the explants cultured at different combinations of 0.5 mg/L 2,4-D and BAP-supplemented MS media (Figure 4.14).

For the last combination of 2,4-D and BAP, i.e. 1.5 mg/L 2.4-D with 0.5, 1.0 or 1.5 mg/L BAP, MS supplemented with 1.5 mg/L 2.4-D + 1.5 mg/L BAP gave the highest percentage (93.3 ± 3.3%) of callus formation, whereas 1.5 mg/L 2,4-D + 1.0 mg/L BAP gave the lowest percentage of callus formation, which was 73.3 ± 3.3% and was the poorest response of callogenesis among all the combinations of 2,4-D and BAP (Table 4.2). The percentage of callus formation was directly proportional to the callus fresh and dry weights and inversely proportional to the day of initial callus formation, i.e. the higher the percentage of callus formation, the higher the amount of callus fresh and dry weights and the shorter the day of initial callus formation. This can be proven by the MS medium supplemented with 1.5 mg/L 2,4-D + 1.5 mg/L BAP, whereby it showed to have the heaviest callus fresh and dry weights (0.3550 ± 0.0033 g and 0.0206 ± 0.0003 g) and the

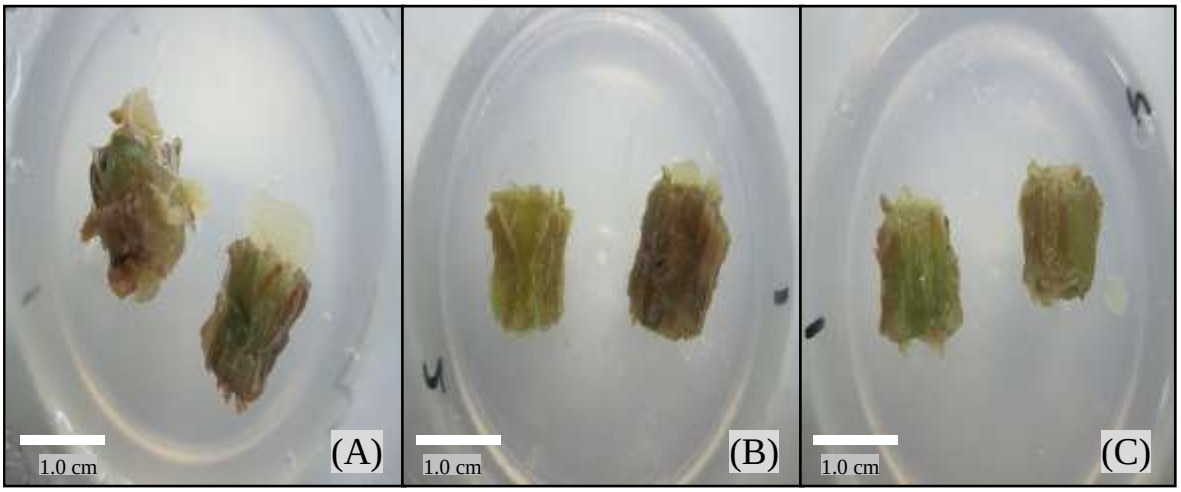

Figure 4.14: Induction of callus from stem explants of *Allamanda cathartica* L. on MS medium supplemented with different combinations of 0.5 mg/L 2,4-D and BAP. The pictures were taken on the fourth week of culture. (A) 0.5 mg/L 2,4-D + 0.5 mg/L BAP; (B) 0.5 mg/L 2,4-D + 1.0 mg/L BAP and (C) 0.5 mg/L 2,4-D + 1.5 mg/L BAP.

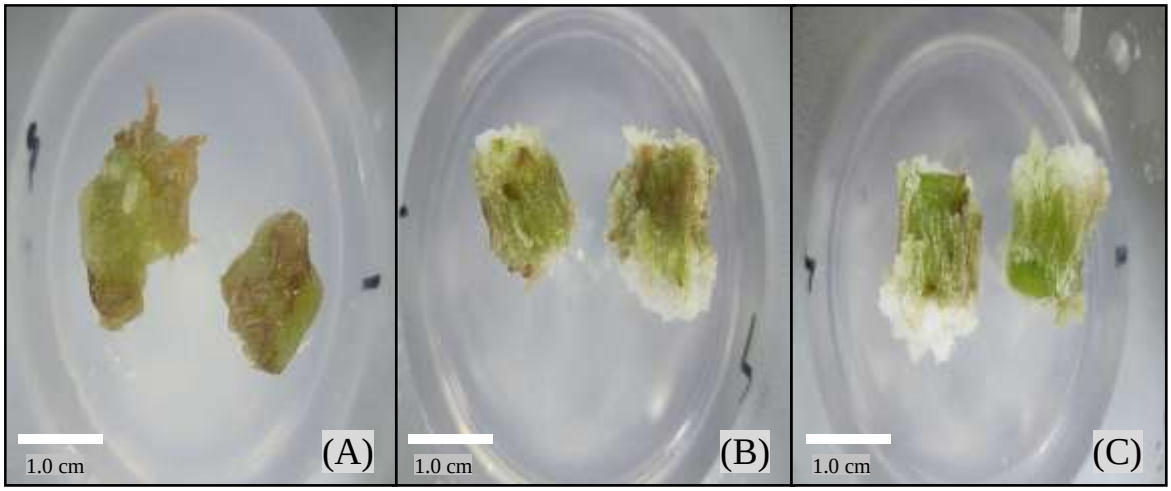

Figure 4.15: Induction of callus from stem explants of *Allamanda cathartica* L. on MS medium supplemented with different combinations of 1.5 mg/L 2,4-D and BAP. The pictures were taken on the fourth week of culture. (A) 1.5 mg/L 2,4-D + 0.5 mg/L BAP; (B) 1.5 mg/L 2,4-D + 1.0 mg/L BAP and (C) 1.5 mg/L 2,4-D + 1.5 mg/L BAP.

shortest period of callus induction (6.0 ± 0.3 days) compared to other two combinations of 1.5 mg/L 2,4-D stated above. The brown-white and greenish friable calli were observed on the whole explants in all the combinations of 1.5 mg/L 2,4-D and BAP (Figure 4.15). However, no significant difference on the day of initial callus formation among the three combinations of 1.5 mg/L 2,4-D but a significant difference was observed on the percentage of callus formation, callus fresh and dry weights between 1.5 mg/L 2,4-D + 1.0 mg/L BAP with the other two combinations, i.e. 1.5 mg/L 2,4-D + 0.5 mg/L BAP and 1.5 mg/L 2,4-D + 1.5 mg/L BAP.

4.1.4 Plant Regeneration

4.1.4.1 Shoot Multiplication

In this study, plant regeneration was divided into two parts, i.e. shoot multiplication and shoot elongation. Shoot multiplication from the nodal parts of *A. cathartica* L. was studied in the treatment of MS medium supplemented with either 1, 3 or 5 mg/L of BAP. According to Table 4.3, PGR-free medium showed no sign of shoot multiplication. There were no significant differences observed for the control medium based on the percentage of shoot formation and number of shoot formation. The highest percentage (100.0 ± 0%) of shoot formation and number (6.0 ± 0.6 shoots) of shoot formation were observed on MS medium supplemented with 5 mg/L of BAP. Meanwhile, the lowest percentage (90.0 ± 5.7%) of shoot formation and number (1.0 ± 0.3 shoots) of shoot formation were noticed on MS medium supplemented with 1 mg/L of BAP. For all concentrations of BAP, multiple shoots were formed from the explants with greenish leaves (Figure 4.16). According to Table 4.3, the results for the percentage of shoot formation were found to

Table 4.3: Shoot multiplication from nodal explants of *Allamanda cathartica* L. after 4 weeks of cultured on MS medium supplemented with BAP at various concentrations.

Plant growth regulator	Concentration (mg/L)	Percentage of shoot formation (%) ± SE[a]	Number of shoot formation (Number of shoots ± SE[a])	Shoot morphology
Control	0	0[a]	0 [a]	No shoot formation was observed
BAP	1	90.0 ± 5.7[b]	1.0 ± 0.3 [a]	Multiple shoots were formed from the explants with greenish leaves
	3	96.7 ± 3.3[b]	4.0 ± 0.3[b]	Multiple shoots were formed from the explants with greenish leaves
	5	100.0 ± 0[b]	6.0 ± 0.6[c]	Multiple shoots were formed from the explants with greenish leaves

SE[a] = Standard Error. The value for the same alphabet is not significantly different (Tukey's multiple range test, $p<0.05$).

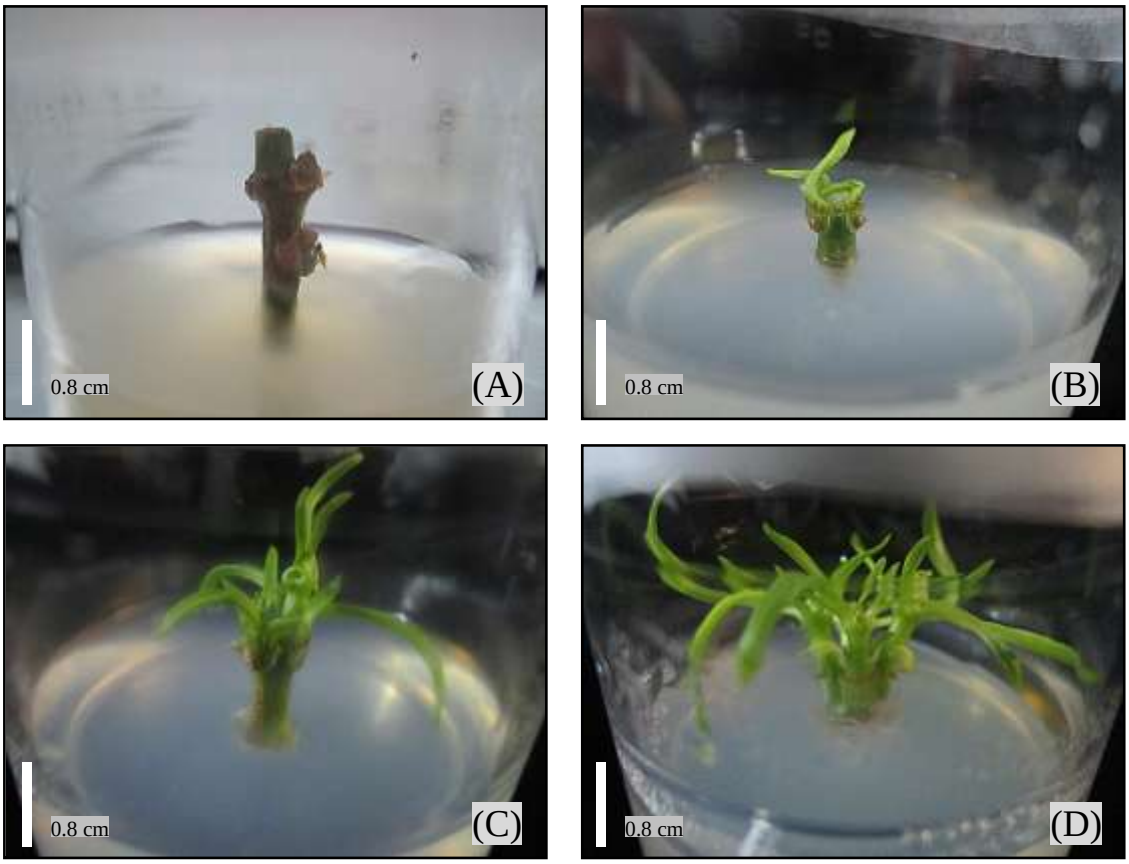

Figure 4.16: Shoot multiplication from nodal explants of *Allamanda cathartica* L. after four weeks of culture on MS supplemented with BAP. (A) Control medium; (B) 1 mg/L BAP; (C) 3 mg/L BAP and (D) 5 mg/L BAP.

have no significant differences with each other when comparing between each treatment. However, a significant difference was observed on the number of shoot formation among the three treatments of BAP.

4.1.4.2 Shoot Elongation

The new shoots formed from nodal explants were then transferred into MS medium without any supplementation of PGRs for shoot elongation. Based on Table 4.4, the lengths of the shoots were gradually increased from Week 0 to Week 4, i.e. 0.50 ± 0 cm, 0.80 ± 0.01 cm, 0.92 ± 0.01 cm, 0.98 ± 0.04 cm and 1.01 ± 0.07 cm, respectively (Figure 4.17). From Week 0 to Week 4, the leaves attached to the shoot were greenish in colour. There were no root formation from Week 0 to Week 3 and the formation of white hairy root was started to be observed on Week 4 (Figure 4.18). Among the fifteen cultured explants, only two explants were observed to have root formation below the media.

Table 4.4: Shoot elongation on MS medium for nodal explants of *Allamanda cathartica* L. from week 0 to 4.

Week	Length of shoot (cm) ± SE[a]	Shoot morphology	Root formation
0	0.50 ± 0	Short shoot with greenish leaves was observed	No root formation was observed
1	0.80 ± 0.01	Elongated shoot with greenish leaves was observed	No root formation was observed
2	0.92 ± 0.01	More elongated shoot with greenish leaves was observed	No root formation was observed
3	0.98 ± 0.04	Longer shoot with greenish leaves was observed	No root formation was observed
4	1.01 ± 0.07	Longest shoot with greenish leaves was observed	Formation of white hairy root was observed from two out of fifteen explants

SE[a] = Standard Error.

Homogenous test was not able to perform because at least one group has fewer than two cases.

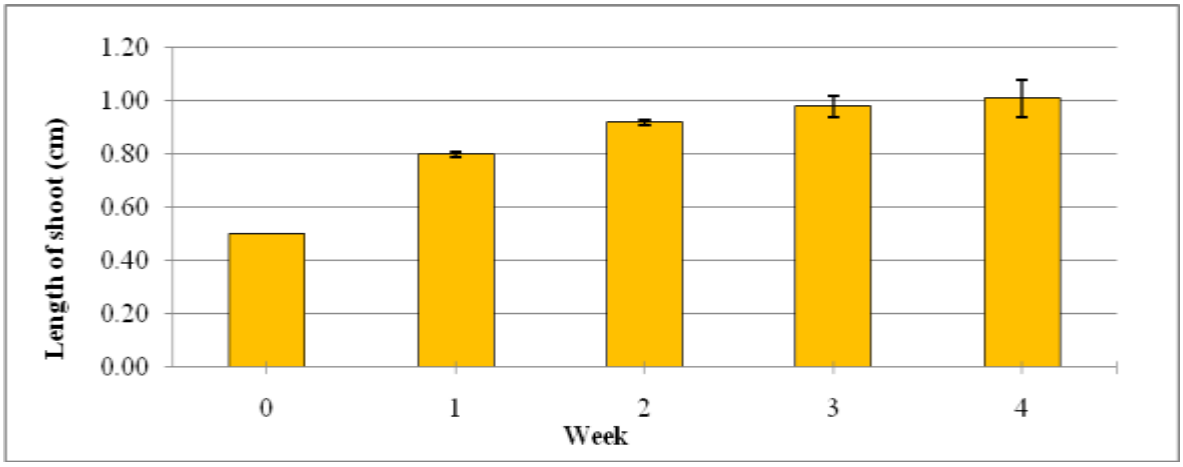

Figure 4.17: The length of shoots for shoot elongation on MS medium for nodal explants of *Allamanda cathartica* L. from week 0 to 4. The error bars indicate the standard error.

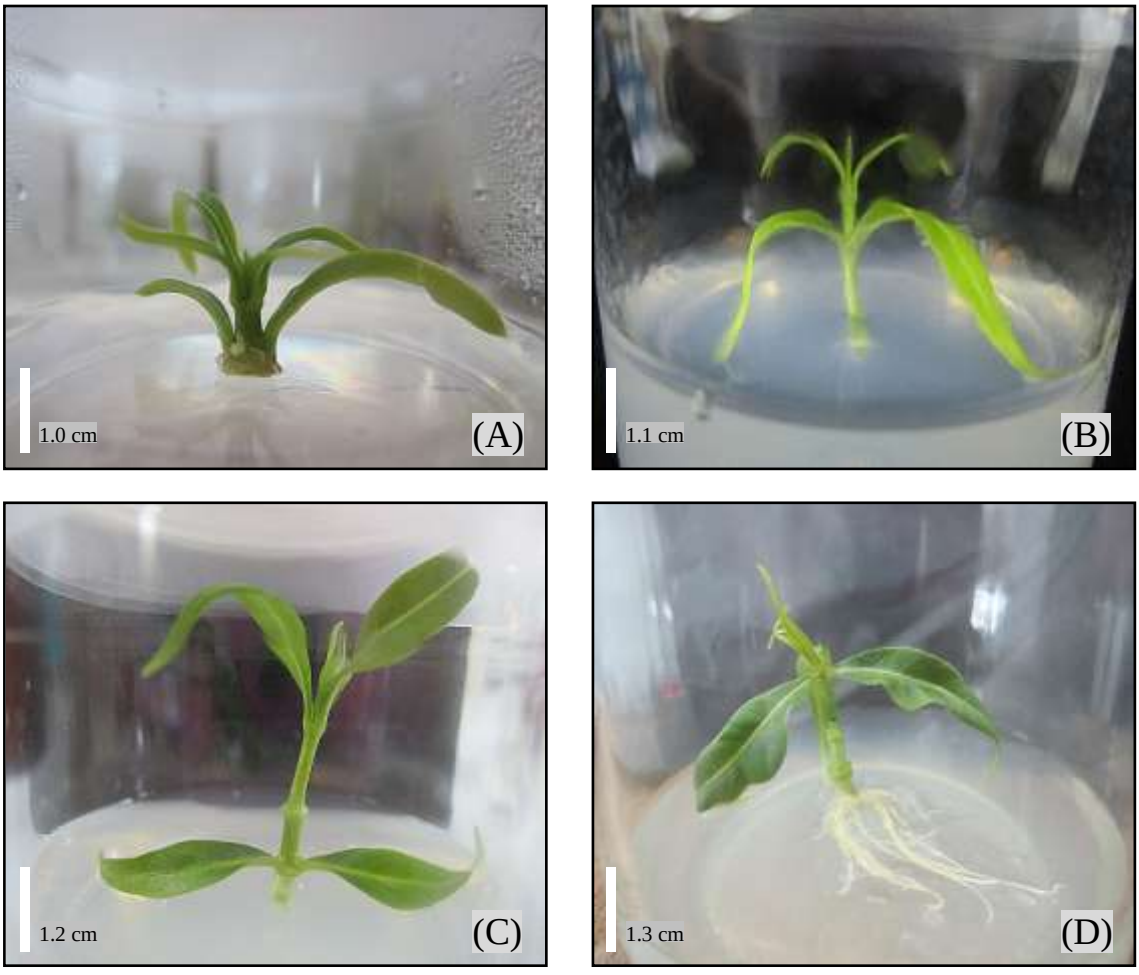

Figure 4.18: Shoot elongation on MS medium for nodal explants *of Allamanda cathartica* L. from Week 1 to 4. (A) Week 1; (B) Week 2; (C) Week 3 and (D) Week 4.

4.1.5 Microscopic Studies (Scanning Electron Microscopy - SEM)

In the microscopic studies, SEM was used to observe the differences between the leaf and stem specimens from *in vitro* and *in vivo* (intact) shoots of *A. cathartica* L.. Figure 4.19 showed abaxial surface of *in vitro* leaf, while Figure 4.20 showed adaxial surface of *in vitro* leaf specimens. Figure 4.21 and 4.22 showed abaxial and adaxial surfaces of *in vivo* leaf, respectively. From the figures, the number of stomata on the abaxial surface was more abundant compared with the adaxial surface for both *in vitro* and *in vivo* leaves. Besides, the surface of *in vitro* leaf was found to be more clean compared to *in vivo* leaf. Structure of stomata on *in vitro* leaf specimen was observed on Figure 4.23 (abaxial surface) and Figure 4.24 (adaxial surface), whereas Figure 4.25 (abaxial surface) showed the structure of stoma on *in vivo* leaf specimen. Trichome was not shown on both *in vitro* and *in vivo* leaves.

The microscopic studies on *in vitro* and *in vivo* (intact) stem specimens of *A. cathartica* L. were also carried out. From Figure 4.26, it showed the surface of *in vitro* stem, meanwhile Figure 4.27 showed the surface of *in vivo* stem. The surface of *in vitro* stem was found to be less contaminated compared to *in vivo* stem. There was no stoma on *in vitro* stem but fewer stomata were found on the *in vivo* stem. However, the amount of stomata observed from the stem specimen was lesser than that on the leaf specimen. Figure 4.28 was the close up of stoma found on *in vivo* stem. Furthermore, the cross sections of *in vitro* and *in vivo* stems were also noticed through the SEM micrographs, i.e. Figure 4.29 and Figure 4.30, respectively. From the cross sections of stem specimens, several structures such as cuticle, epidermis, cortex, interfascular region and pith can be determined. Trichome was not shown on both *in vitro* and *in vivo* stems.

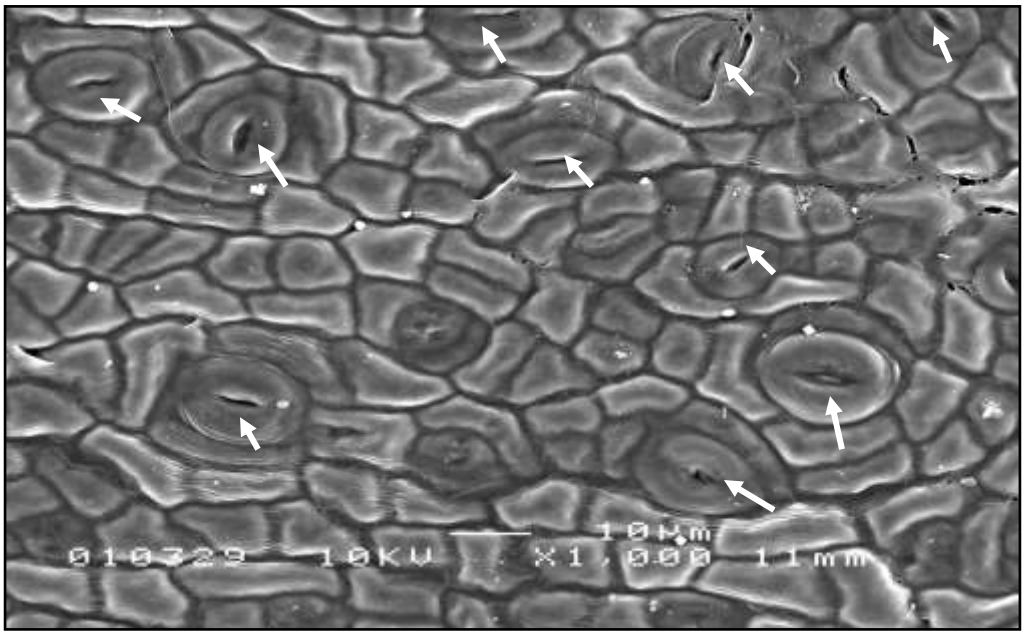

Figure 4.19: SEM micrograph showing abaxial surface of *in vitro* leaf specimen of *Allamanda cathartica* L. More stomata (white arrows) were seen clearly on the *in vitro* leaf.

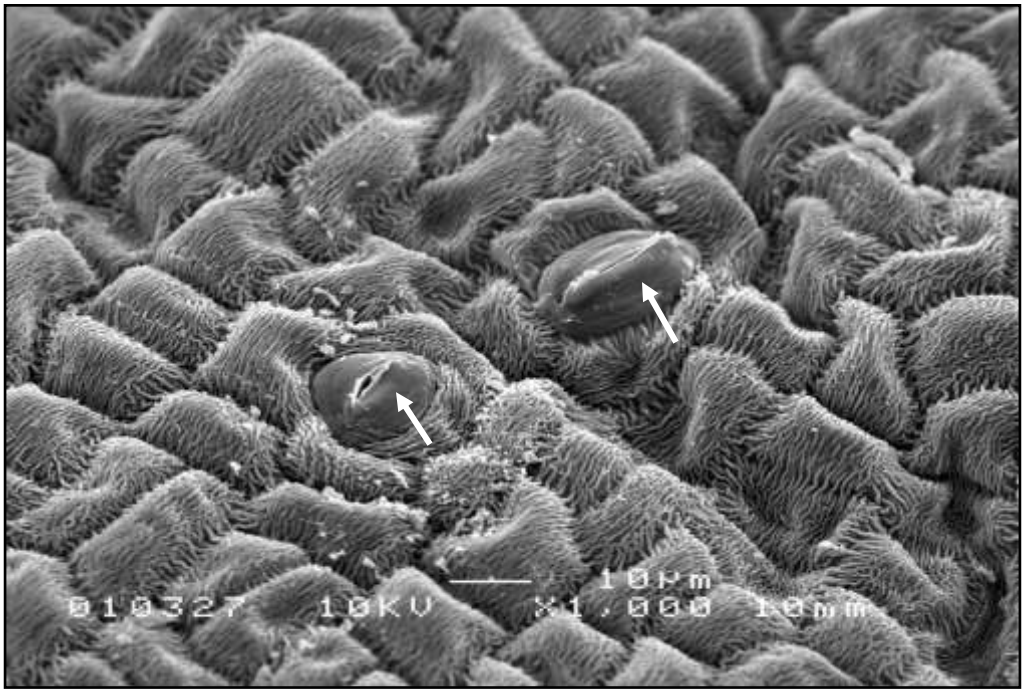

Figure 4.20: SEM micrograph showing adaxial surface of *in vitro* leaf specimen of *Allamanda cathartica* L. Less stomata (white arrows) were seen clearly on the *in vitro* leaf.

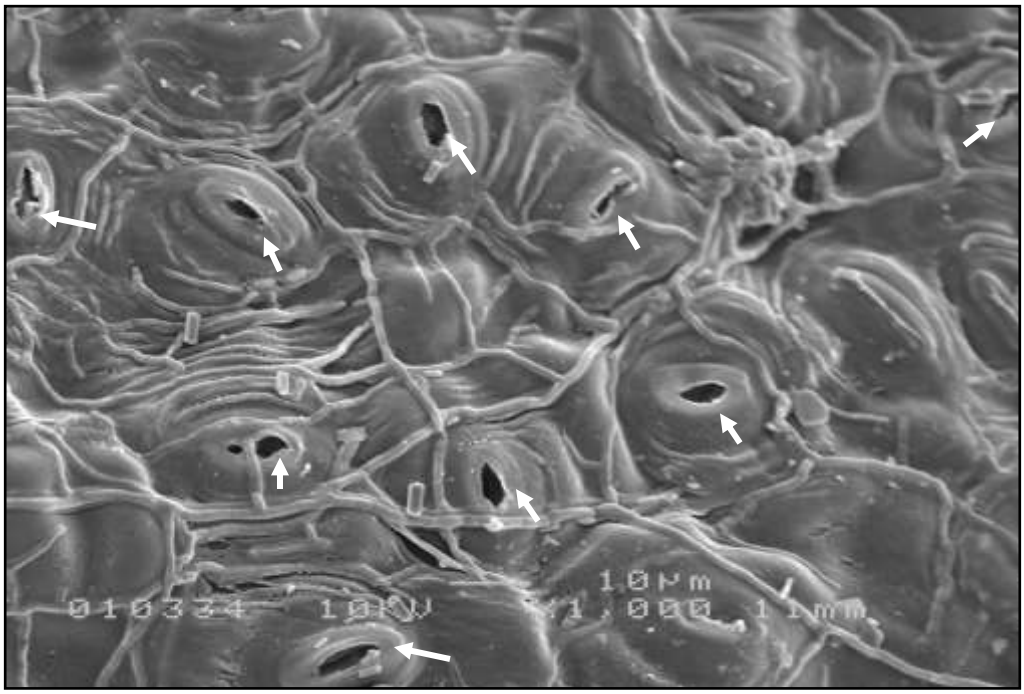

Figure 4.21: SEM micrograph showing abaxial surface of *in vivo* leaf specimen of *Allamanda cathartica* L. More stomata (white arrows) were seen clearly on the *in vivo* leaf.

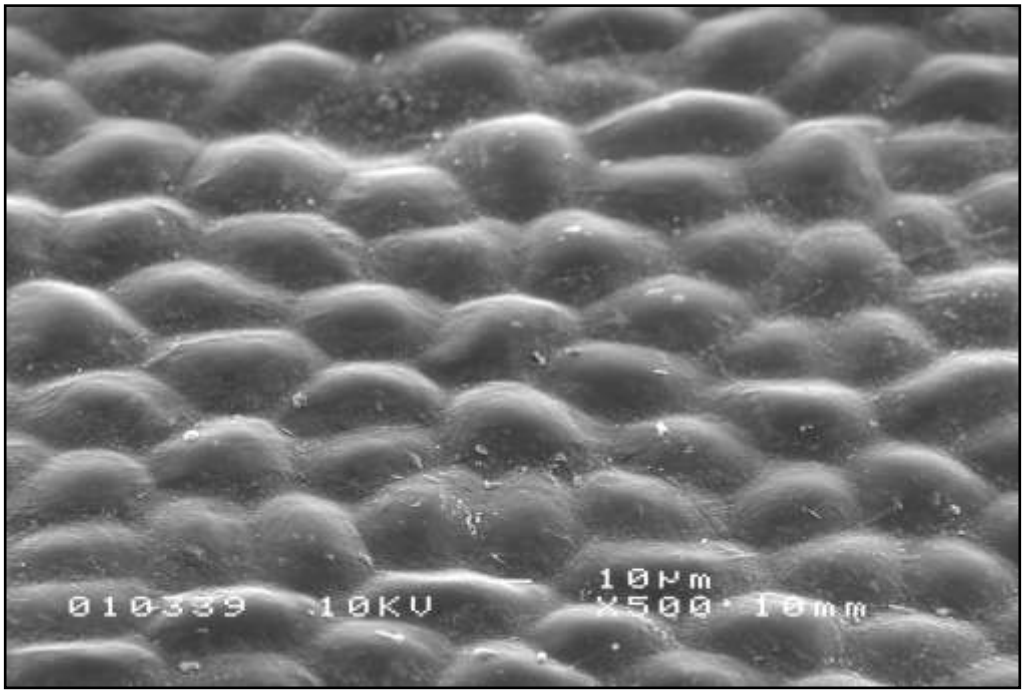

Figure 4.22: SEM micrograph showing adaxial surface of *in vivo* leaf specimen of *Allamanda cathartica* L. No stomata were observed on the *in vivo* leaf.

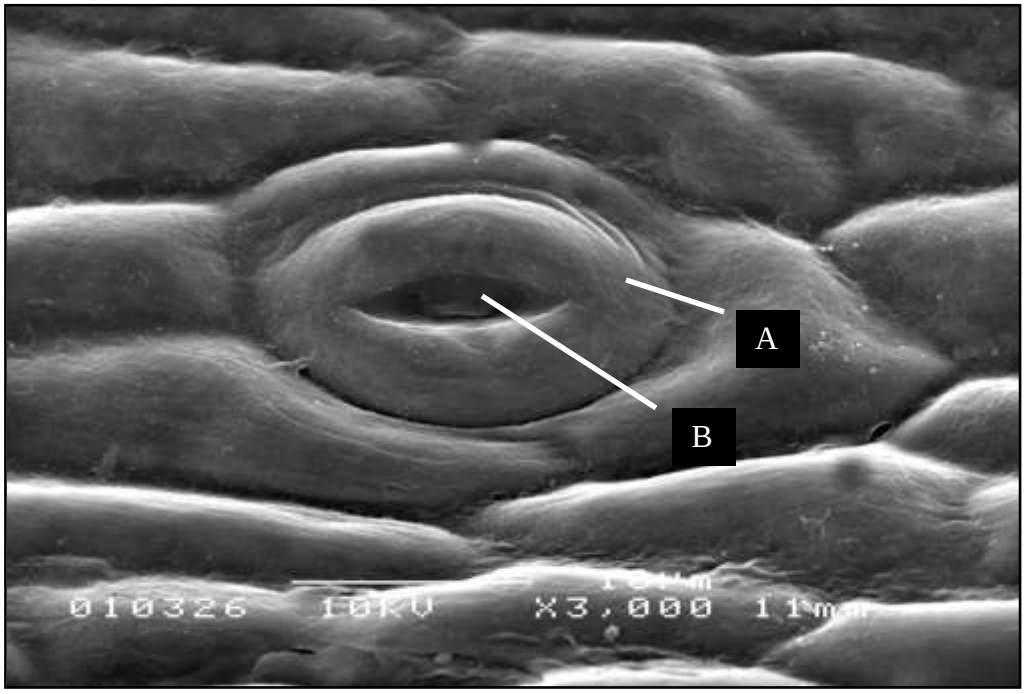

Figure 4.23: SEM micrograph showing the close up of stoma on abaxial surface of *in vitro* leaf of *Allamanda cathartica* L. (A) Guard cell and (B) Stomatal opening.

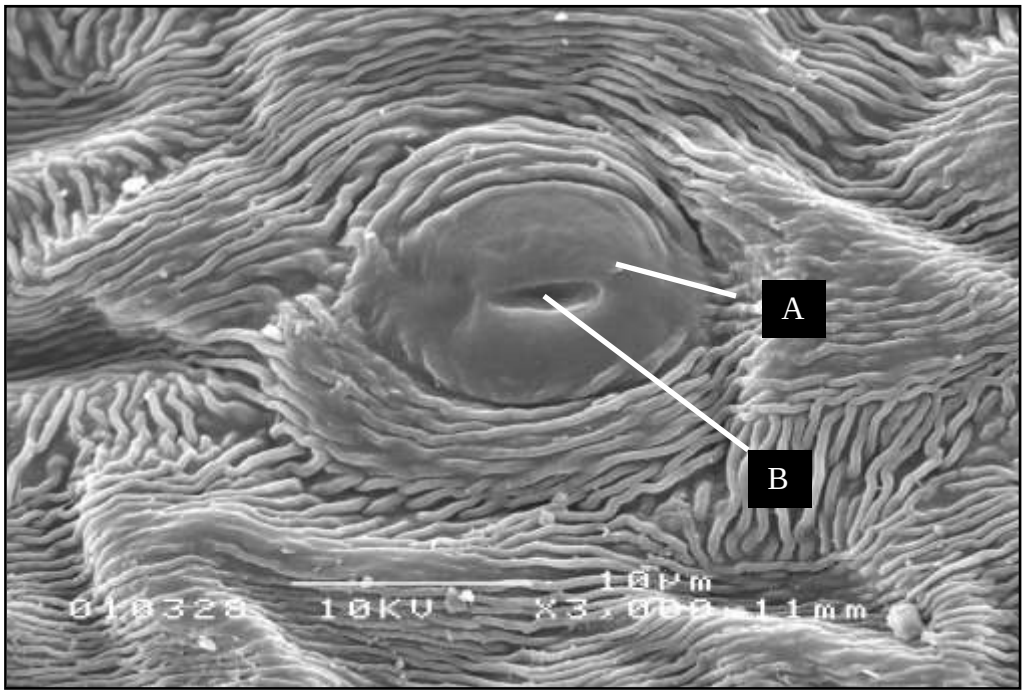

Figure 4.24: SEM micrograph showing the close up of stoma on adaxial surface of *in vitro* leaf of *Allamanda cathartica* L. (A) Guard cell and (B) Stomatal opening.

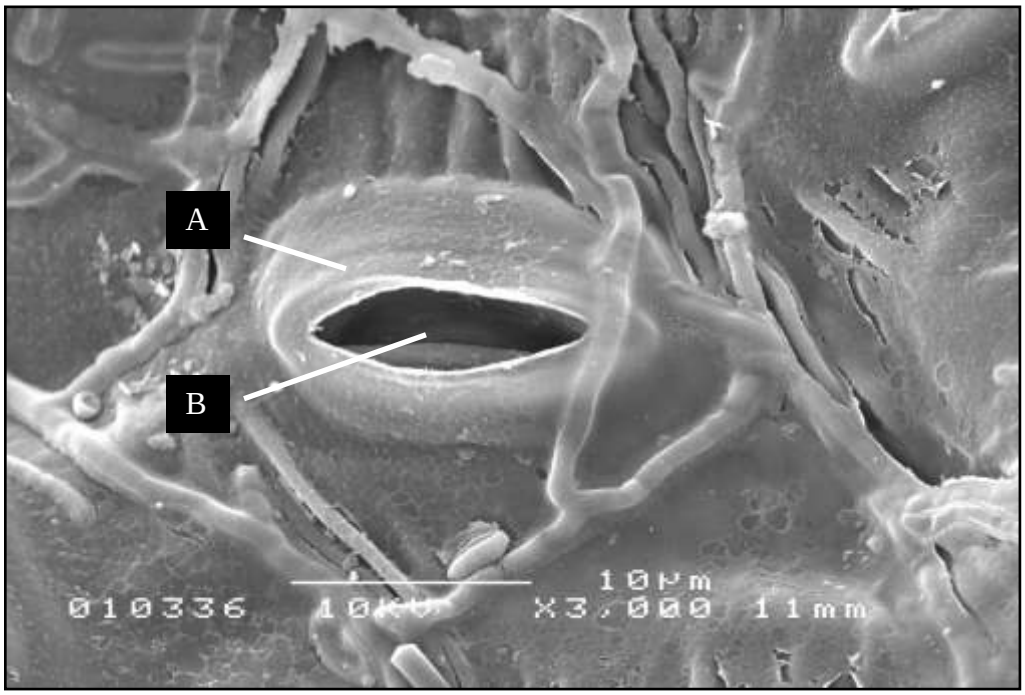

Figure 4.25: SEM micrograph showing the close up of stoma on abaxial surface of *in vivo* leaf of *Allamanda cathartica* L. (A) Guard cell and (B) Stomatal opening.

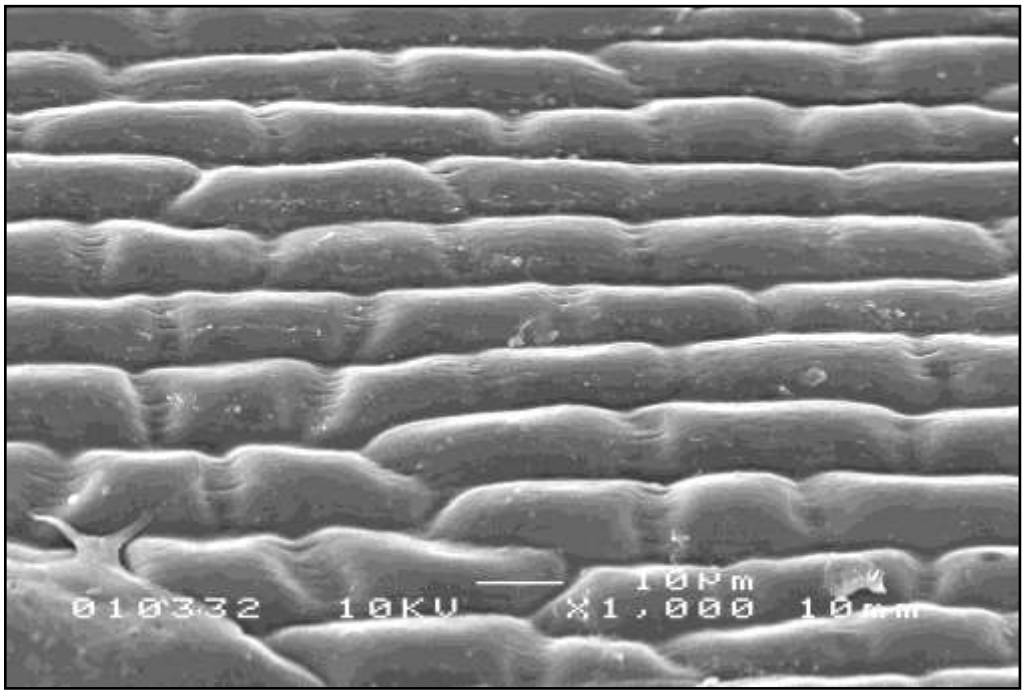

Figure 4.26: SEM micrograph showing the surface of *in vitro* stem of *Allamanda cathartica* L. No stoma was observed on *in vitro* stem.

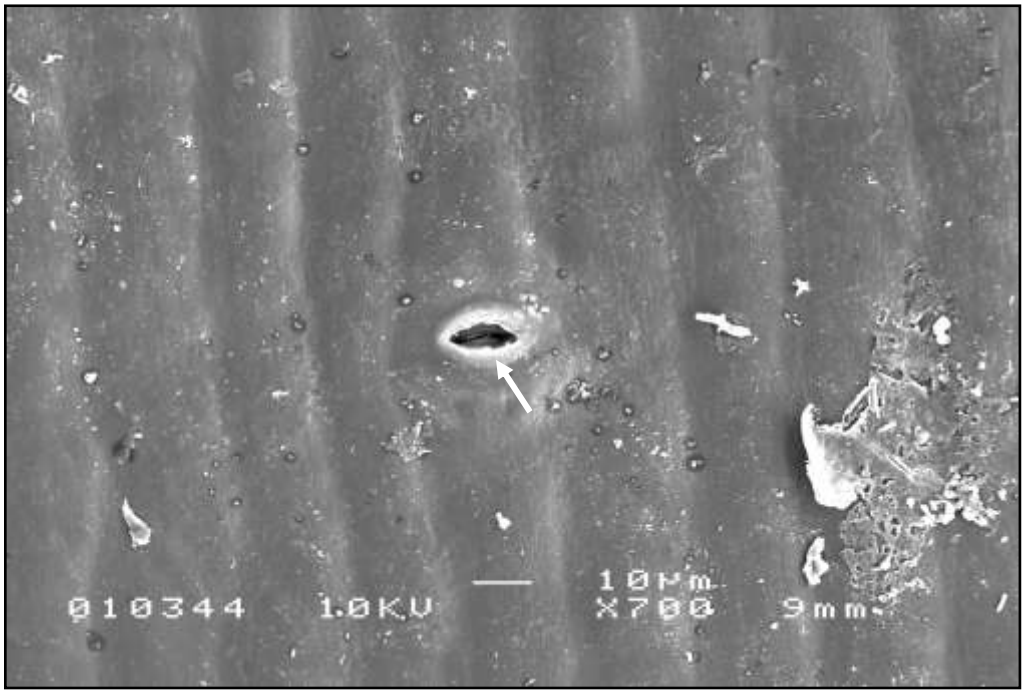

Figure 4.27: SEM micrograph showing the surface of *in vivo* stem of *Allamanda cathartica* L. A stoma (white arrow) was observed on *in vivo* stem.

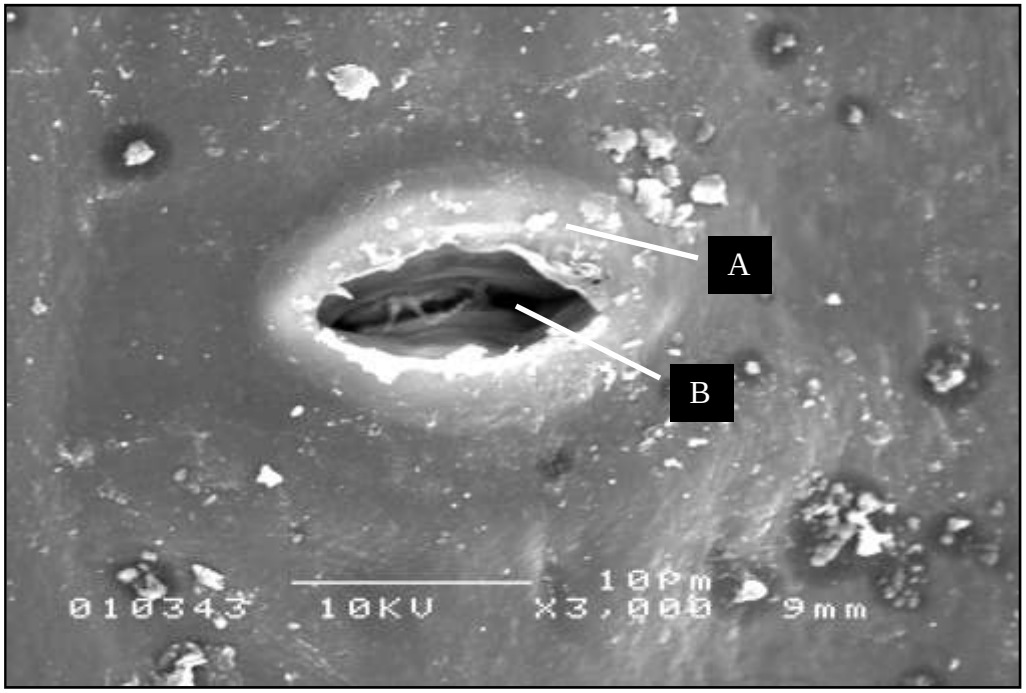

Figure 4.28: SEM micrograph showing the close up of stoma found on *in vivo* stem of *Allamanda cathartica* L. (A) Guard cell and (B) Stomatal opening.

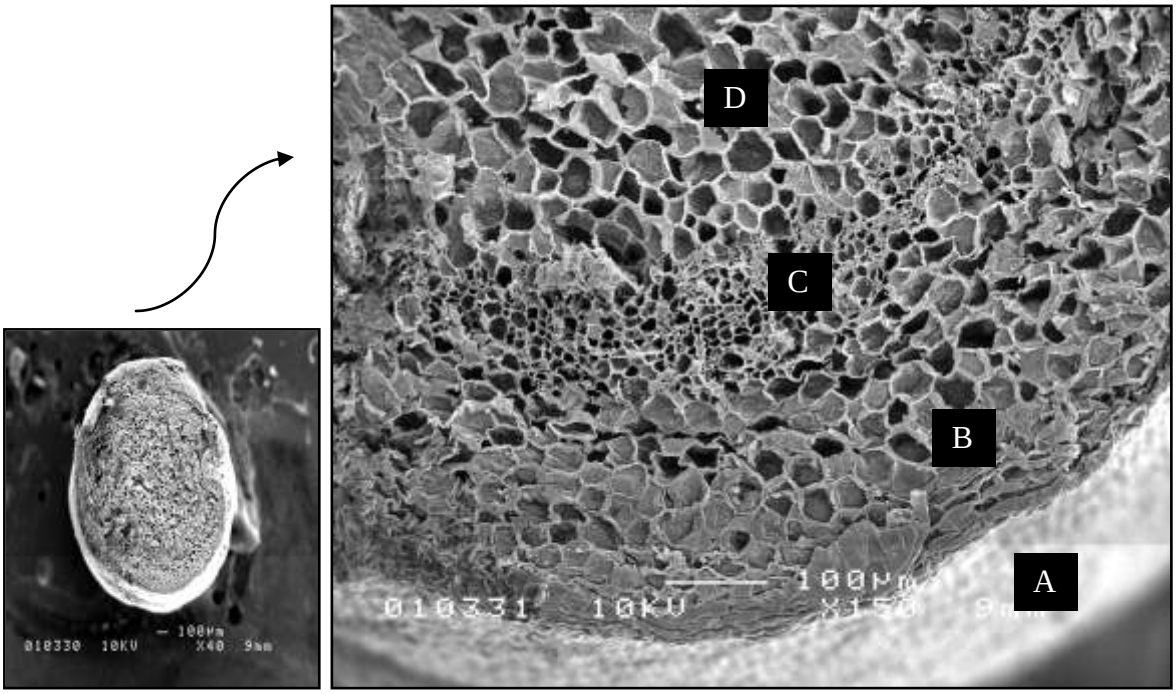

Figure 4.29: SEM micrograph showing the cross section of *in vitro* stem of *Allamanda cathartica* L. (A) Epidermis; (B) Cortex; (C) Interfascicular region and (D) Pith.

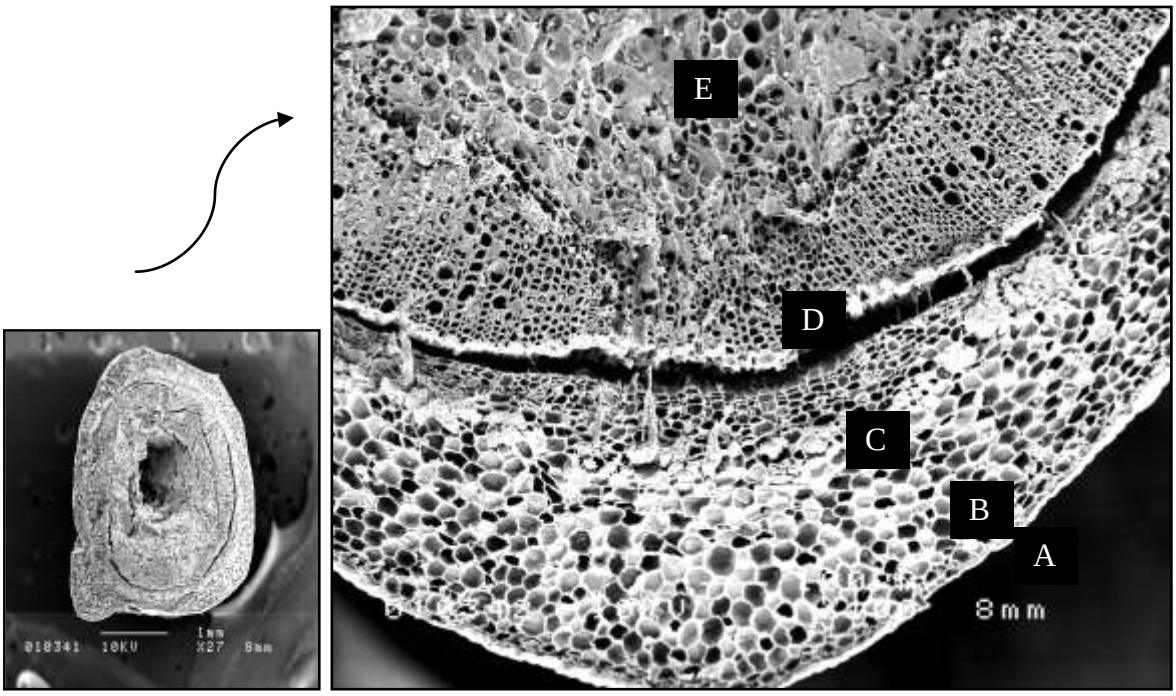

Figure 4.30: SEM micrograph showing the cross section of *in vivo* stem of *Allamanda cathartica* L. (A) Cuticle; (B) Epidermis; (C) Cortex; (D) Interfascicular region and (E) Pith.

4.2 INVESTIGATION ON SECONDARY METABOLITES OF *Allamanda cathartica* L.

4.2.1 Phytochemical Screening

Phytochemical screening on reducing sugars, anthraquinones, terpenoids and steroids, saponins, tannins, glycosides, essential oils and flavonoids were carried out. The phytochemical screening of ethanolic extracts of both dried and fresh plant materials, i.e. leaves, stems, petals and roots of *A. cathartica* L. were shown in Table 4.5 and 4.6, respectively.

4.2.1.1 Ethanolic Extracts from Dried Plant Materials

Table 4.5 showed the results of phytochemical screening of ethanolic extracts from dried plant materials (leaves, stems, petals and roots) of *A. cathartica* L. and Figure 4.31 was the extracts before the phytochemical tests were performed. According to the results, all the leaf, stem, petal and root extracts were observed to have reducing sugars (formation of a brick red precipitate) (Figure 4.32), anthraquinones (changed of rose pink to red colouration) (Figure 4.33), terpenoids and steroids (red violet colouration for terpenoids and to green bluish colouration for steroids were observed on the interface) (Figure 4.34), glycosides (formation of a red precipitate) (Figure 4.35) and essential oils (formation of a white precipitate) (Figure 4.36), which gave the positive responses when tested with specific phytochemical tests as stated in Chapter 3. Saponins and flavonoids were absent in the leaf extract but present in the stem, petal and root extracts of *A. cathartica* L., in which an emulsion was formed for the presence of saponins (Figure 4.37) and a yellow colouration (Method I) or a golden reddish precipitate (Method II) was produced for the

presence of flavonoids (Figure 4.38 and Figure 4.39). Meanwhile, the leaf, stem and petal extracts indicated the presence of tannins when tested with few drops of 0.1% $FeCl_3$ except for root extract (Figure 4.40).

Table 4.5: Phytochemical screening of ethanolic extracts from the dried plant materials (leaves, stems, petals and roots) of *Allamanda cathartica* L.

Test	Positive indicator	Leaf	Stem	Petal	Root
Reducing sugars	Formation of a brick red precipitate	+	+	+	+
Anthraquinones	A rose pink to red colouration	+	+	+	+
Terpenoids and steroids	A red violet colour for terpenoids and green bluish colour for steroids	+ (steroids)	+ (terpenoids)	+ (steroids and terpenoids)	+ (terpenoids)
Saponins	Formation of an emulsion	-	+	+	+
Tannins	A blue colour for gallic tannins and green black for catecholic tannins	+ (catecholic)	+ (catecholic)	+ (catecholic)	-
Glycosides	Formation of a red precipitate	+	+	+	+
Essential oils	Formation of a white precipitate	+	+	+	+
Flavonoids (I)	A yellow colouration	-	+	+	+
(II)	Formation of a golden reddish precipitate	-	-	+	+

Legends: (+) = Presence of phytochemical constituent; (-) = Absence of phytochemical constituent.

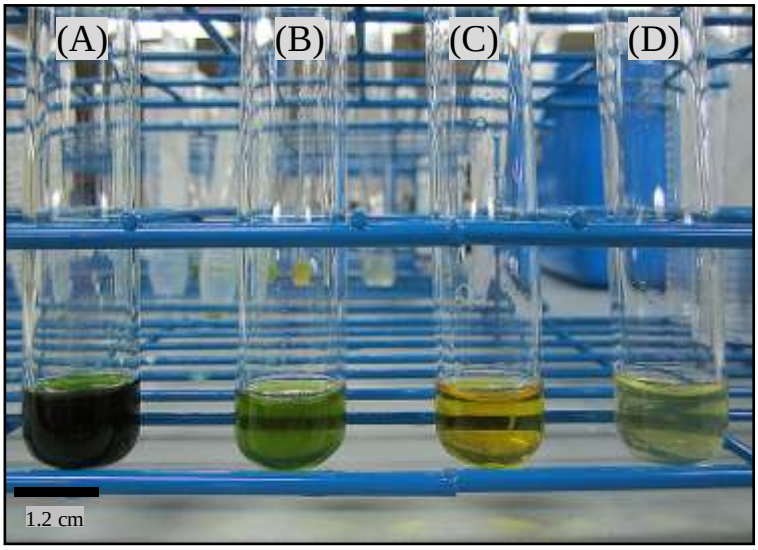

Figure 4.31: Ethanolic extracts of dried plant materials of *Allamanda cathartica* L. before the test of phytochemical screening. (A) Leaf; (B) Stem; (C) Petal and (D) Root.

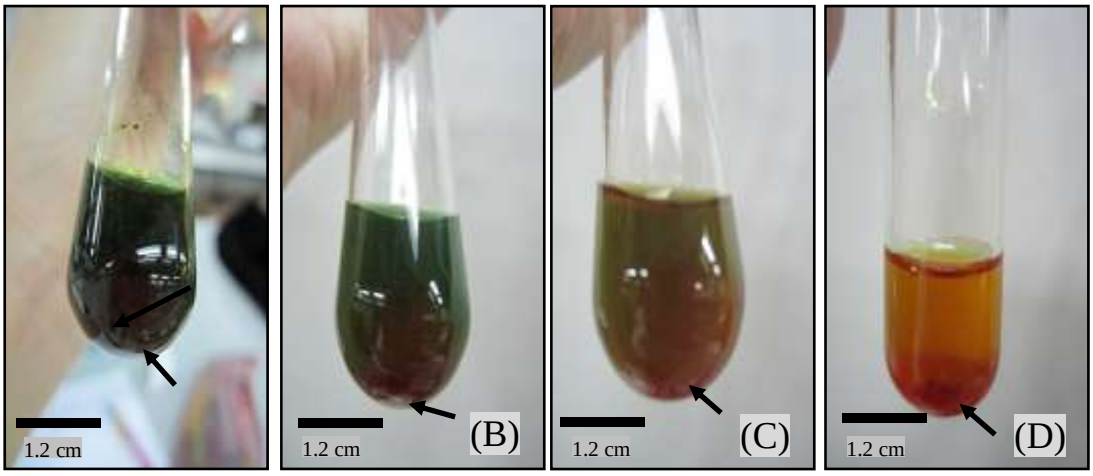

Figure 4.32: Observations for phytochemical screening on reducing sugars from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A brick red precipitate (arrows) was formed for all the extracts.

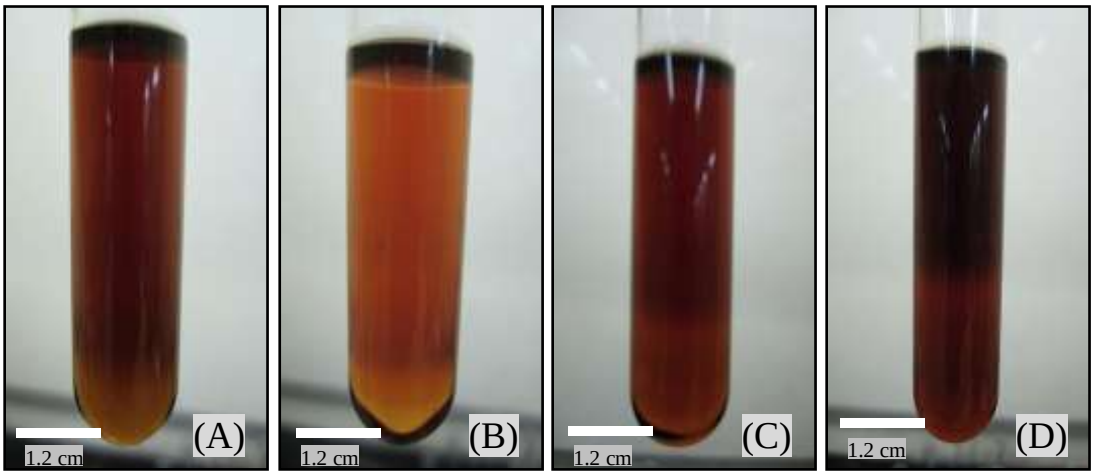

Figure 4.33: Observations for phytochemical screening on anthraquinones from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A rose pink changed to red colouration was observed for all the extracts.

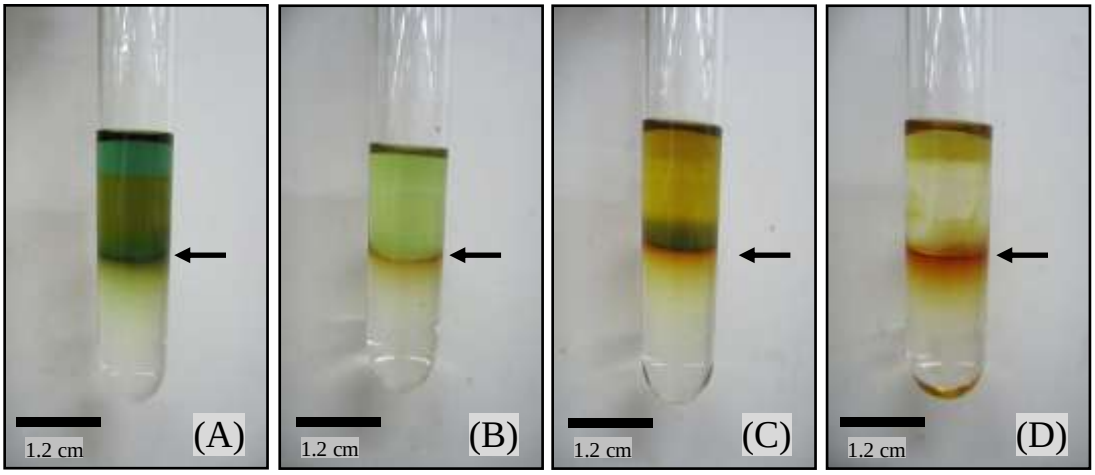

Figure 4.34: Observations for phytochemical screening on terpenoids and steroids from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. Red violet and green bluish colourations (arrows) were observed on the interface for terpenoids and steroids, respectively for all the extracts.

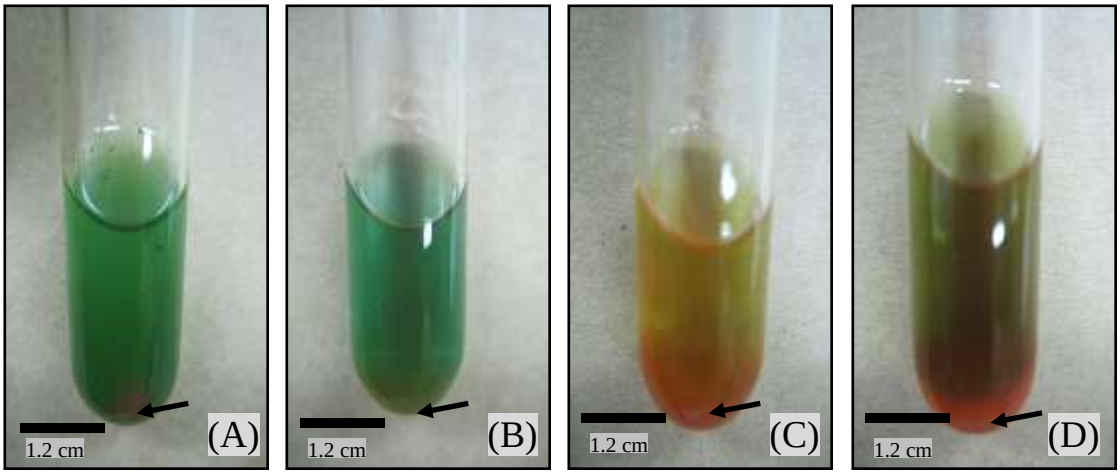

Figure 4.35: Observations for phytochemical screening on glycosides from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A red precipitate (arrows) was formed for all the extracts.

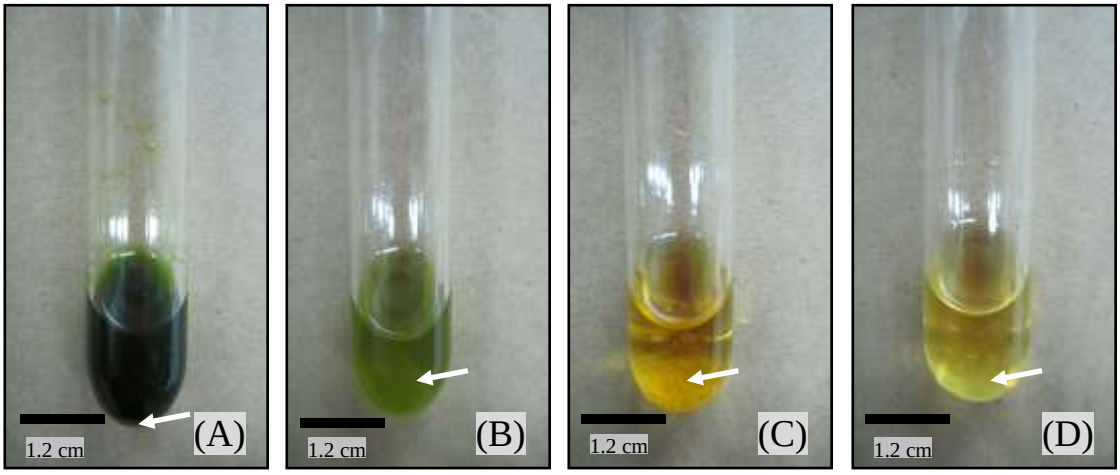

Figure 4.36: Observations for phytochemical screening on essential oils from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A white precipitate (arrows) was formed for all the extracts.

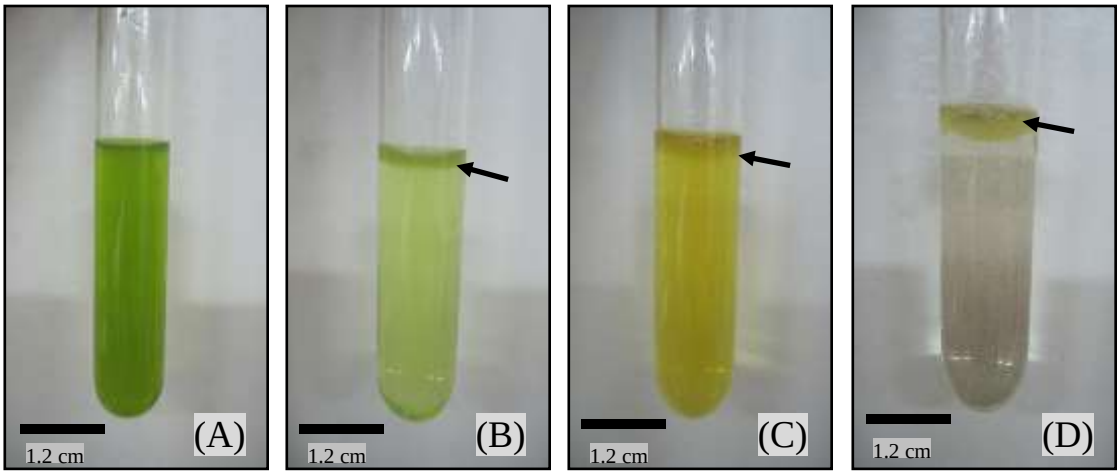

Figure 4.37: Observations for phytochemical screening on saponins from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. An emulsion (arrows) was formed for the extracts of stems, petals and roots.

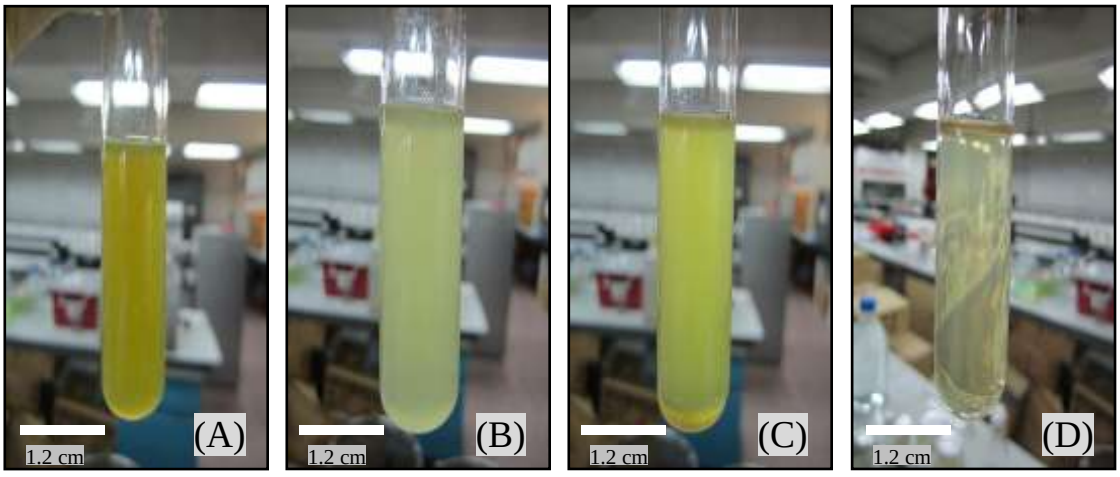

Figure 4.38: Observations for phytochemical screening on flavonoids (Method I) from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A yellow colouration was observed for the extracts of stems, petals and roots.

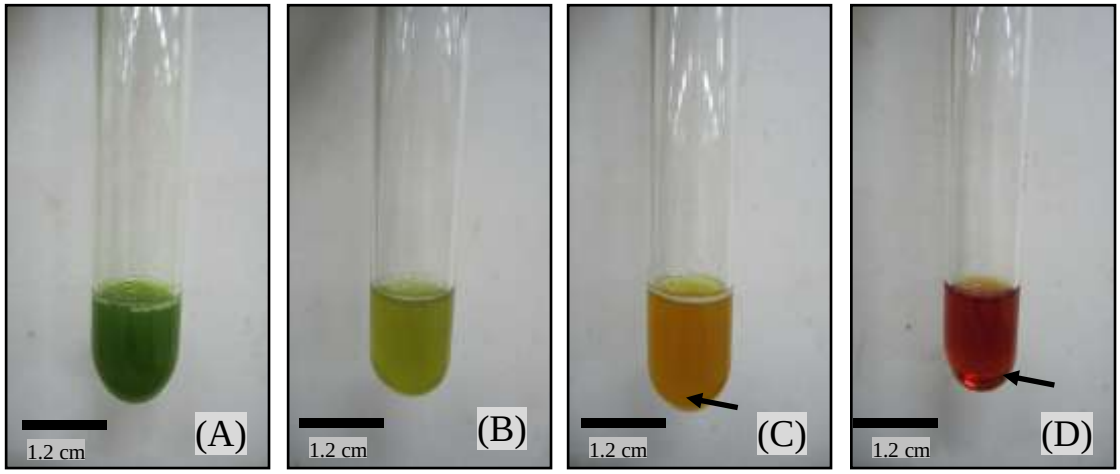

Figure 4.39: Observations for phytochemical screening on flavonoids (Method II) from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A golden reddish precipitate (arrows) was formed for the extracts of petals and roots.

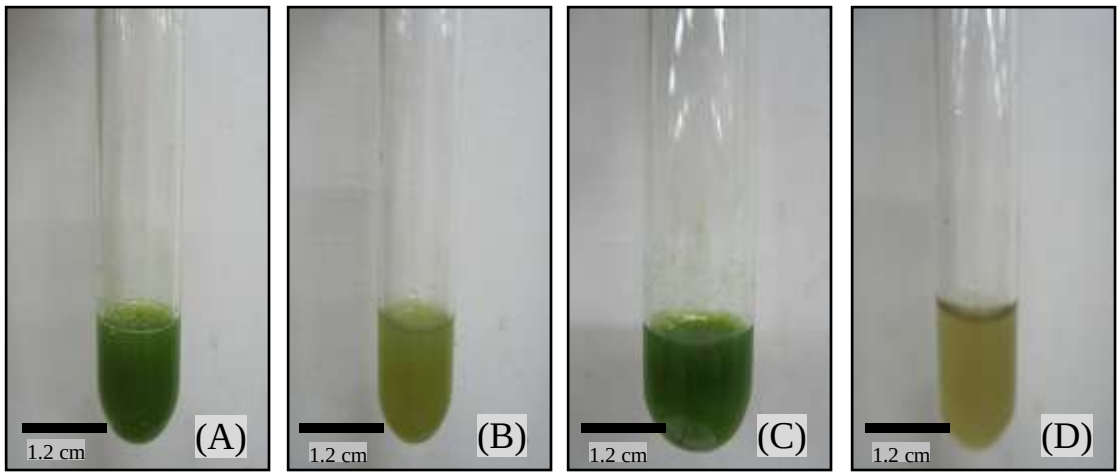

Figure 4.40: Observations for phytochemical screening on catecholic tannins from ethanolic extracts of *Allamanda cathartica* L. (dried plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. Green black colouration was observed for the extracts of leaves, stems and petals.

4.2.1.2 Ethanolic Extracts from Fresh Plant Materials

Table 4.6 showed the results of phytochemical screening of ethanolic extracts from fresh plant materials, i.e. leaves, stems, petals and roots of *A. cathartica* L. and Figure 4.41 showed the extracts before the phytochemical tests were carried out. Based on the results, all the leaf, stem, petal and root extracts were observed to have reducing sugars (formation of a brick red precipitate) (Figure 4.42), anthraquinones (changed of rose pink to red colouration) (Figure 4.43), terpenoids and steroids (red violet colouration for terpenoids and to green bluish colouration for steroids were observed on the interface) (Figure 4.44), glycosides (formation of a red precipitate) (Figure 4.45) and essential oils (formation of a white precipitate) (Figure 4.46), which gave the positive responses when tested with particular phytochemical tests as stated in Chapter 3. Phytochemicals such as saponins (Figure 4.47) and flavonoids (Method I and Method II) (Figure 4.48 and Figure 4.49) were not shown in the ethanolic extract of leaf. Except for the extracts of leaf, petal and root, the ethanolic extracts of stem gave the negative response when tested with few drops of 0.1% $FeCl_3$, indicated the absence of tannins (Figure 4.50).

Table 4.6: Phytochemical screening of ethanolic extracts from the fresh plant materials (leaves, stems, petals and roots) of *Allamanda cathartica* L.

Test	Positive indicator	Leaf	Stem	Petal	Root
Reducing sugars	Formation of a brick red precipitate	+	+	+	+
Anthraquinones	A rose pink to red colouration	+	+	+	+
Terpenoids and steroids	A red violet colour for terpenoids and green bluish colour for steroids	+ (steroids)	+ (terpenoids)	+ (steroids and terpenoids)	+ (terpenoids)
Saponins	Formation of an emulsion	-	+	+	+
Tannins	A blue colour for gallic tannins and green black for catecholic tannins	+ (catecholic)	-	+ (catecholic)	+ (catecholic)
Glycosides	Formation of a red precipitate	+	+	+	+
Essential oils	Formation of a white precipitate	+	+	+	+
Flavonoids (I)	A yellow colouration	-	+	+	+
(II)	Formation of a golden reddish precipitate	-	-	+	+

Legends: (+) = Presence of phytochemical constituent; (-) = Absence of phytochemical constituent.

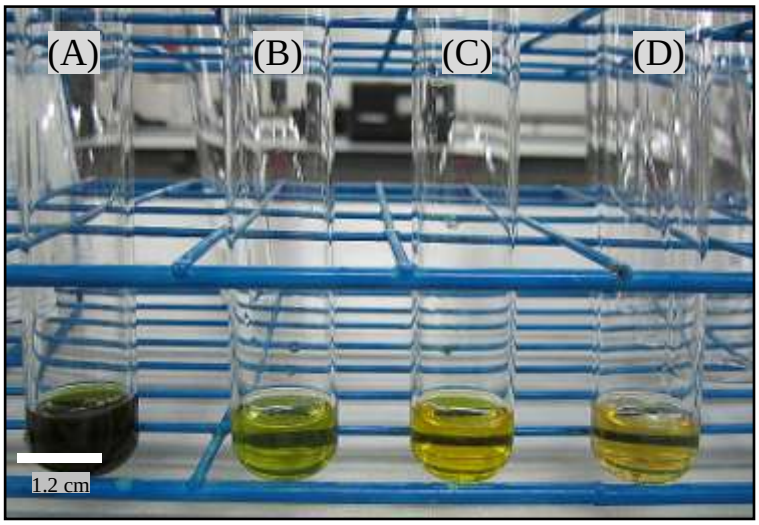

Figure 4.41: Ethanolic extracts of fresh plant materials of *Allamanda cathartica* L. before the test of phytochemical screening. (A) Leaf; (B) Stem; (C) Petal and (D) Root.

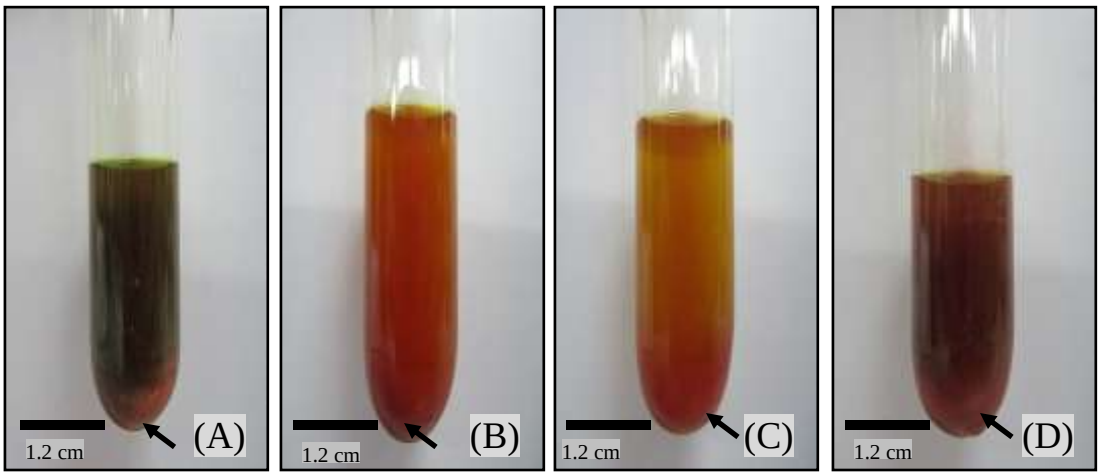

Figure 4.42: Observations for phytochemical screening on reducing sugars from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A brick red precipitate (arrows) was formed for all the extracts.

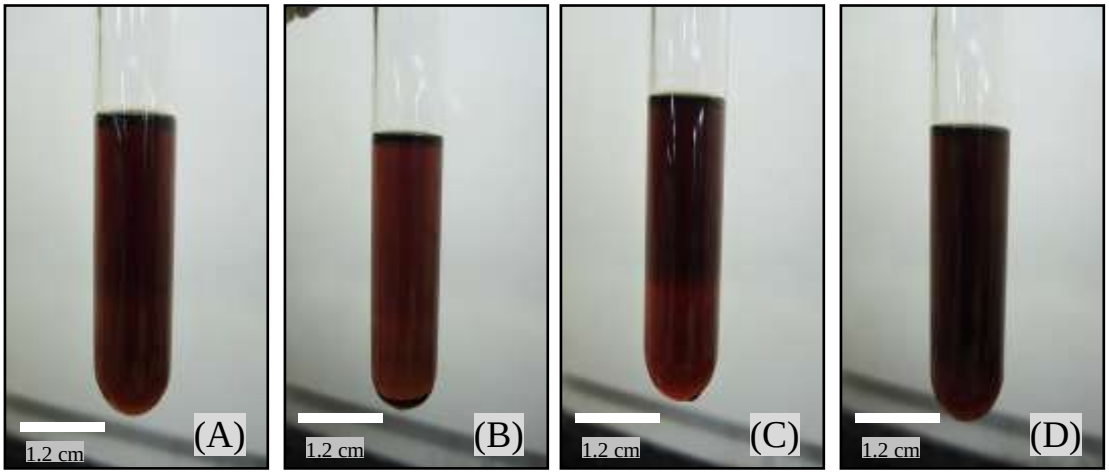

Figure 4.43: Observations for phytochemical screening on anthraquinones from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A rose pink changed to red colouration was observed for all the extracts.

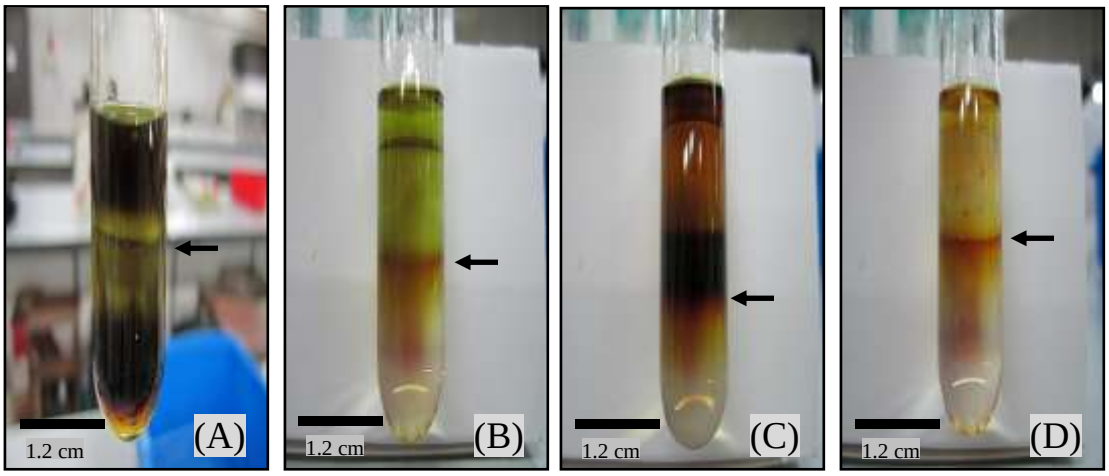

Figure 4.44: Observations for phytochemical screening on terpenoids and steroids from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. Red violet and green bluish colourations (arrows) were observed on the interface for terpenoids and steroids, respectively for all the extracts.

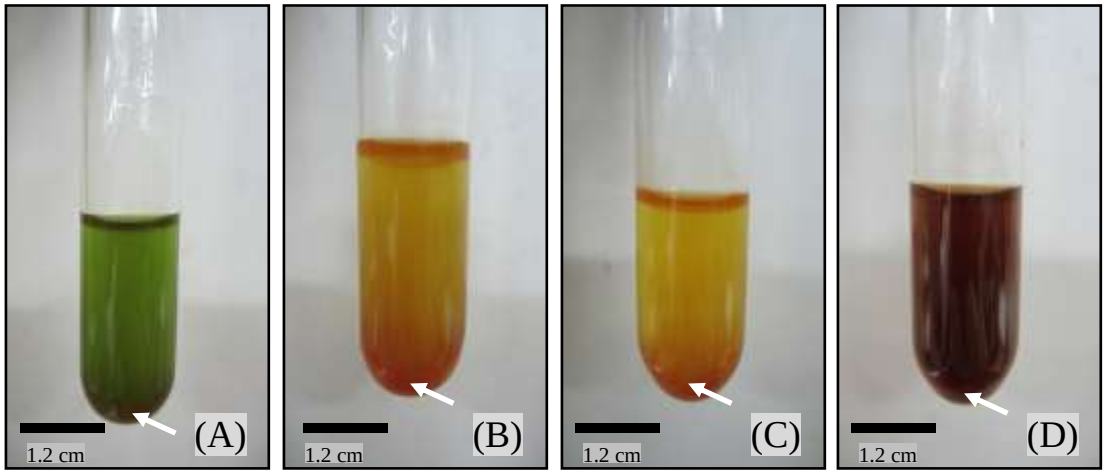

Figure 4.45: Observations for phytochemical screening on glycosides from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A red precipitate (arrows) was formed for all the extracts.

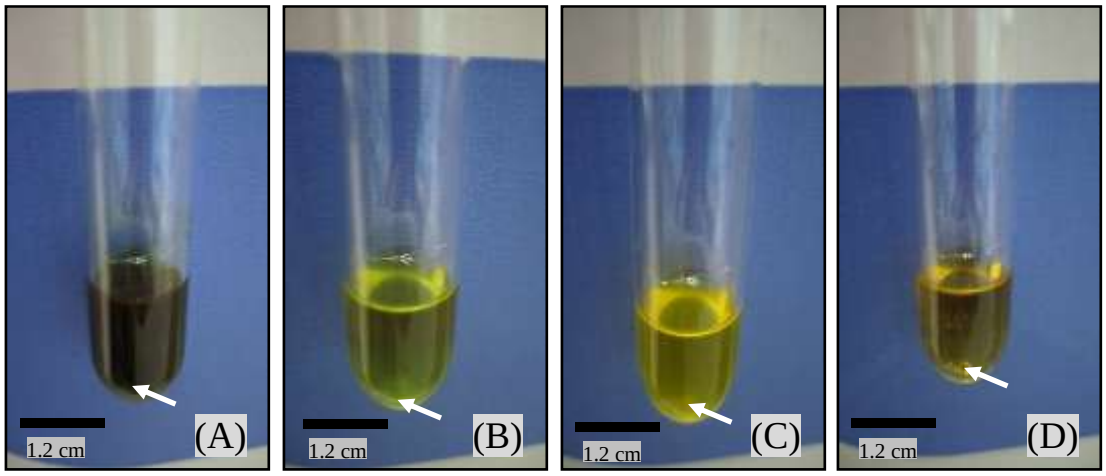

Figure 4.46: Observations for phytochemical screening on essential oils from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A white precipitate (arrows) was formed for all the extracts.

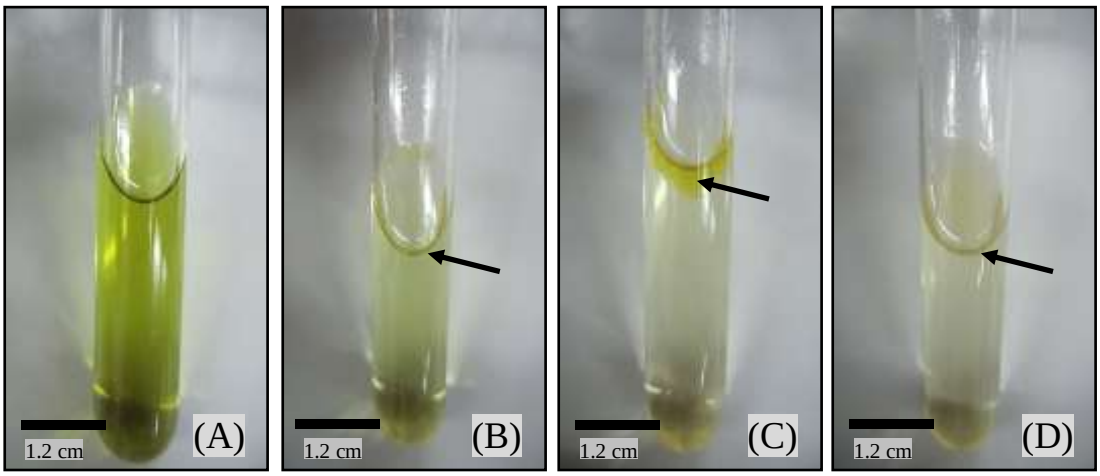

Figure 4.47: Observations for phytochemical screening on saponins from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. An emulsion (arrows) was formed for the extracts of stems, petals and roots.

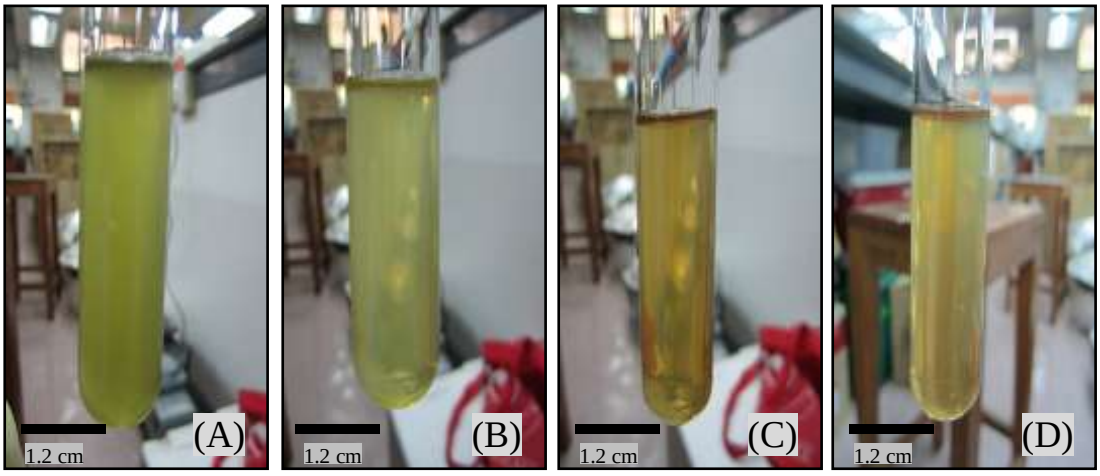

Figure 4.48: Observations for phytochemical screening on flavonoids (Method I) from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A yellow colouration was observed for the extracts of stems, petals and roots.

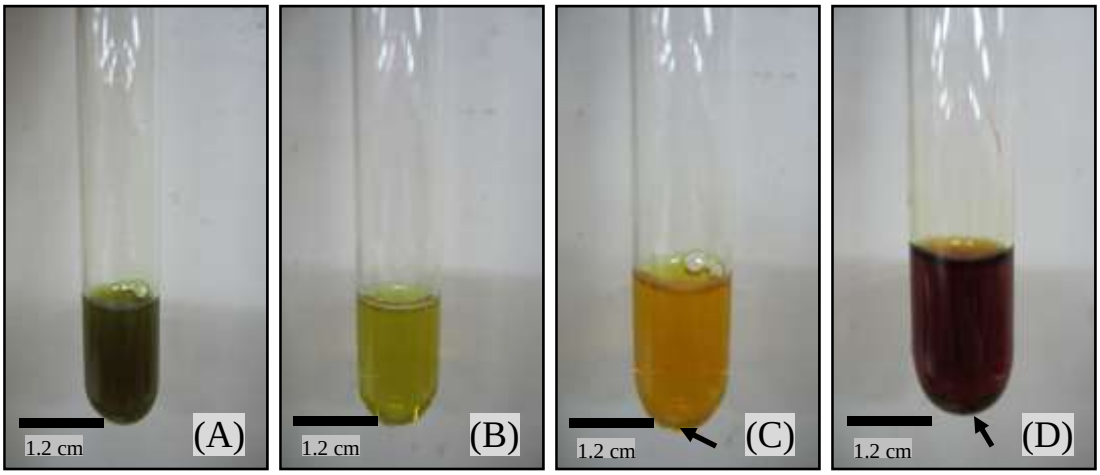

Figure 4.49: Observations for phytochemical screening on flavonoids (Method II) from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. A golden reddish precipitate (arrows) was formed for the extracts of petals and roots.

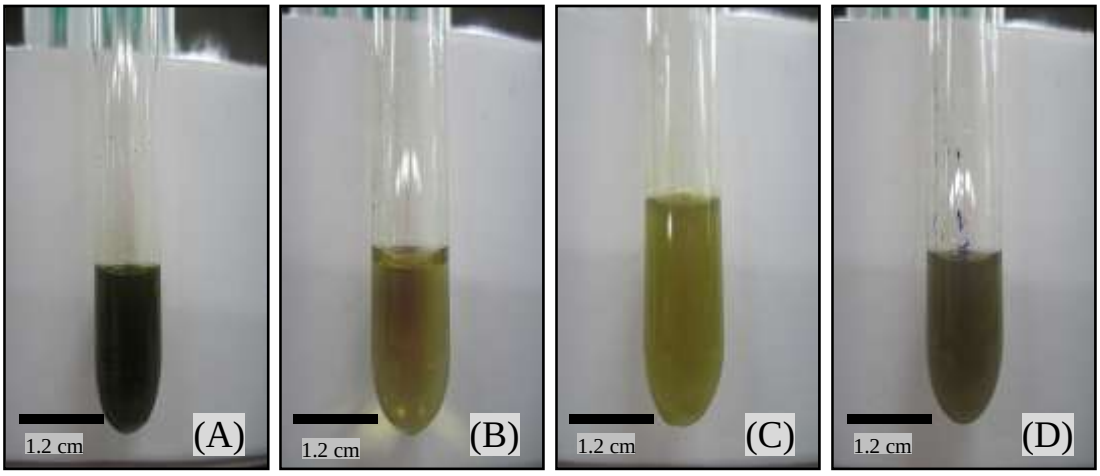

Figure 4.50: Observations for phytochemical screening on catecholic tannins from ethanolic extracts of *Allamanda cathartica* L. (fresh plant materials). (A) Leaf; (B) Stem; (C) Petal and (D) Root. Green black colouration was observed for the extracts of leaves, petals and roots.

4.2.2 Determination of Antioxidant Activities

The antioxidant activities of leaf, stem, petal and root extracts (ethanolic) of *A. cathartica* L. for both dried and fresh plant materials were determined using the DPPH free radical scavenging assay.

4.2.2.1 Ethanolic Extracts from Dried Plant Materials

Table 4.7 showed the percentage yield of ethanolic crude extracts of leaves, stems, petals and roots of *A. cathartica* L. with stem extract produced the highest percentage yield, i.e. 24.22%, followed by the extracts of petals (16.30%), leaves (8.26%) and roots (0.54%).

According to Table 4.8, the free radical scavenging activities of crude ethanolic extracts of leaves, stems, petals and roots were directly proportional to its concentration, i.e. the percentage scavenging activities of DPPH were increased as the concentrations of extracts increased from 0 to 100 μg/ml (Figure 4.51). Among the ethanolic extracts tested, root extract from *A. cathartica* L. gave the highest percentage of scavenging of DPPH at 100 μg/ml (89.21 ± 0.65%), followed by the extracts of petals, stems and leaves which showed the percentage scavenging activities of 88.41 ± 1.58%, 85.22 ± 2.16% and 70.10 ± 12.55%, respectively at the concentration of 100 μg/ml. Moreover, ascorbic acid was the positive control in this study and showed to have a value of 88.40 ± 1.02% scavenging activity of DPPH at 100 μg/ml which was a strong antioxidant compared to the extracts of leaves and stems but a weak antioxidant as compared to petal and root extracts.

Table 4.7: Percentage yield of ethanolic extracts from the dried plant materials (leaves, stems, petals and roots) of *Allamanda cathartica* L.

Plant material	Weight of raw material (g)	Weight of the extract (g)	Percentage yield of extract (%)
Leaf	17.32	1.43	8.26
Stem	29.48	7.14	24.22
Petal	10.43	1.70	16.30
Root	24.00	0.13	0.54

Table 4.8: Percentage scavenging activity of DPPH by the ethanolic extracts of leaves, stems, petals and roots (dried plant materials) of *Allamanda cathartica* L.

Concentration (µg/ml)	Percentage scavenging activity of DPPH (%) ±SE[a]				
	Ascorbic acid	Leaf	Stem	Petal	Root
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
20	18.44 ± 6.02^{b}	9.2 ± 0.82^{ab}	7.22 ± 4.24^{a}	63.21 ± 1.54^{b}	65.97 ± 3.60^{b}
40	50.85 ± 6.42^{c}	32.05 ± 7.07^{abc}	34.44 ± 5.03^{b}	67.20 ± 0.74^{b}	75.97 ± 3.26^{bc}
60	82.40 ± 2.44^{d}	44.89 ± 10.74^{bc}	42.45 ± 6.58^{b}	78.00 ± 0.76^{c}	80.38 ± 2.18^{cd}
80	85.20 ± 1.76^{d}	52.47 ± 10.90^{c}	72.00 ± 2.11^{c}	84.00 ± 0.77^{d}	82.79 ± 1.50^{cd}
100	88.40 ± 1.02^{d}	70.10 ± 12.55^{c}	85.22 ± 2.16^{c}	88.41 ± 1.58^{d}	89.21 ± 0.65^{d}

SE^{a} = Standard Error.

The value for the same alphabet is not significant different (Tukey's multiple range test, $p<0.05$).

There were no significant differences on the percentage scavenging activity of DPPH among the five different sources of antioxidants at the control sample (0 µg/ml). For leaf extract, a significant difference was observed on the percentage scavenging activity of DPPH at concentrations of 20, 40 and 60 µg/ml but no significant difference was observed between the concentrations of 80 and 100 µg/ml. For stem extract, no significant difference was observed on the percentage scavenging activity of DPPH between concentrations of 40 and 60 µg/ml, 80 and 100 µg/ml, but a significant difference was noticed among all the concentrations with 20 µg/ml. Furthermore, no significant difference was observed on the percentage scavenging activity of DPPH between concentrations of 20 and 40 µg/ml, 80 and 100 µg/ml but a significant difference was showed among all the concentrations with

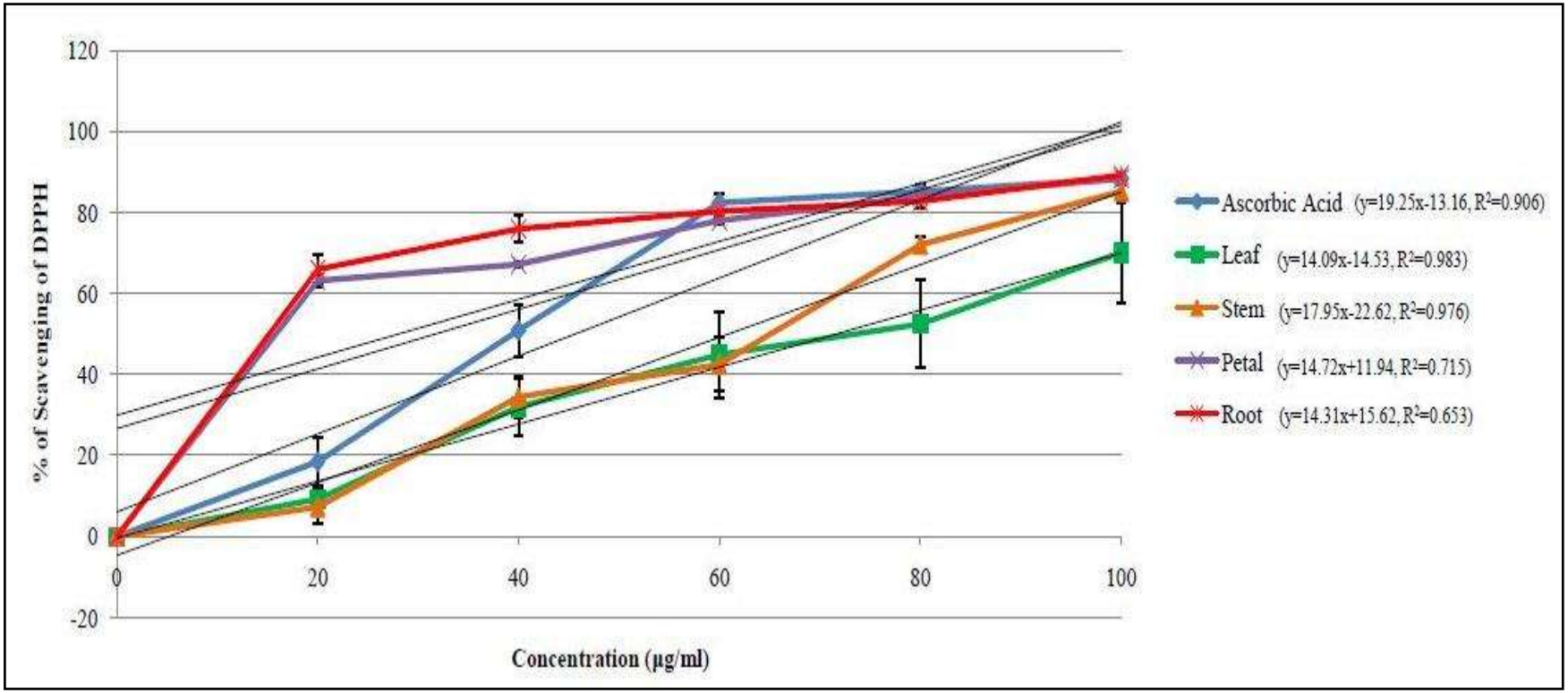

Figure 4.51: Determination of IC_{50} values for positive control (ascorbic acid) and crude ethanol extracts of leaf, stem, petal and root (dry plant materials) of *Allamanda cathartica* L. from linear correlation between concentrations (µg/ml) versus percentage of scavenging of DPPH (%).The error bars indicate the standard error.

60 µg/ml for the extract of petals. For root extract at concentrations of 60 and 80 µg/ml, no significant difference was observed on the percentage of scavenging activity compared to other three concentrations. As for ascorbic acid, a significant difference was observed on the percentage of DPPH activity at 20 and 40 µg/ml among the three other concentrations, i.e. 60, 80 and 100 µg/ml.

The results of IC_{50} values of ethanolic crude extracts of leaves, stems, petals and roots of *Allamanda cathartica* L. (dried plant materials) was presented in Figure 4.52. Among all the extracts, ethanol extract of roots (2.40 µg/ml) showed the lowest value of IC_{50}, followed by petal (2.59 µg/ml) extract. As can be observed from Figure 4.52, the leaf (4.58 µg/ml) and stem (4.05 µg/ml) extracts showed the values of IC_{50} were significantly higher than other two extracts. Ethanol extract of leaves showed the highest IC_{50} value among all the extracts which means the free radical scavenging activity was the lowest. In addition, ascorbic acid (3.28 µg/ml) showed the better IC_{50} value compared to the extracts of leaves and stems, but it was in contrast to the extracts of petals and roots.

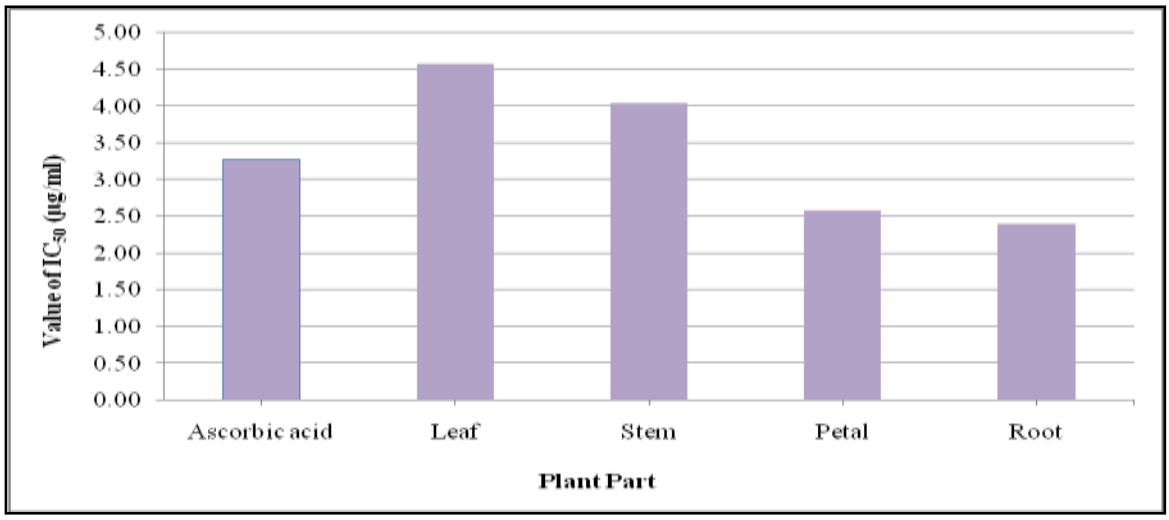

Figure 4.52: IC_{50} values in DPPH assay of ethanolic leaf, stem, petal and roots extracts (dried plant materials) of *Allamanda cathartica* L.

4.2.2.2 Ethanolic Extracts from Fresh Plant Materials

The ethanol extracts of fresh plant materials were prepared directly by soaking the plant materials in 99.9% ethanol without air-dried at room temperature. Table 4.9 showed the percentage yield of ethanol extracts from leaves, stems, petals and roots of *Allamanda cathartica* L. The petal extract of *A. cathartica* L. produced the highest yield (39.70%) as compared to other types of extracts, which followed by the extracts of leaves, stems and roots with the percentage yield of 38.53%, 14.86% and 3.21%, respectively.

The percentage scavenging activity for each of the plant extract was dependant on their capacity to scavenge the free radical of DPPH. According to Table 4.10, none of the extracts were significantly comparable with positive control (88.40 ± 1.02% at 100 μg/ml) in terms of scavenging capacity. The best scavenging activity among the examined extracts was achieved from ethanolic extract of roots (83.60 ± 1.00% at 100 μg/ml) which was significantly higher than other extracts. Meanwhile, among all the extracts, ethanolic exract of leaves showed the lowest scavenging activity at 100 μg/ml (62.84 ± 12.70%). Ethanolic extracts of stems (73.20 ± 1.76%) and petals (64.73 ± 11.21%) gave the intermediate scavenging activity. Apart from that, the graph of percentage scavenging activity of DPPH versus concentration of extracts (Figure 4.53) also showed a similar trend as the antioxidant activities determination from the ethanolic extracts of dry plant materials, i.e. the percentage scavenging activities for all the plant extracts increased as their concentrations increased from 0 to 100 μg/ml.

Table 4.9: Percentage yield of ethanolic extracts from the fresh plant materials (leaves, stems, petals and roots) of *Allamanda cathartica* L.

Plant sample	Weight of raw material (g)	Weight of the extract (g)	Percentage yield of extract (%)
Leaf	11.03	4.25	38.53
Stem	11.17	1.66	14.86
Petal	11.51	4.57	39.70
Root	11.53	0.37	3.21

Table 4.10: Percentage scavenging activity of DPPH by the ethanolic extracts of leaves, stems, petals and roots (fresh plant materials) of *Allamanda cathartica* L.

Concentration (µg/ml)	Percentage scavenging activity of DPPH (%) ±SE[a]				
	Ascorbic acid	Leaf	Stem	Petal	Root
0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
20	18.44 ± 6.02^{b}	8.41 ± 1.41^{ab}	4.81 ± 1.40^{a}	16.75 ± 7.66^{ab}	13.64 ± 6.00^{a}
40	50.85 ± 6.42^{c}	27.66 ± 13.36^{ab}	43.54 ± 7.90^{b}	31.20 ± 2.09^{bc}	46.05 ± 6.40^{b}
60	82.40 ± 2.44^{d}	34.06 ± 13.14^{ab}	49.96 ± 5.37^{bc}	38.0 ± 1.05^{bcd}	77.60 ± 2.44^{c}
80	85.70 ± 1.76^{d}	49.67 ± 18.55^{ab}	66.78 ± 2.35^{cd}	50.79 ± 4.59^{cd}	80.40 ± 1.76^{c}
100	88.40 ± 1.02^{d}	62.84 ± 12.70^{b}	73.20 ± 1.76^{d}	64.73 ± 11.21^{d}	83.60 ± 1.00^{c}

SE^{a} = Standard Error.

The value for the same alphabet is not significant different (Tukey's multiple range test, $p<0.05$).

There were no significant differences on the percentage scavenging activity of DPPH among the five different extracts of *A. cathartica* L. at the control sample (0 µg/ml). For the ethanolic extract of leaves, a significant difference was observed on the DPPH's scavenging activity at the concentration of 100 µg/ml but no significant differences was noticed between the concentrations of 20, 40, 60 and 80 µg/ml. For the extracts of stems and petal, a significant difference on the percentage scavenging activity of DPPH was observed for all concentrations of extracts. Furthermore, for the extract of roots and ascorbic acid, a significant difference on the DPPH's scavenging activity was shown at the concentrations of 20 and 40 µg/ml but no significant difference was found at extract concentrations of 60 to 100 µg/ml.

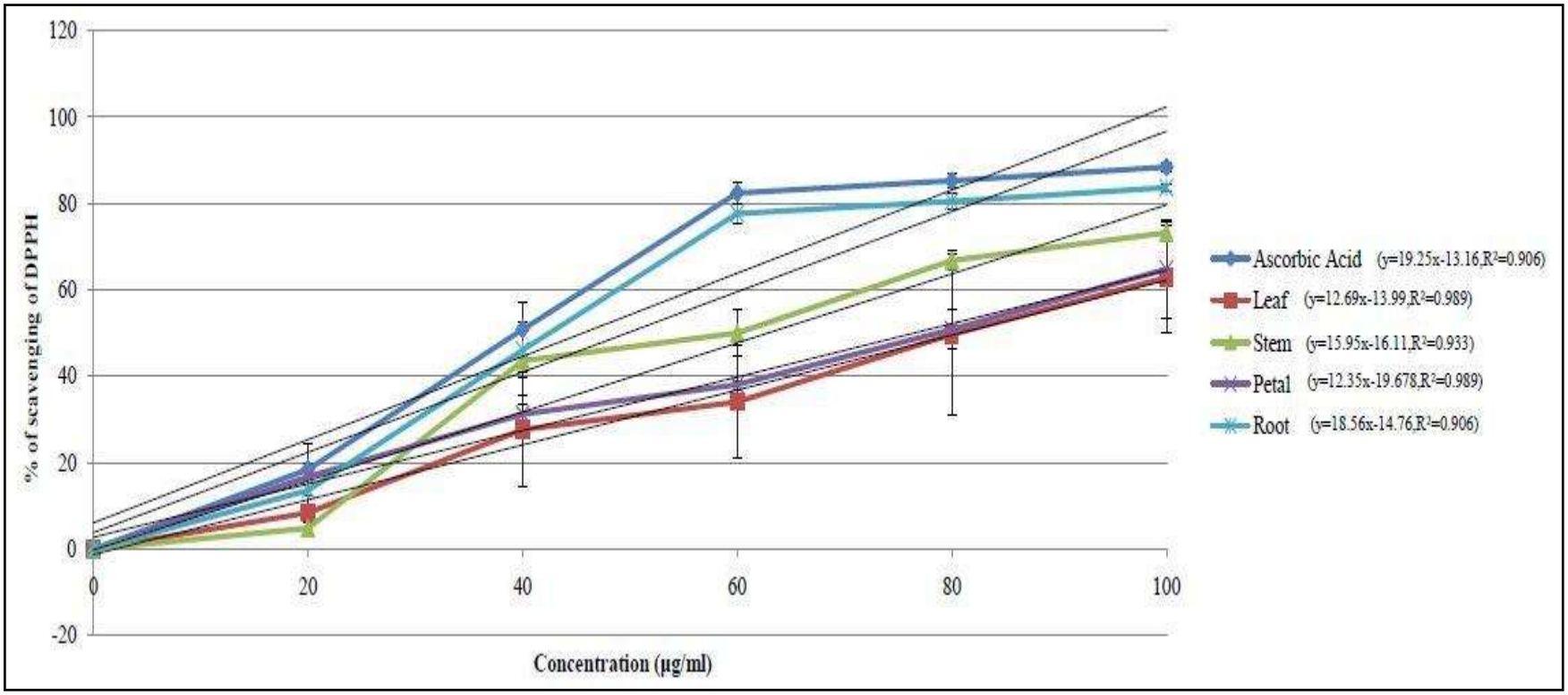

Figure 4.53: Determination of IC_{50} values for positive control (ascorbic acid) and crude ethanol extracts of leaf, stem, petal and root (fresh plant materials) of *Allamanda cathartica* L. from linear correlation between concentrations (µg/ml) versus percentage of scavenging of DPPH (%).The error bars indicate the standard error.

Figure 4.54 was the IC_{50} values in DPPH assay of ethanolic extracts of *A. cathartica* L. leaves, stems, petals and roots (fresh plant materials). Among all the extracts, ethanol extract of roots (3.49 µg/ml) showed the lowest value of IC_{50}, followed by stem (4.14 µg/ml) extract. The petal (4.83 µg/ml) and leaf (5.04 µg/ml) extracts showed the values of IC_{50} were significantly higher than other two extracts. Ethanol extract of leaves showed the highest IC_{50} value among all the extracts which means the free radical scavenging activity was the lowest. In addition, ascorbic acid (3.28 µg/ml) showed the better IC_{50} value compared to the extracts of leaves, stems, petals and roots of *A. cathartica* L.

Figure 4.54: IC_{50} values in DPPH assay of ethanolic leaf, stem, petal and roots extracts (fresh plant materials) of *Allamanda cathartica* L.

4.3 Pigment Extraction and Coloured Coating Development from Natural Pigments (Chlorophylls and Carotenoids)

4.3.1 Pigment Extraction

The plant pigments such as chlorophyll *a*, chlorophyll *b* and carotenoids present in the leaves (*in vivo*), stems (*in vivo*), petals (*in vivo*) and calli (*in vitro*) were extracted in three different extracting solvents of 0.15% HCl in 99.9% methanol, 0.01% HCl in 70%

acetone and 95% ethanol at different volumes, i.e. 5.0, 12.5, 20 .0and 25.0 ml. The extracts were measured by UV-VIS spectrophotometer for determination of spectral characteristics and compared. For all the plant materials (leaves, stems, petals and calli), the most suitable extracting solvent that gave maximum absorption for chlorophyll *a* (absorption peak around 660 and 420 nm), chlorophyll *b* (absorption peak around 643 and 453 nm) and carotenoids (absorption peak around 435-480 nm) were identified in this study. Those determined extracting solvents for extracting the leaves (green colour) and petals (orange yellowish colour) were then used to develop the natural coloured coating.

4.3.1.1 Pigment Extraction from the Leaves

Leaves contain two important pigments involved in the process of photosynthesis, i.e. chlorophyll *a* and chlorophyll *b*. Figure 4.55, 4.56, 4.57 and 4.58 showed the adsorption spectral characteristics for the leaf extract of *A. cathartica* L. in 0.15% HCl + 99.9% methanol at the volumes of 5.0, 12.5, 20.0 and 25.0 ml, respectively. From the figures, the pigments of chlorophyll *a* (Absorbance of 2.443 at 663 nm and Absorbance of 2.914 at 437 nm) and chlorophyll *b* (Absorbance of 2.914 at 468 nm) showed the maximum absorption in 20.0 ml methanol extract of leaves (Figure 4.57) among the different volumes of solvents. Methanol solvent at the volume of 5.0 ml gave the lowest absorption for the pigments of chlorophyll *a* (Absorbance of 0.485 at 666 nm) and chlorophyll *b* (Absorbance of 0.539 at 469 nm) as shown on Figure 4.55. Whereas, methanol solvents at the volumes of 12.5 (Figure 4.56) and 25.0 ml (Figure 4.58) were observed to give the intermediate absorption for the pigments of chlorophyll *a* and chlorophyll *b* among the extracts.

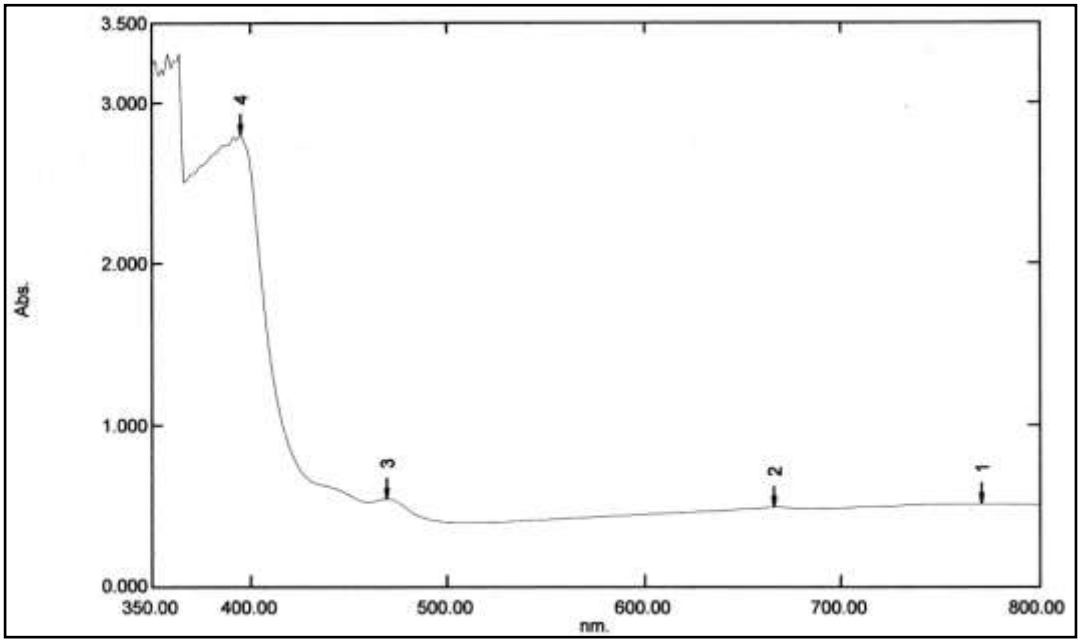

Figure 4.55: Absorption spectral for methanolic leaf extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 2 indicated chlorophyll *a* at 666 nm (0.485) and Peak 3 indicated chlorophyll *b* at 469 nm (0.539).

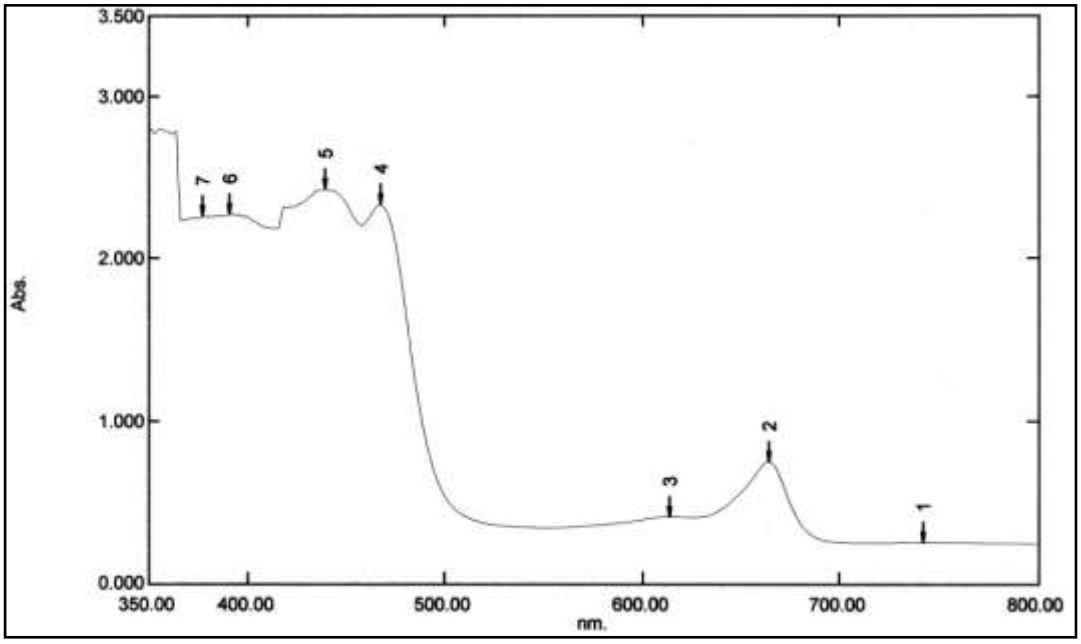

Figure 4.56: Absorption spectral for methanolic leaf extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 2 indicated chlorophyll *a* at 664 nm (0.747) and Peak 4 indicated chlorophyll *b* at 467 nm (2.334).

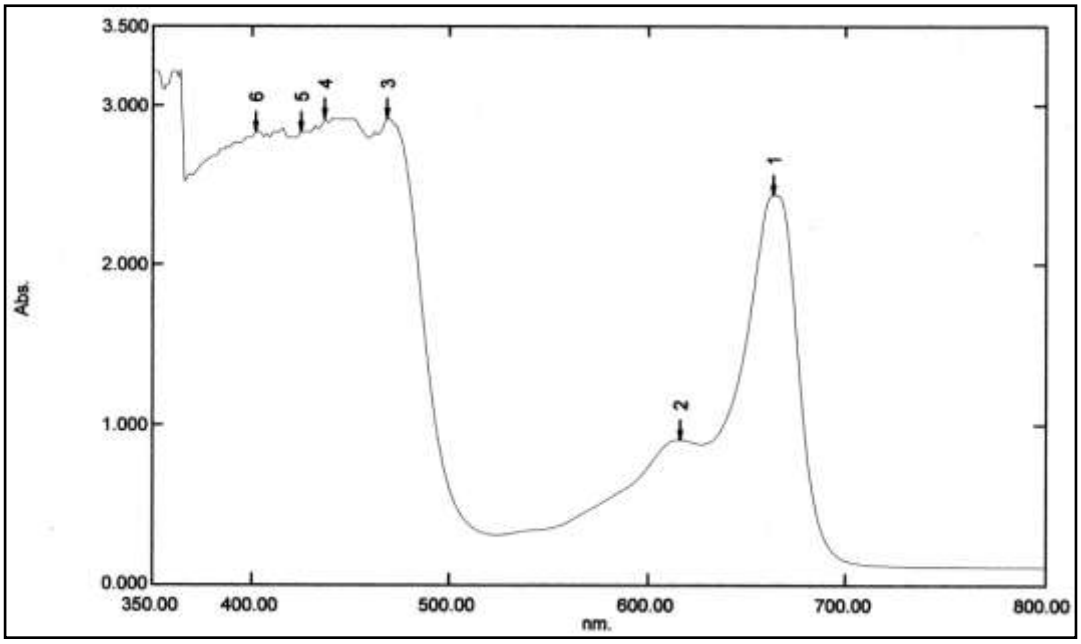

Figure 4.57: Absorption spectral for methanolic leaf extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 2 and 4 indicated chlorophyll *a* at 663 nm (2.443) and 437 (2.914). Peak 3 indicated chlorophyll *b* at 468 nm (2.914).

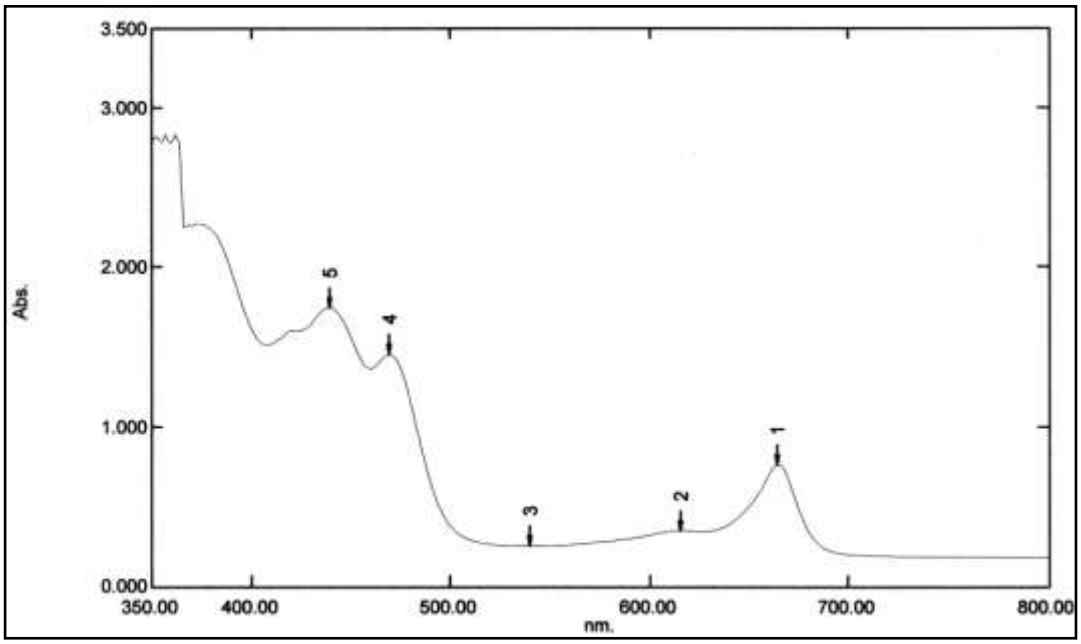

Figure 4.58: Absorption spectral for methanolic leaf extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 1 and 5 indicated chlorophyll *a* at 664 nm (0.761) and 439 (1.747). Peak 4 indicated chlorophyll *b* at 469 nm (1.451).

For the extracting solvent of 0.01% HCl in 70% acetone, a volume at 25.0 ml was able to extract the pigments of chlorophyll *a* (Absorbance of 0.656 at 664 nm and Absorbance of 1.782 at 438 nm) and chlorophyll *b* (Absorbance 1.438 at 470 nm) which showed the most intense absorption among the other volumes (Figure 4.62). Besides, only chlorophyll *a* was extracted out from the leaves at the volumes of 5.0,12.5 and 20.0 ml of acetone at 672 nm (0.877) (Figure 4.59), 666 nm (0.137) (Figure 4.60) and 665 (0.263) & 441 (1.294) (Figure 4.61), respectively.

Among the different volumes of 95% ethanol tested, chlorophyll *a* (Absorbance of 2.312 at 664 nm and Absorbance of 2.817 at 440 nm) and chlorophyll *b* (Absorbance of 2.659 at 468 nm) were noticed to have most intense absorption at the volume of 20.0 ml (Figure 4.65). At 5.0 ml of ethanol, chlorophyll *a* was not extracted out from the leaves but chlorophyll *b* (Absorbance 3.068 at 462 nm and Absorbance of 2.613 at 655 nm) showed to have the maximum absorption compared with other volumes of ethanol (Figure 4.63). Figure 4.64 and 4.66 were the absorption spectral for the pigments extracted at 12.5 and 25.0 ml of ethanol, respectively, with 12.5 ml gave the lowest absorption for chlorophyll *a* (Absorbance of 0.411 at 672 nm and Absorbance 0.878 at 440 nm) and chlorophyll *b* (Absorbance 0.762 at 469 nm). Among the three extracting solvents used, 0.15% HCl in 99.9% methanol (20.0 ml) gave the better absorption characteristic for pigment extraction from the leaves of *A. cathartica* L.

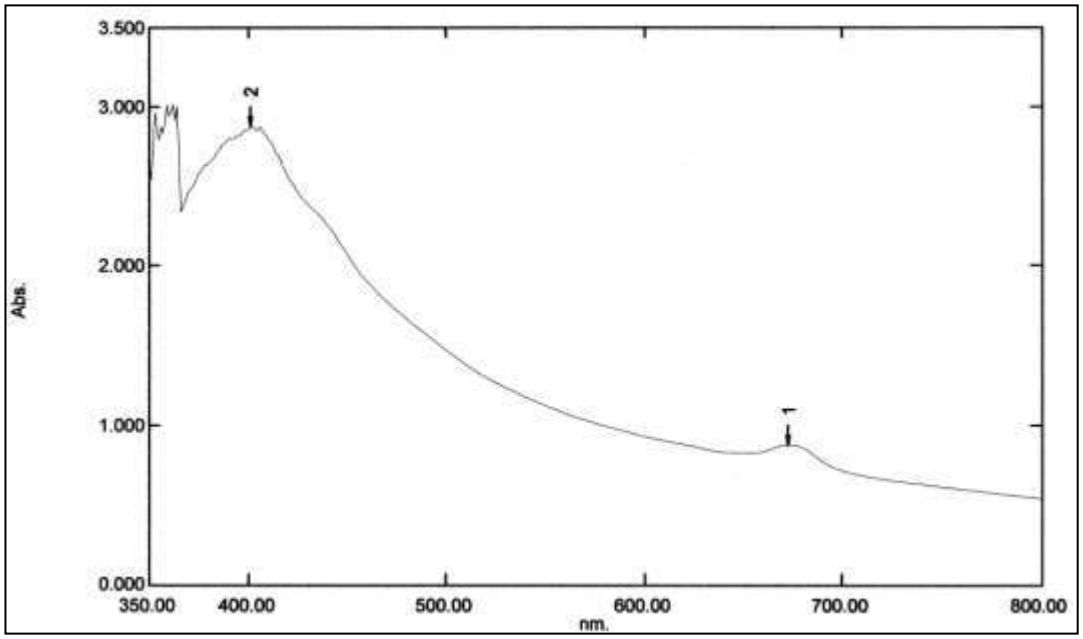

Figure 4.59: Absorption spectral for acetone leaf extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 1 indicated chlorophyll *a* at 672 nm (0.877).

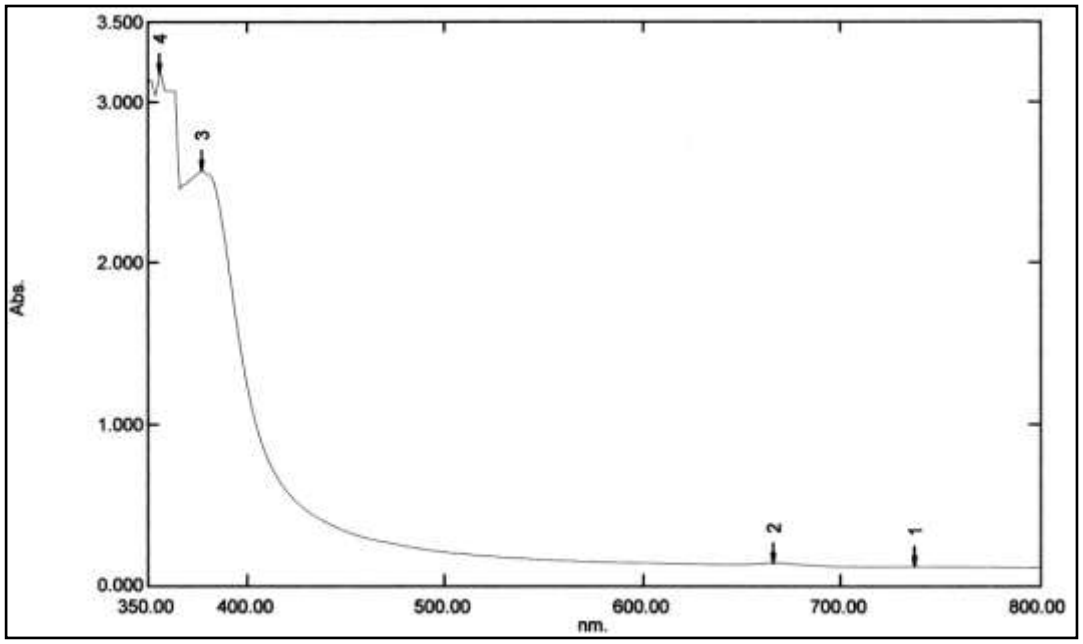

Figure 4.60: Absorption spectral for acetone leaf extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 2 indicated chlorophyll *a* at 666 nm (0.137).

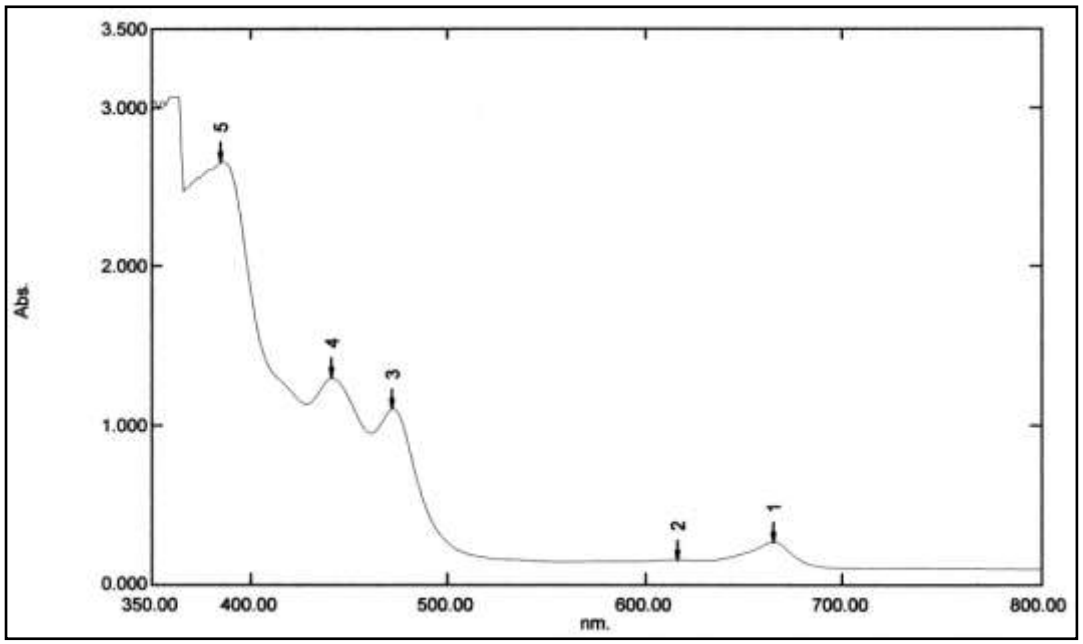

Figure 4.61: Absorption spectral for acetone leaf extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 1 and 4 indicated chlorophyll *a* at 665 nm (0.263) and 441 nm (1.294).

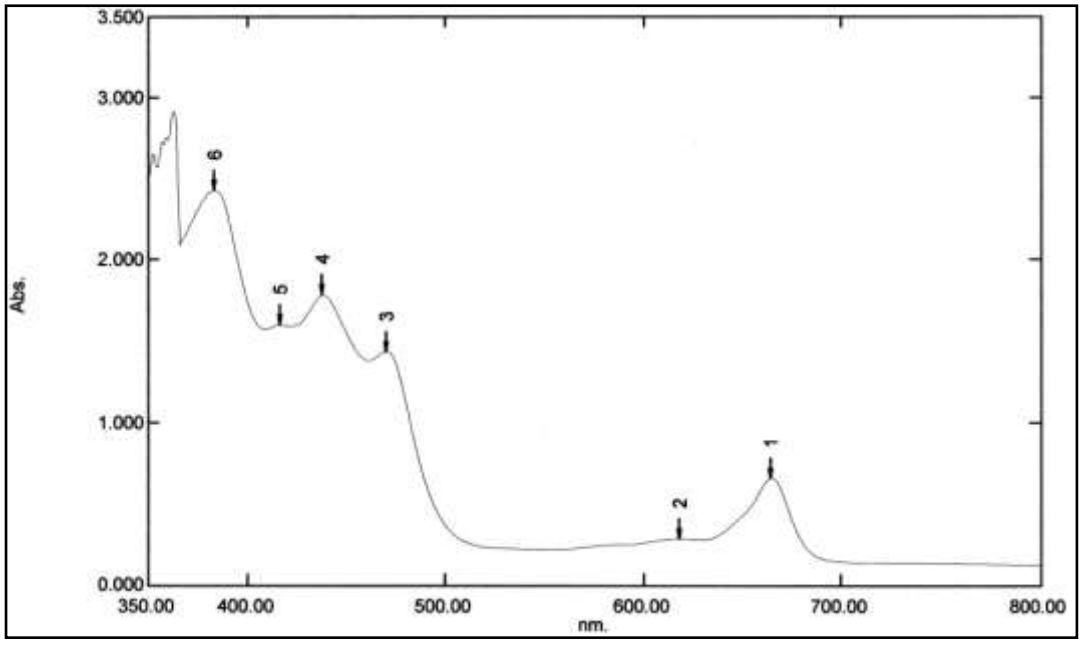

Figure 4.62: Absorption spectral for acetone leaf extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 1 and 4 indicated chlorophyll *a* at 664 nm (0.656) and 438 (1.782). Peak 3 indicated chlorophyll *b* at 470 nm (1.438).

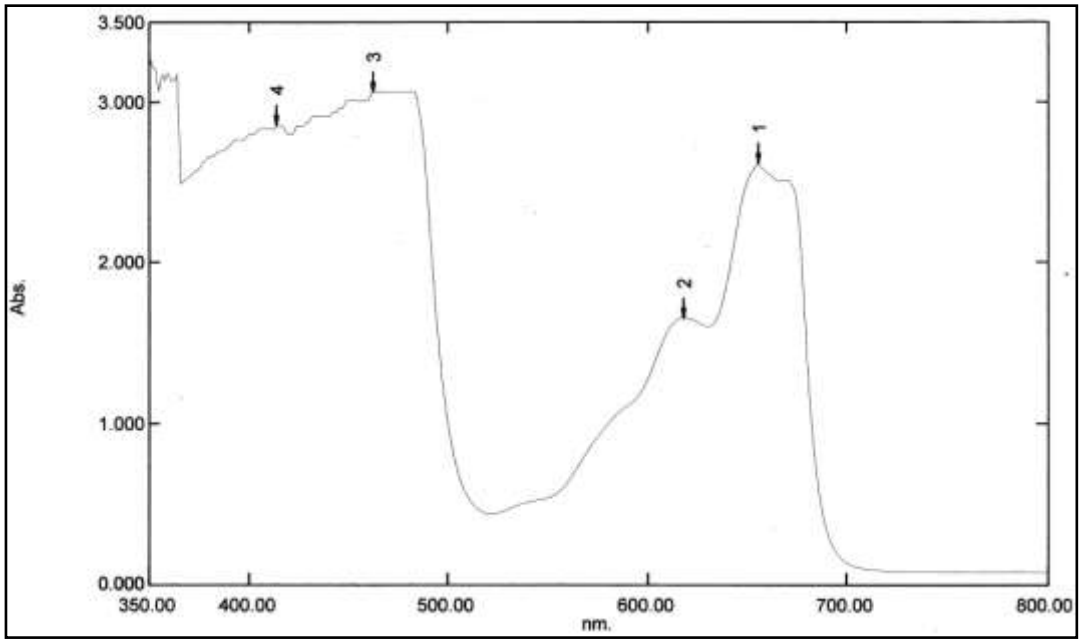

Figure 4.63: Absorption spectral for ethanolic leaf extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 1 and 3 indicated chlorophyll *b* at 655 nm (2.613) and 462 nm (3.675).

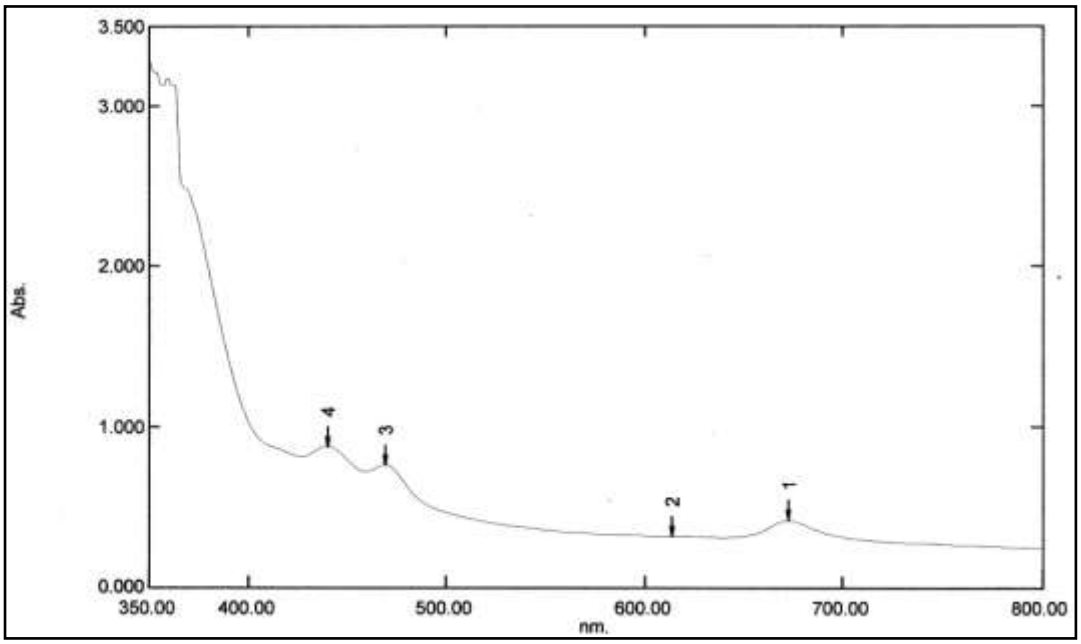

Figure 4.64: Absorption spectral for ethanolic leaf extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 1 and 4 indicated chlorophyll *a* at 672 nm (0.411) and 440 (0.878). Peak 3 indicated chlorophyll *b* at 469 nm (0.762).

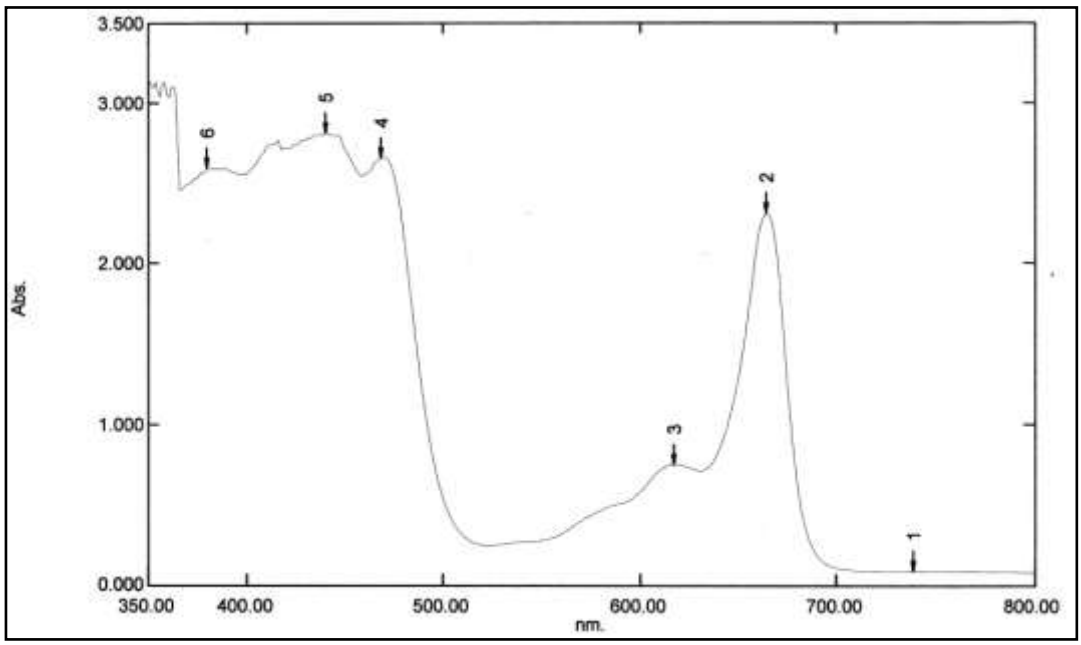

Figure 4.65: Absorption spectral for ethanolic leaf extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 2 and 5 indicated chlorophyll *a* at 664 nm (2.312) and 440 (2.817). Peak 4 indicated chlorophyll *b* at 468 nm (2.659).

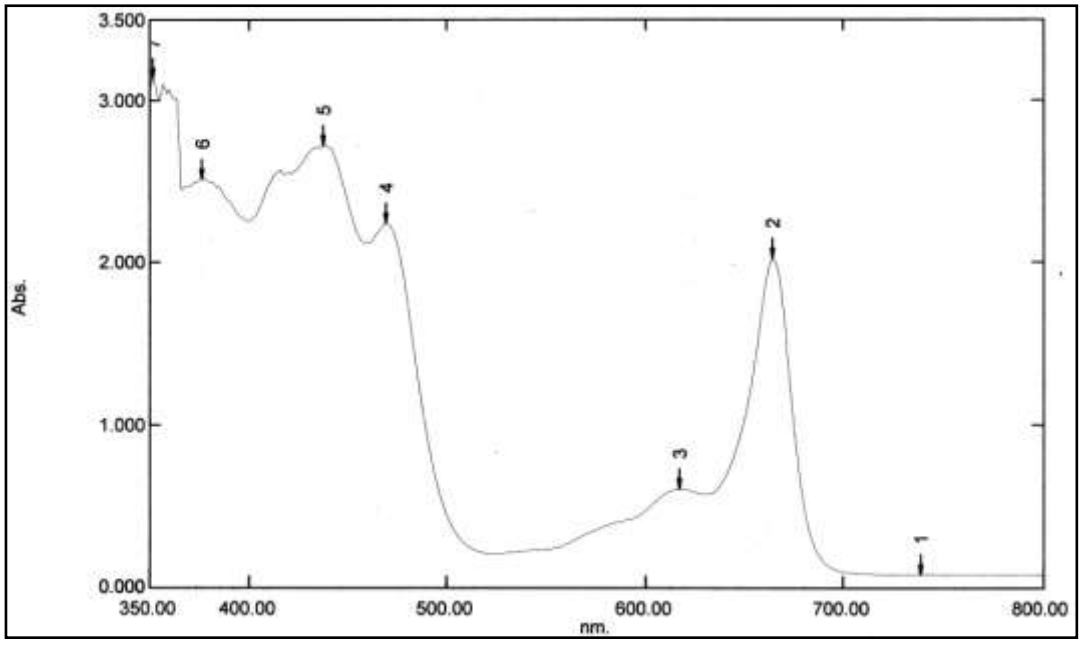

Figure 4.66: Absorption spectral for ethanolic leaf extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 2 and 5 indicated chlorophyll *a* at 664 nm (2.022) and 438 (2.723). Peak 4 indicated chlorophyll *b* at 469 nm (2.241).

4.3.1.2 Pigment Extraction from the Stems

Chlorophyll *a* and chlorophyll *b* were also extracted from the stems of *A. cathartica* L. using three different extracting solvents, i.e. 0.15% HCl in 99.9% methanol, 0.01% HCl in 70% acetone and 95% ethanol at the volumes of 5.0, 12.5, 20.0 and 25.0 ml. For the extracting solvent of 0.15% HCl + 99.9% methanol, the pigments of chlorophyll *a* (Absorbance of 0.743 at 664 nm and Absorbance of 1.221 at 437 nm) and chlorophyll *b* (Absorbance of 0.898 at 470 nm) (Figure 4.69) showed the maximum absorption in 20.0 ml methanol extract of stems. For 5.0 and 25.0 ml of methanol solvents, only chlorophyll *a* was available in the stem extract with the absorbance of 0.206 (Figure 4.67) and 0.224 (Figure 4.70) at 668 nm, respectively. Meanwhile, methanol solvent at 12.5 ml gave the average response for the extraction of chlorophyll *a* (Absorbance of 0.390 at 665 nm [138] Absorbance of 0.671 at 435 nm) and chlorophyll *b* (Absorbance of 0.613 at 467 nm) (Figure 4.68).

As for the extracting solvent of 0.01% HCl + 70% acetone, only the pigment of chlorophyll *a* was extracted from the stems at 5.0, 12.5 and 25.0 ml, i.e. at 665 (Absorbance = 0.134) (Figure 4.71), 669 (Absorbance = 0.354) (Figure 4.72) and 666 (Absorbance = 0.274) and 436 (Absorbance = 0.705) (Figure 4.74), respectively. However, acetone solvent at 20.0 ml was able to extract both chlorophyll *a* and chlorophyll *b* from the stems at the wavelengths of 666 nm (Absorbance = 0.274) and 467 nm (Absorbance = 0.403) (Figure 4.73), respectively.

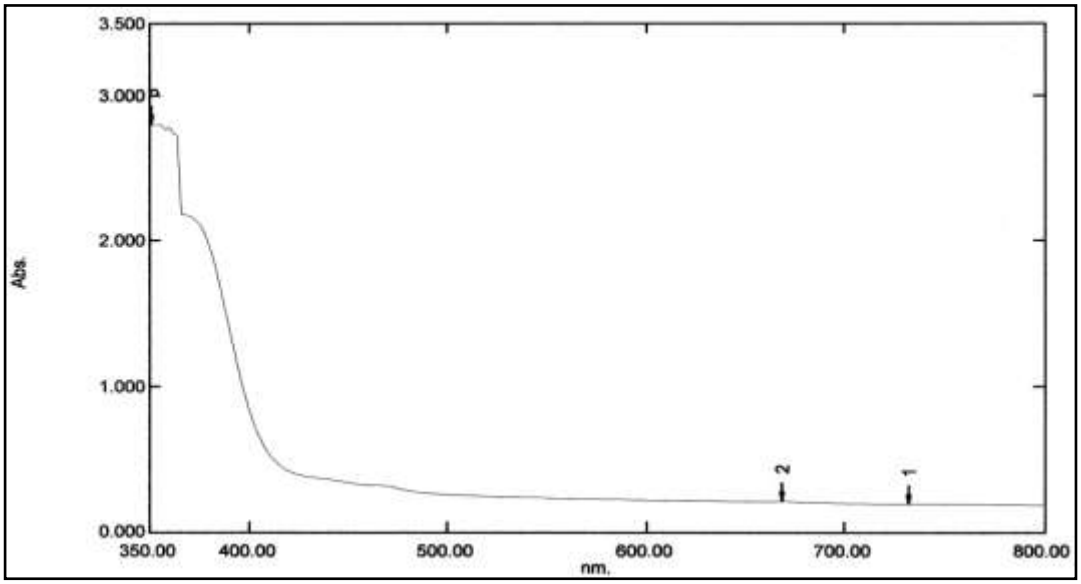

Figure 4.67: Absorption spectral for methanolic stem extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 2 indicated chlorophyll *a* at 668 nm (0.206).

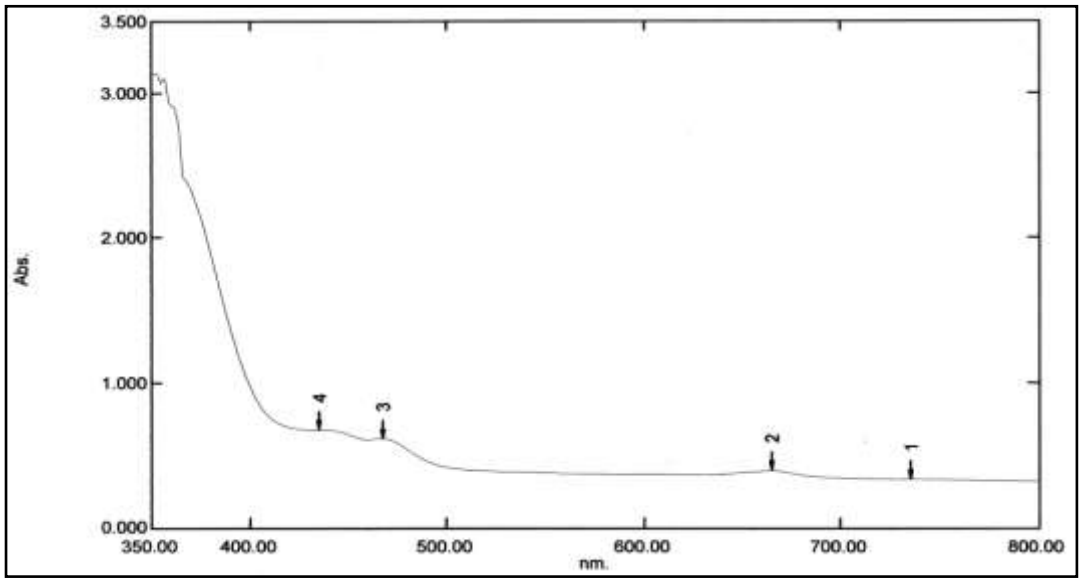

Figure 4.68: Absorption spectral for methanolic stem extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 2 and 4 indicated chlorophyll *a* at 665 nm (0.390) and 435 (0.671). Peak 3 indicated chlorophyll *b* at 467 nm (0.613).

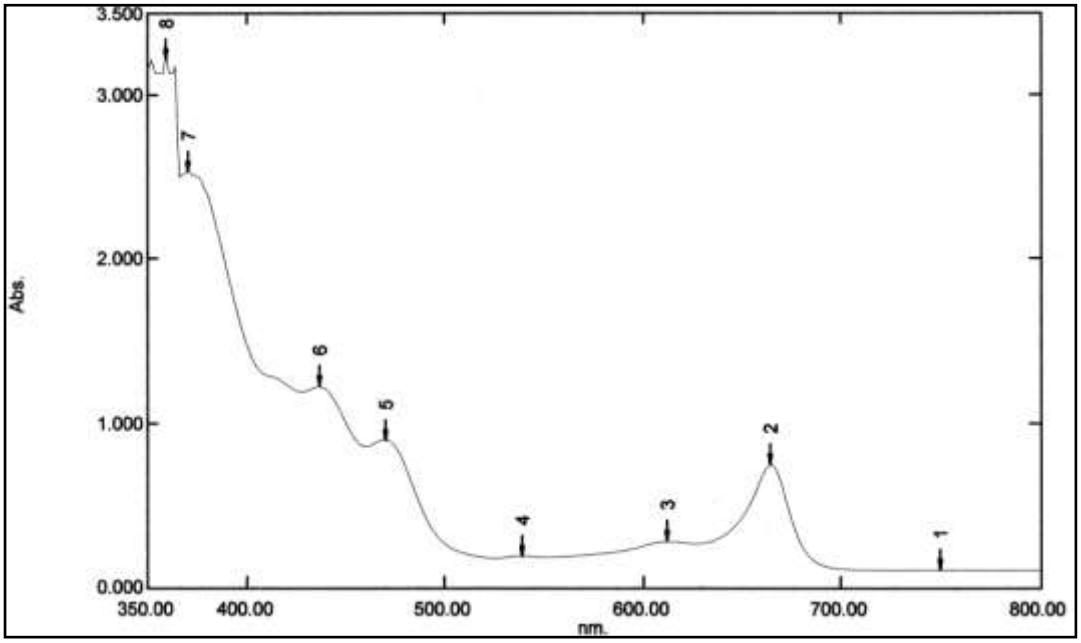

Figure 4.69: Absorption spectral for methanolic stem extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 2 and 6 indicated chlorophyll *a* at 664 nm (0.743) and 437 (1.221). Peak 5 indicated chlorophyll *b* at 470 nm (0.898).

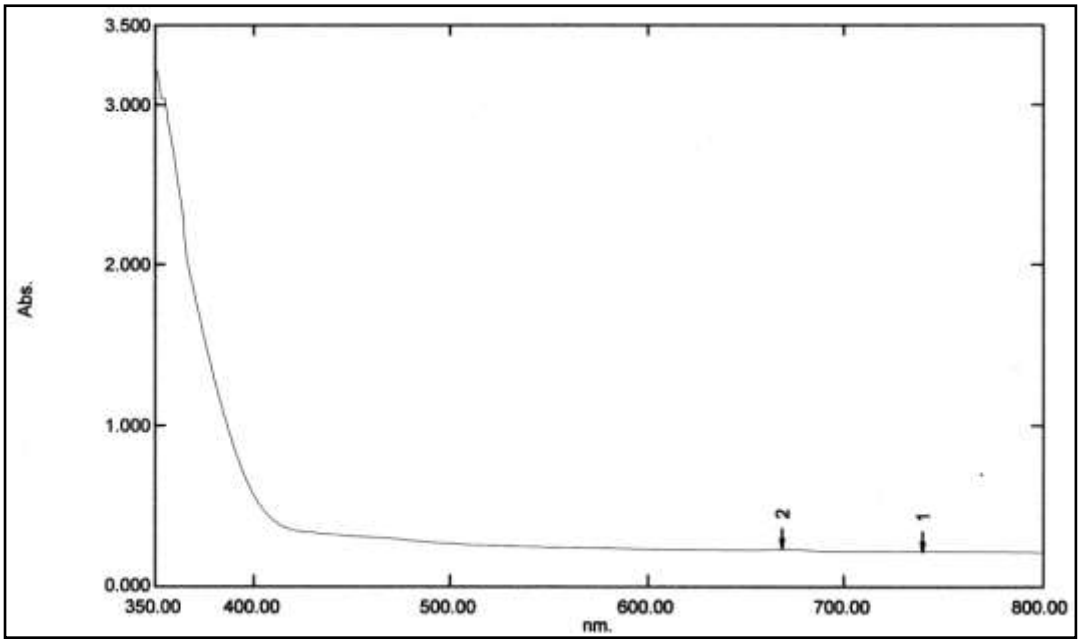

Figure 4.70: Absorption spectral for methanolic stem extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 2 indicated chlorophyll *a* at 668 nm (0.224).

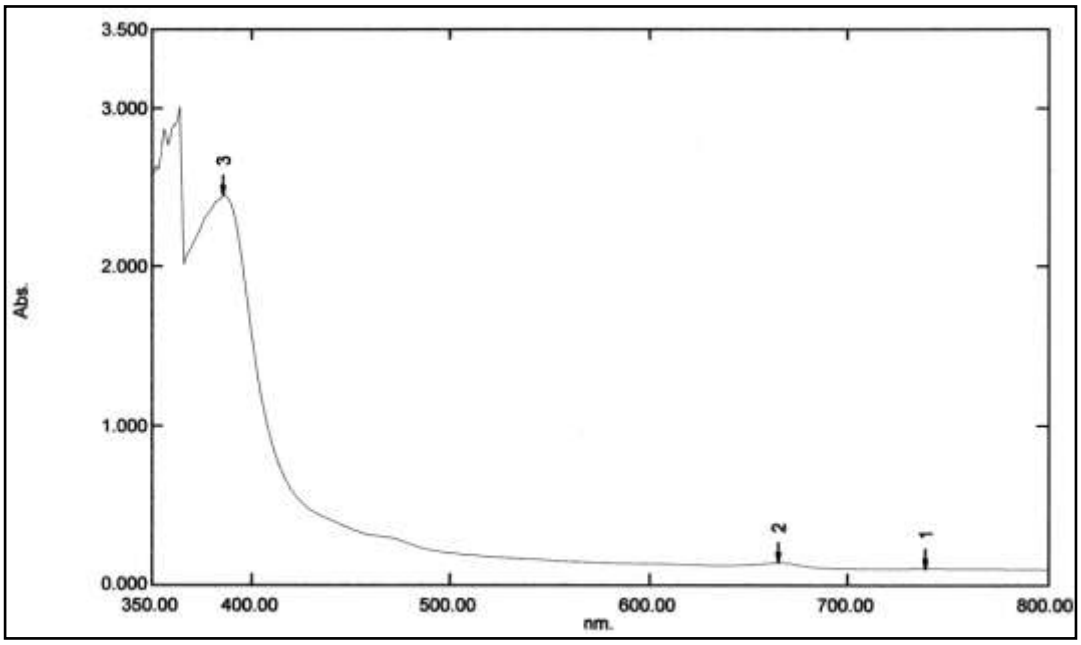

Figure 4.71: Absorption spectral for acetone stem extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 2 indicated chlorophyll *a* at 665 nm (0.134).

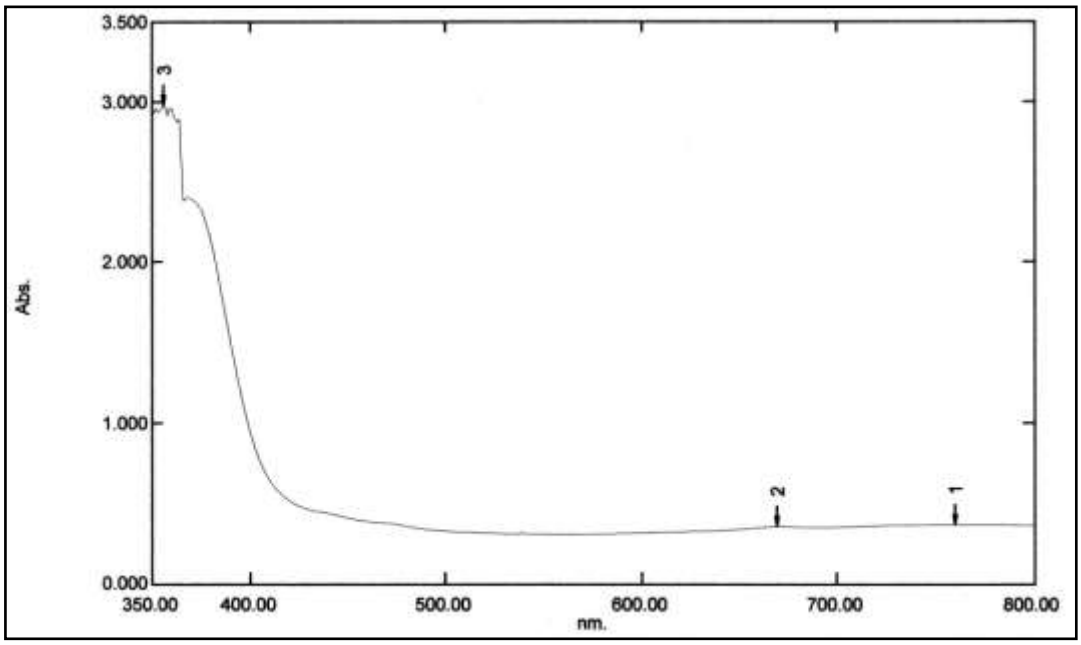

Figure 4.72: Absorption spectral for acetone stem extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 2 indicated chlorophyll *a* at 669 nm (0.354).

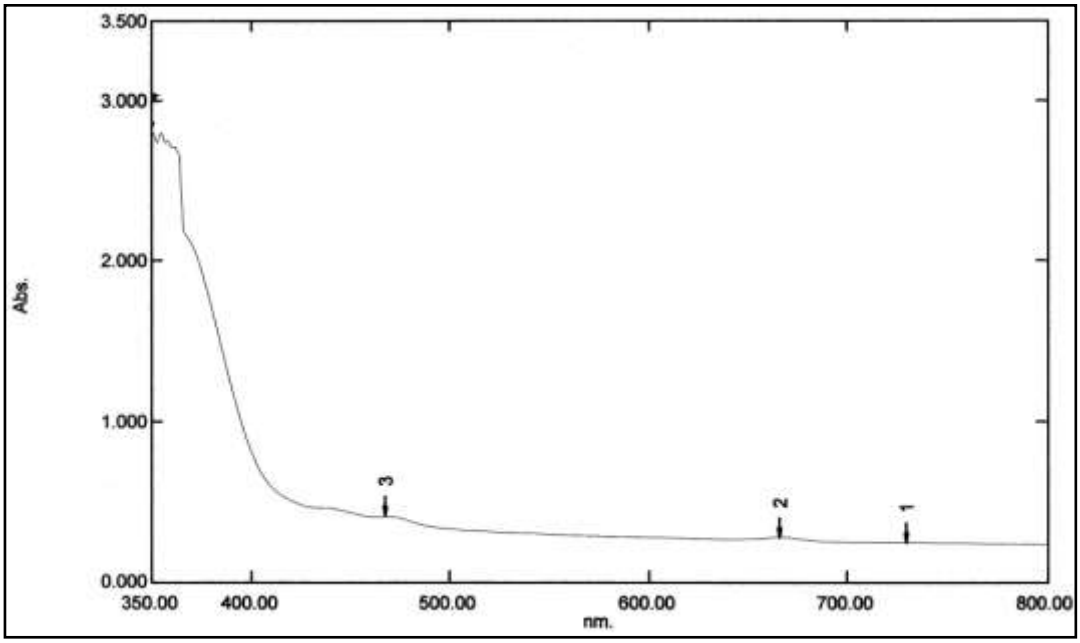

Figure 4.73: Absorption spectral for acetone stem extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 2 indicated chlorophyll *a* at 666 nm (0.274). Peak 3 indicated chlorophyll *b* at 467 (0.403).

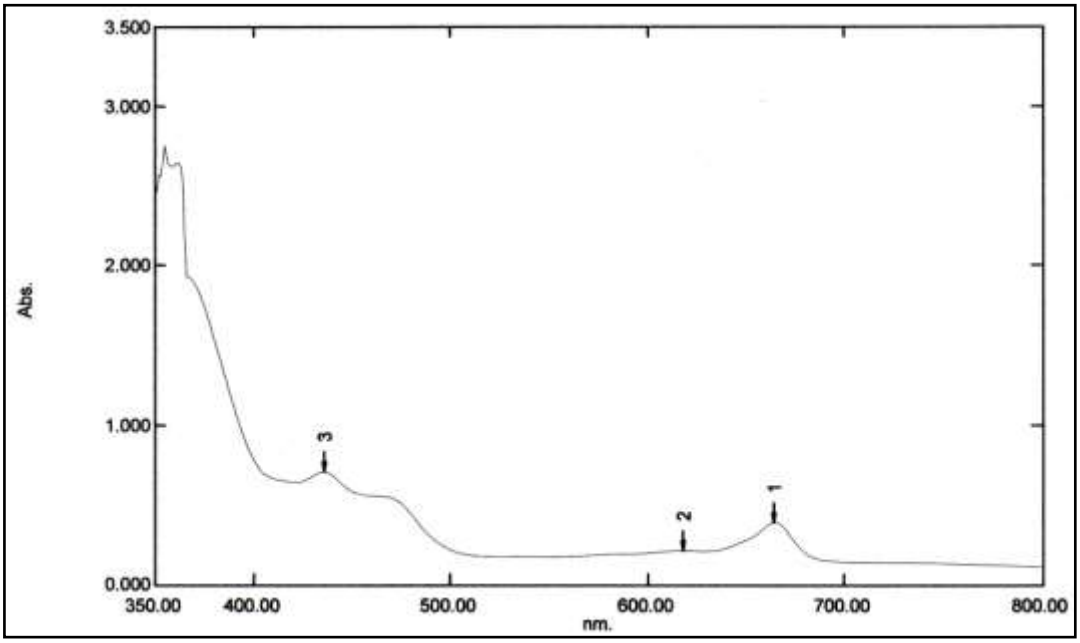

Figure 4.74: Absorption spectral for acetone stem extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 1 and 3 indicated chlorophyll *a* at 664 nm (0.388) and 436 nm (0.705).

For the extracting solvent of 95% ethanol at 5.0 ml, maximum absorption characteristic on the spectrum was observed at 665 nm (Absorbance = 1.719) and 433 nm (Absorbance = 2.515) (Figure 4.75) for chlorophyll *a*, but not for chlorophyll *b*. Similar with 5.0 ml ethanol, only chlorophyll *a* (Absorbance of 1.371 at 664 nm and Absorbance of 2.135 at 434 nm) (Figure 4.76) was extracted out from the stems at 12.5 ml. As compared between 20.0 and 25.0 ml of ethanol extracting solvents, more chlorophyll *a* (Absorbance of 0.864 at 664 nm and Absorbance of 1.399 at 435) and chlorophyll *b* (Absorbance of 0.943 at 467) (Figure 4.77) can be extracted out at the volume of 20.0 ml, whereas, chlorophyll *a* at 664 nm (Absorbance = 0.684) and 435 nm (Absorbance = 1.050) and chlorophyll *b* at 468 nm (Absorbance = 0.727) (Figure 4.78) were extracted out from the stems in 25.0 ml of ethanol solvent. Among the three extracting solvents used, 95% ethanol (20 ml) gave the better absorption characteristic for pigment extraction from the stems *A. cathartica* L.

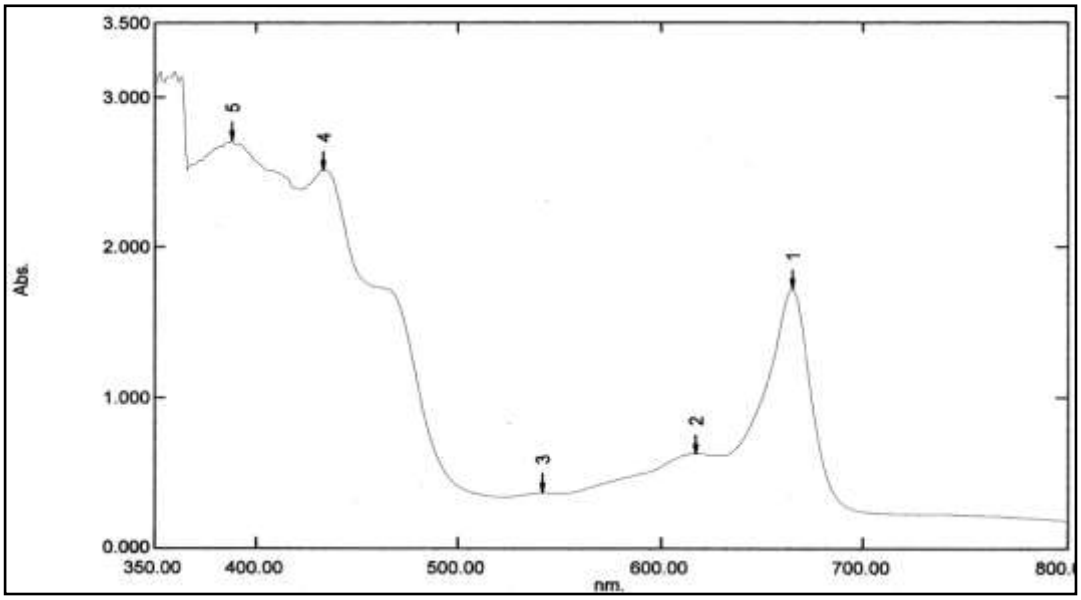

Figure 4.75: Absorption spectral for ethanolic stem extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 1 and 4 indicated chlorophyll *a* at 665 nm (1.719) and 433 nm (2.515).

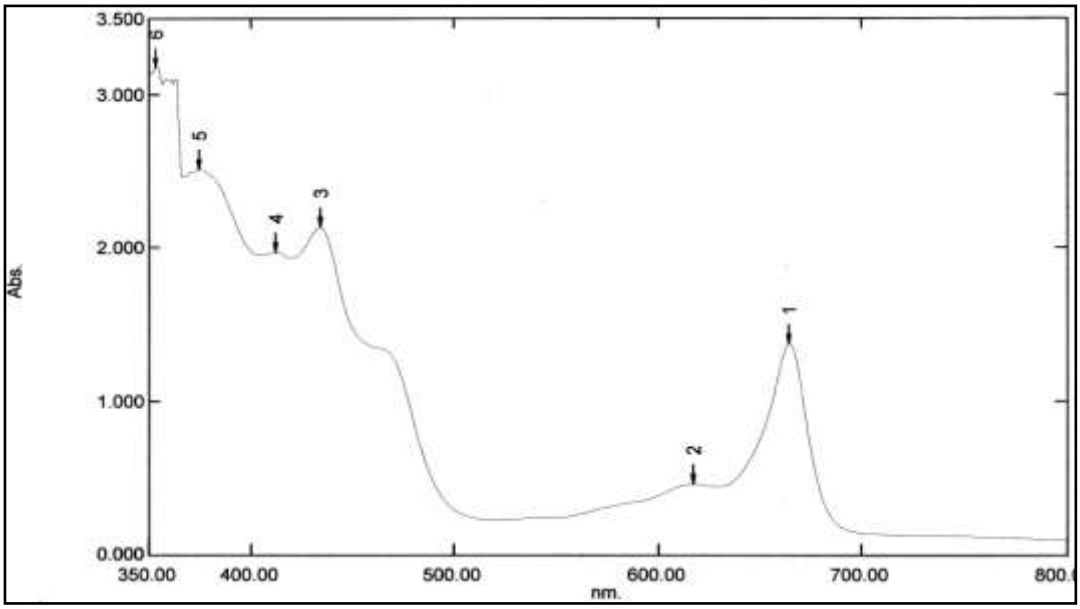

Figure 4.76: Absorption spectral for ethanolic stem extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 1 and 3 indicated chlorophyll *a* at 664 nm (1.371) and 434 nm (2.135).

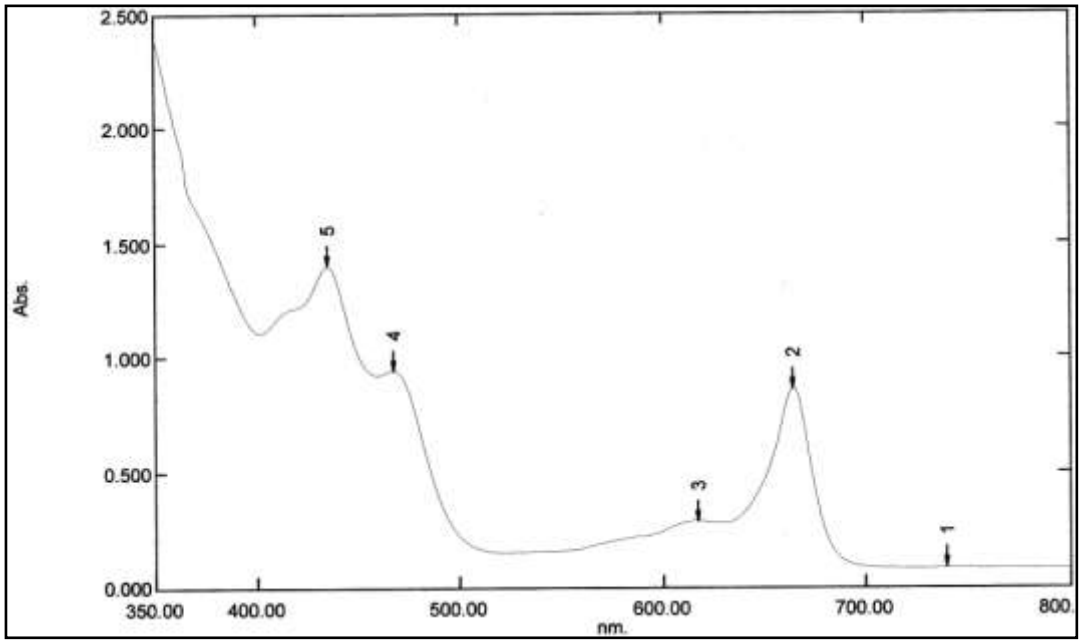

Figure 4.77: Absorption spectral for ethanolic stem extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 2 and 5 indicated chlorophyll *a* at 664 nm (0.864) and 435 (1.399). Peak 4 indicated chlorophyll *b* at 467 nm (0.943).

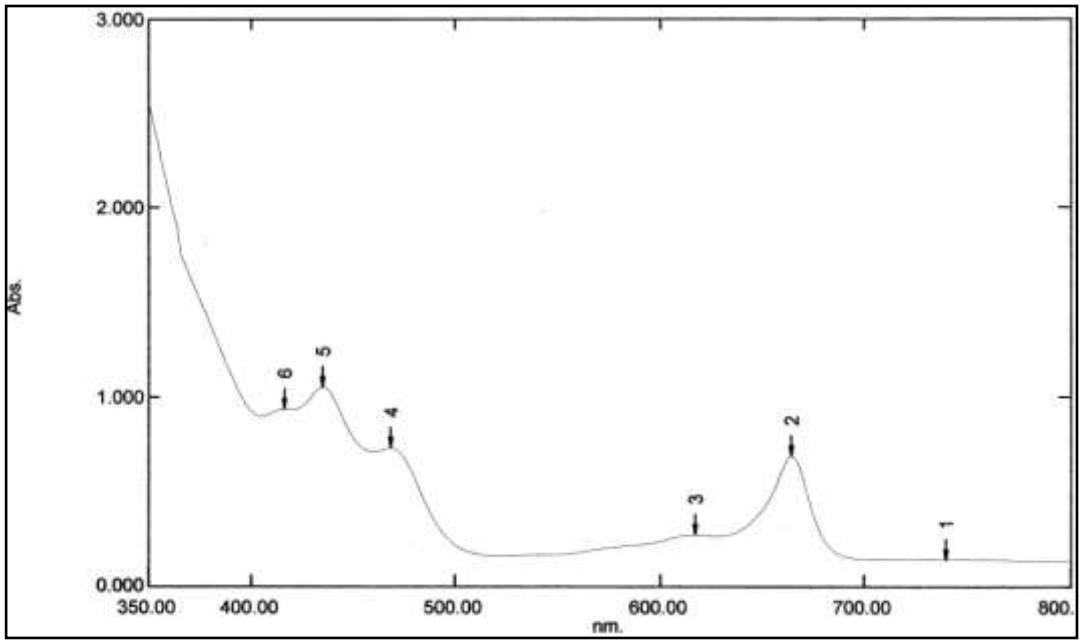

Figure 4.78: Absorption spectral for ethanolic stem extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 2 and 5 indicated chlorophyll *a* at 664 nm (0.684) and 435 (1.050). Peak 4 indicated chlorophyll *b* at 468 nm (0.727).

4.3.1.3 Pigment Extraction from the Petals

In petals, the pigment of carotenoids was more significant compared to chlorophyll *a* and chlorophyll *b*, due to the bright yellow bell-like flowers of *A. cathartica* L. In the extracting solvent of 0.15% HCl + 99.9% methanol, the carotenoid pigment (Absorbance of 1.138 at 438 nm and Asorbance of 0.980 at 466 nm) (Figure 4.81) showed to have the maximum absorption in 20.0 ml methanol extract of petals, followed by methanol extracting solvent at the volumes of 25.0, 12.5 and 5.0 ml with the carotenoids' absorption peaks at 438 nm (Absorbance = 0.975) and 466 nm (Absorbance = 0.810) (Figure 4.82), 436 nm (Absorbance = 0.665) and 467 nm (Absorbance = 0.550) (Figure 4.80), 469 nm (Absorbance = 0.539) (Figure 4.79), respectively.

Among all the volumes of 0.01% HCl in 70% acetone, the volume at 12.5 ml was the best extracting solvent for the carotenoid pigment (Absorbance of 1.603 at 449 nm and Absorbance of 1.486 at 480 nm) (Figure 4.84) from petals of *A. cathartica* L. The extracting capacity of acetone at 5.0 ml (Absorbance of 0.543 at 470 nm) (Figure 4.83) was better than that of 20.0 (Absorbance of 0.394 at 478 nm) (Figure 4.85) and 25.0 ml (Absorbance of 0.441 at 471 nm) (Figure 4.86) of acetone for the extraction of carotenoid pigment from the petal.

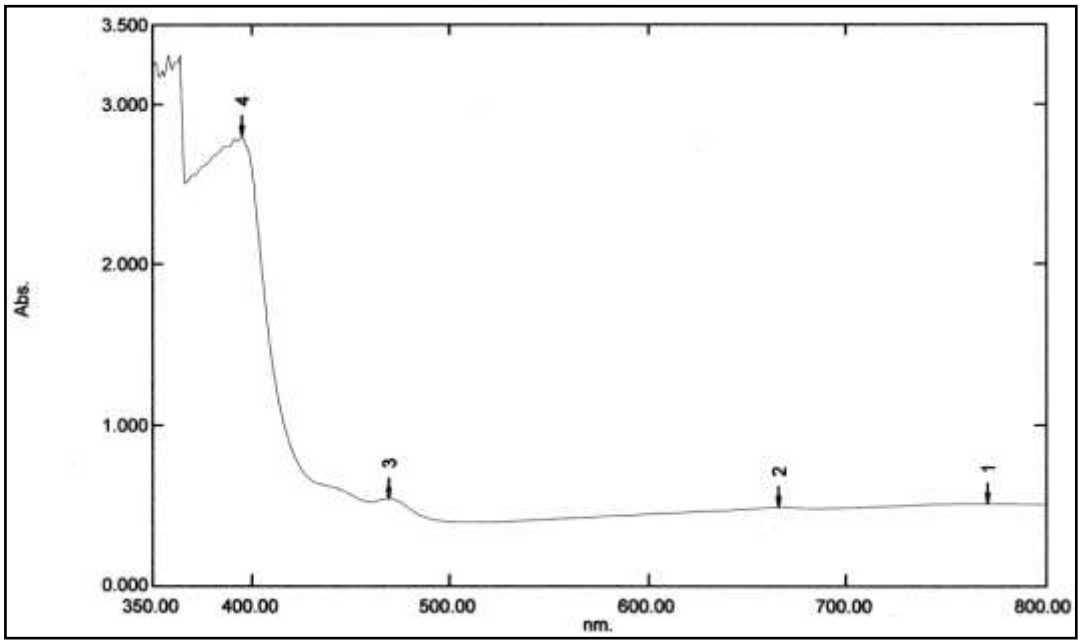

Figure 4.79: Absorption spectral for methanolic petal extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 3 indicated carotenoid at 469 nm (0.539).

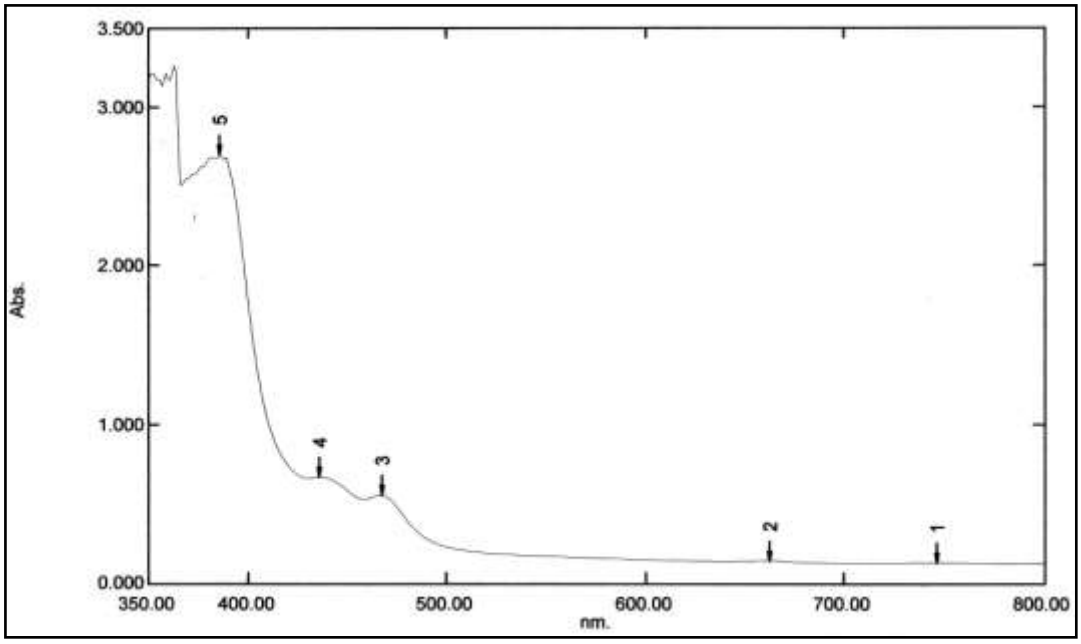

Figure 4.80: Absorption spectral for methanolic petal extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 3 and 4 indicated carotenoid at 467 nm (0.550) and 436 nm (0.665).

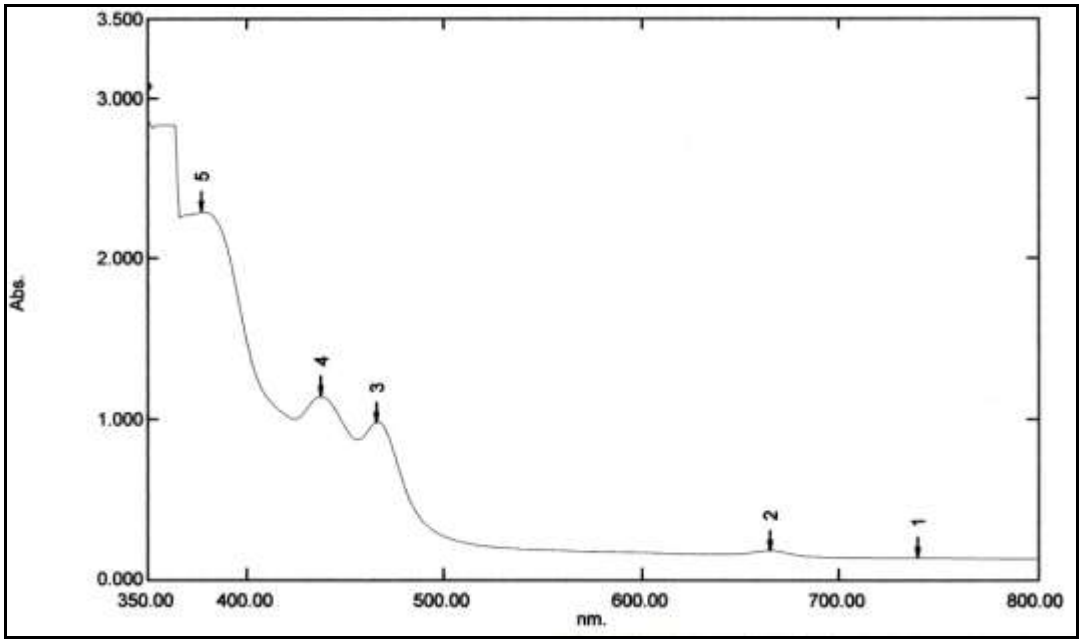

Figure 4.81: Absorption spectral for methanolic petal extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 3 and 4 indicated carotenoid at 466 nm (0.980) and 438 nm (1.138).

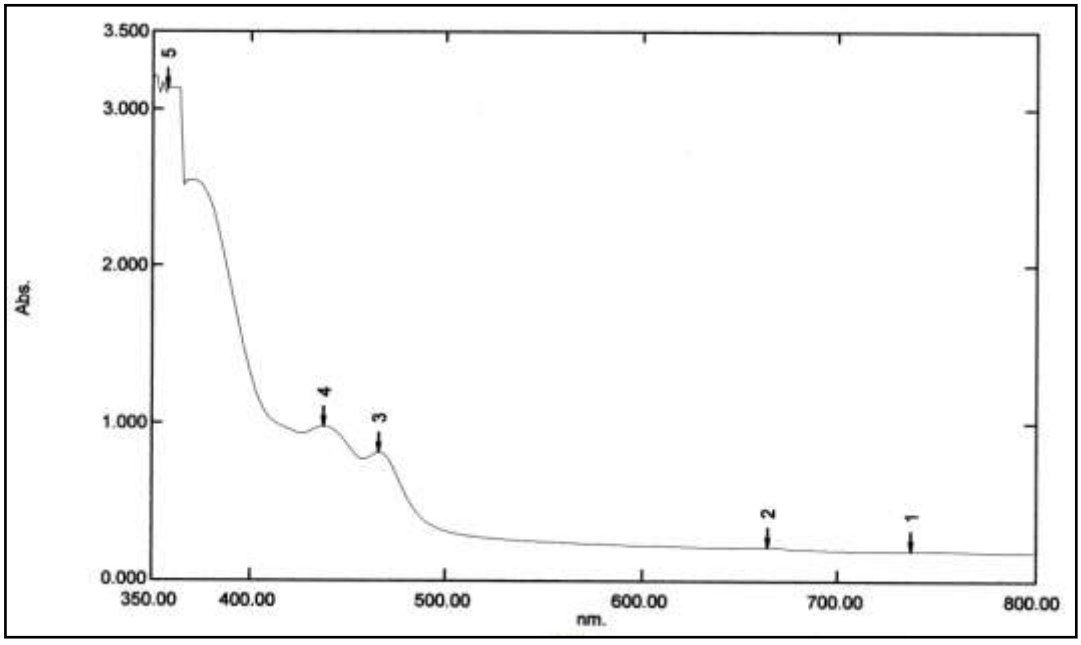

Figure 4.82: Absorption spectral for methanolic petal extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 3 and 4 indicated carotenoid at 466 nm (0.810) and 438 nm (0.975).

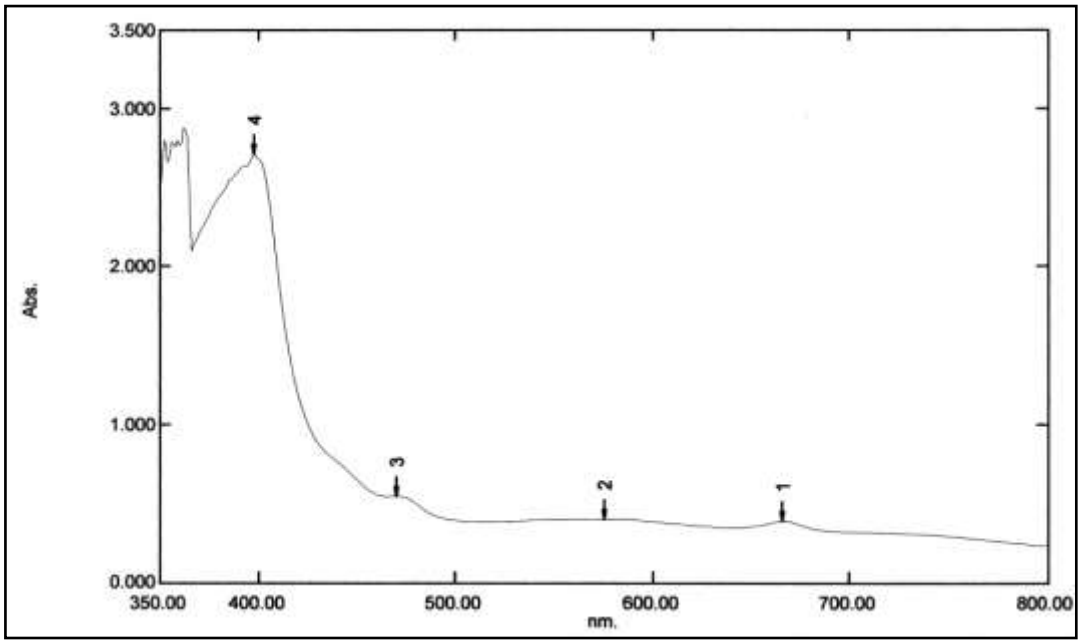

Figure 4.83: Absorption spectral for acetone petal extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 3 indicated carotenoid at 470 nm (0.543).

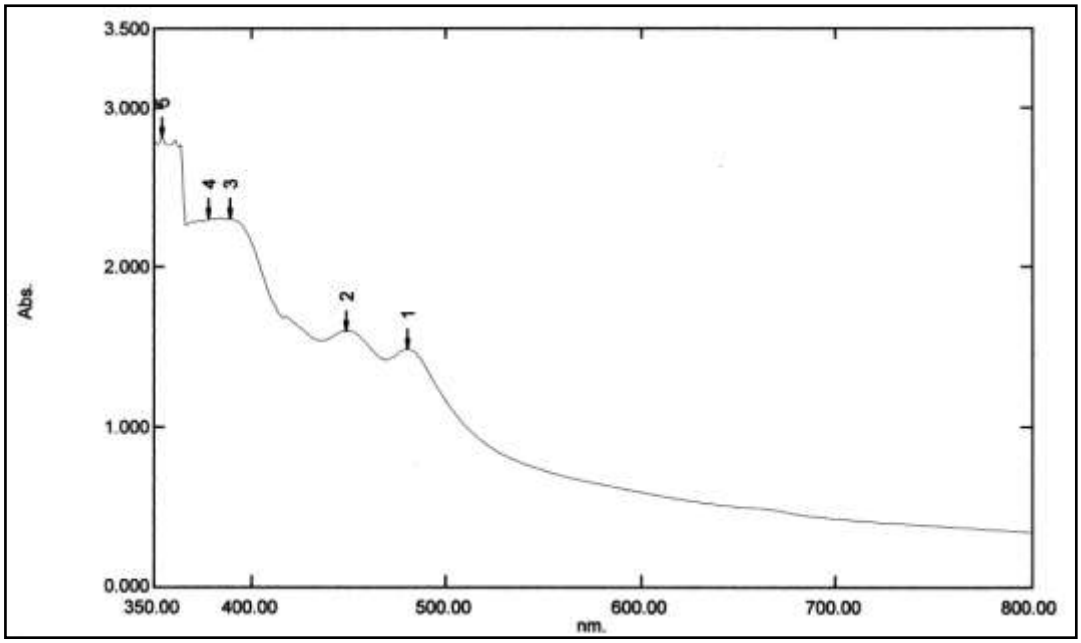

Figure 4.84: Absorption spectral for acetone petal extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 1 and 2 indicated carotenoid at 480 nm (1.486) and 449 nm (1.603).

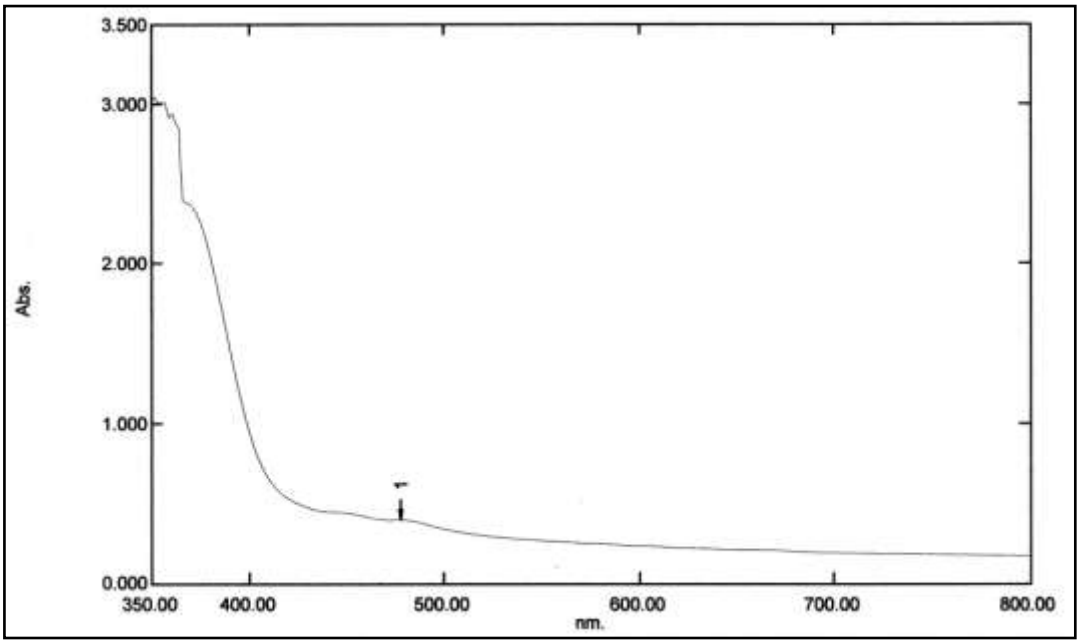

Figure 4.85: Absorption spectral for acetone petal extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 1 indicated carotenoid at 478 nm (0.394).

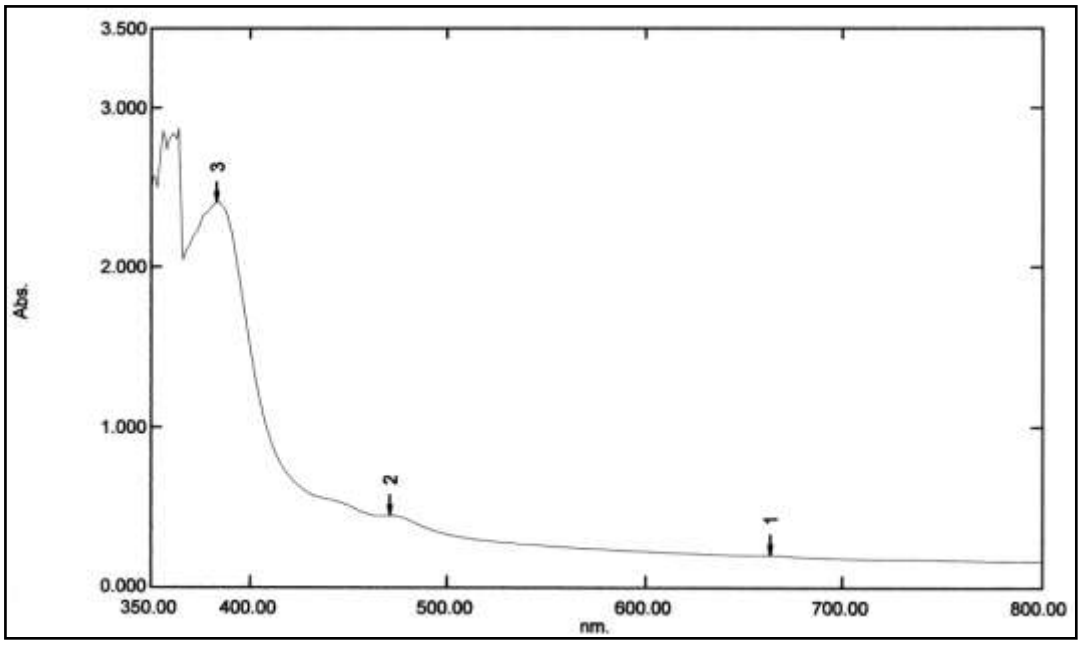

Figure 4.86: Absorption spectral for acetone petal extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 2 indicated carotenoid at 471 nm (0.441).

For the extracting solvent of 95% ethanol, the carotenoid pigment (Absorbance of 1.940 at 439 nm and Absorbance of 1.679 at 469 nm) (Figure 4.87) showed a maximum absorption in 5.0 ml ethanol extract of petals, followed by 20.0 and 12.5 ml of ethanol solvents, whereby the carotenoid pigment showed an absorption peaks of 1.649 and 1.454 at 440 nm and 1.458 and 1.247 at 469 nm (Figure 4.88 and 4.89) for both 20.0 and 12.5 ml of ethanol, respectively. In addition, ethanol extracting solvent at the volume of 25.0 ml (Absorbance of 1.198 at 440 nm and Absorbance of 1.056 at 469 nm) (Figure 4.90) showed the shortest absorption peak for carotenoid pigment among all volumes of ethanol. Among the three extracting solvents used, 95% ethanol (5 ml) gave the better absorption characteristic for pigment extraction from the petals *A. cathartica* L.

4.3.1.4 Pigment Extraction from the Callus

Callus are dedifferentiated cells produced as a result of wounding or of culturing tissues in the presence of PGRs. As compared with the extracts of leaves, stems and petals, none or less chlorophyll *a*, chlorophyll *b* and carotenoid pigments were observed in the callus extract. For the extracting solvent of 0.15% HCl + 99.9% methanol, only 12.5 ml of methanol showed to have the absorption peak for chlorophyll *a* at 663 nm with the absorbance value of 0.018 (Figure 4.91) among all methanol extracts of calli. Meanwhile, for the extracting solvent of 0.01% HCl in 70% acetone, 20.0 ml of acetone was the only extracting solvent which gave the absorption peak of chlorophyll *a* at 441 nm with the absorbance value of 0.678 (Figure 4.92). For the extracting solvent of 95% ethanol, absorption peaks were noticed at the volumes of 5.0 and 20.0 ml for chlorophyll *b* (Absorbance of 0.334 at 453 nm) (Figure 4.93) and chlorophyll *a* (Absorbance of 0.066 at

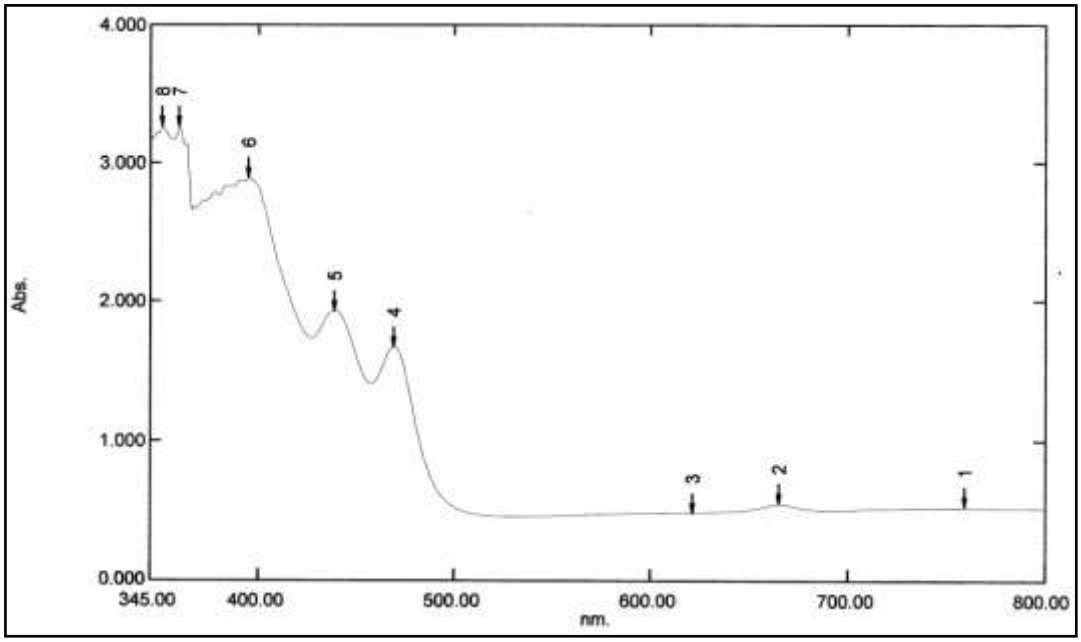

Figure 4.87: Absorption spectral for ethanolic petal extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 4 and 5 indicated carotenoid at 469 nm (1.679) and 439 nm (1.940).

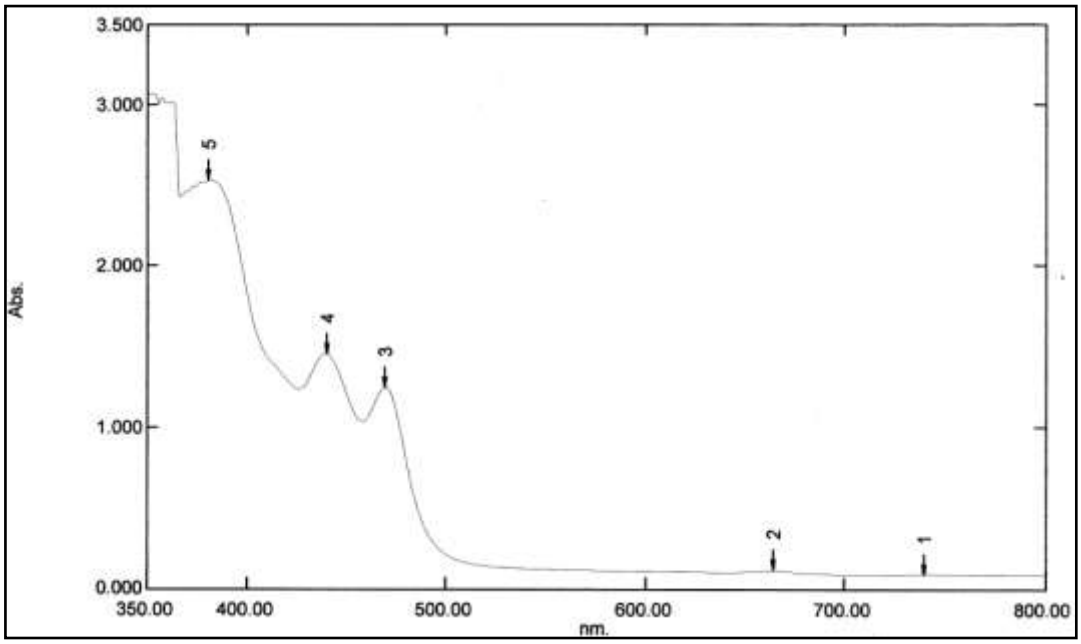

Figure 4.88: Absorption spectral for ethanolic petal extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 3 and 4 indicated carotenoid at 469 nm (1.247) and 440 nm (1.454).

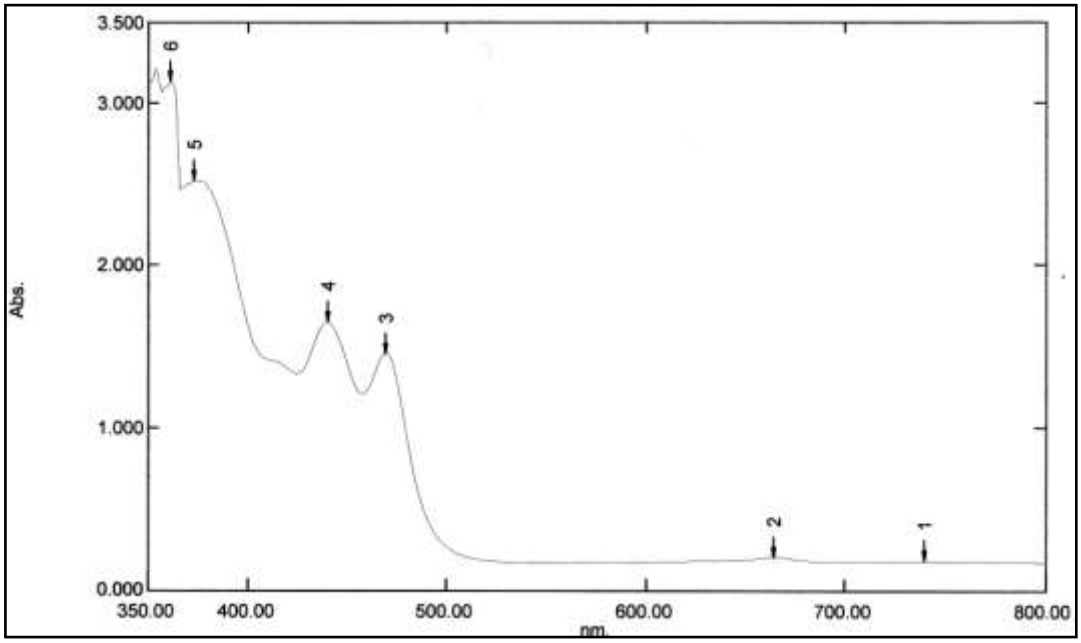

Figure 4.89: Absorption spectral for ethanolic petal extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 3 and 4 indicated carotenoid at 469 nm (1.458) and 440 nm (1.649).

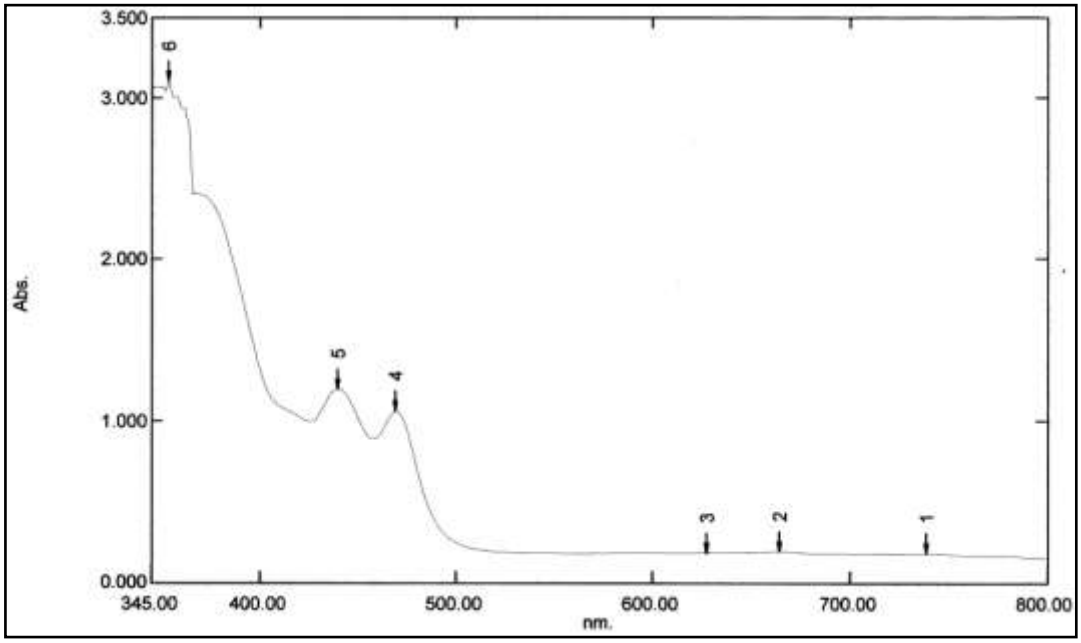

Figure 4.90: Absorption spectral for ethanolic petal extract of *Allamanda cathartica* L. at volume of 25.0 ml. Peak 4 and 5 indicated carotenoid at 469 nm (1.056) and 440 nm (1.198).

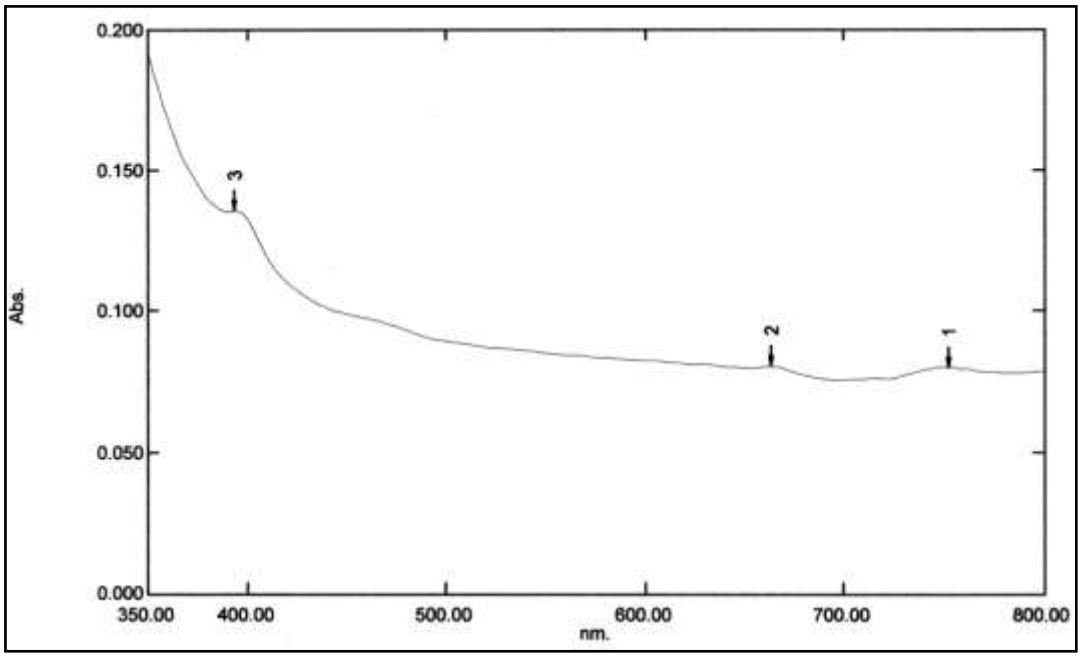

Figure 4.91: Absorption spectral for methanolic callus extract of *Allamanda cathartica* L. at volume of 12.5 ml. Peak 2 indicated chlorophyll *a* at 663 nm (0.081).

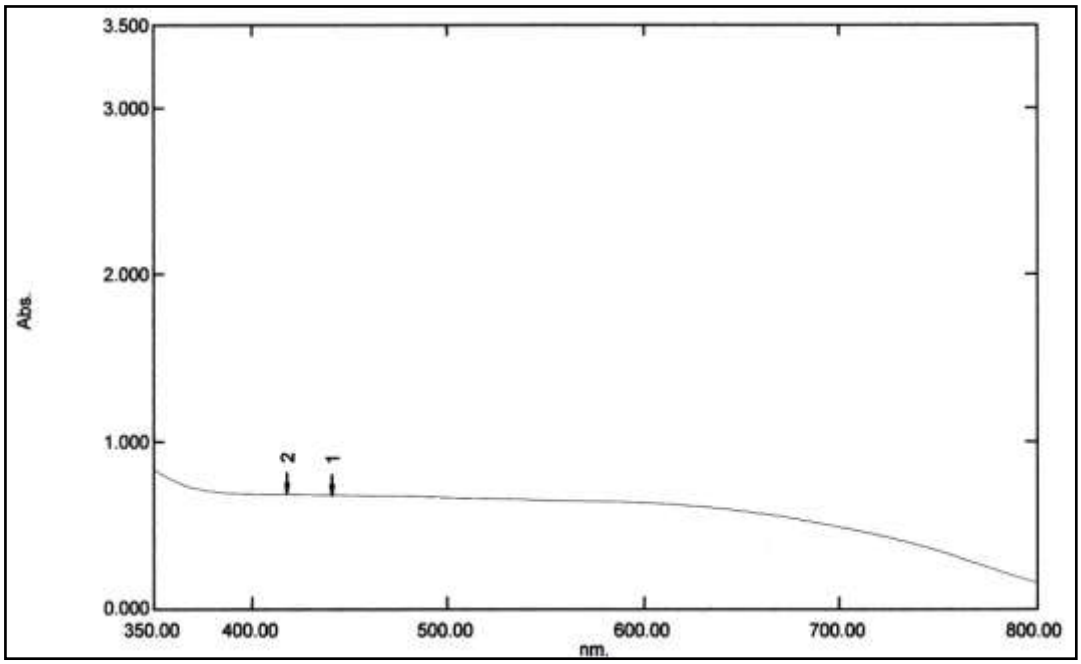

Figure 4.92: Absorption spectral for acetone callus extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 1 indicated chlorophyll *a* at 441 nm (0.678).

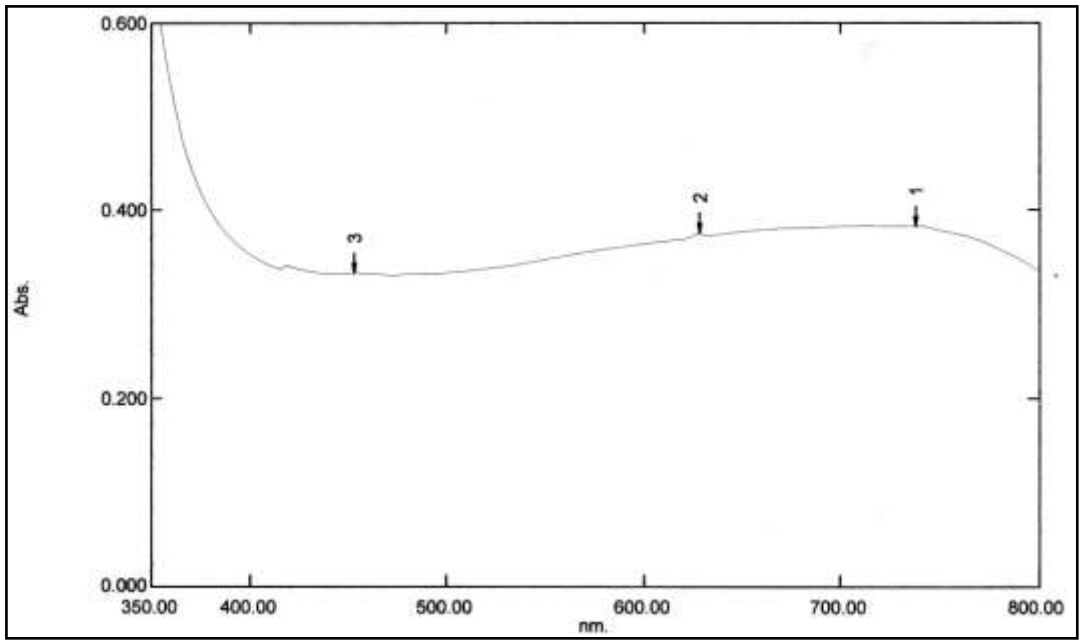

Figure 4.93: Absorption spectral for ethanolic callus extract of *Allamanda cathartica* L. at volume of 5.0 ml. Peak 2 indicated chlorophyll *b* at 453 nm (0.334).

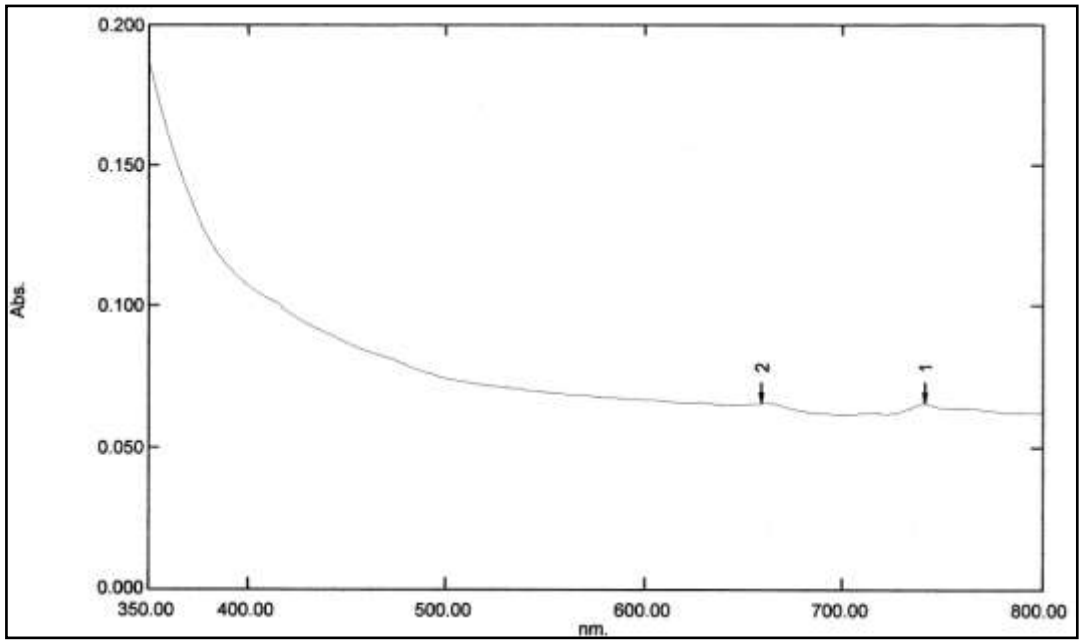

Figure 4.94: Absorption spectral for ethanolic callus extract of *Allamanda cathartica* L. at volume of 20.0 ml. Peak 2 indicated chlorophyll *a* at 659 nm (0.066).

659 nm) (Figure 4.94), respectively. Among the three extracting solvents used, 0.01% HCl in 70% acetone (20 ml) gave the better absorption characteristic for pigment extraction from the calli that developed from the explants of *A. cathartica* L.

4.3.2 Coloured Coating Development from Natural Pigments

The natural colours of pigments, such as the green colour in chlorophylls and orange yellowish colour in caretenoids can be found in the leaves and petals, respectively. Those natural colourants were found to be as potentially new alternative sources of synthetic colourants for the industries of paint and textile. In the present study, the natural colourants were extracted from the leaves and petals of *A. cathartica* L. using two different types of extracting solvents, i.e. 95% ethanol and 0.15% HCl + 99.9% methanol, which given the better colouration compared to 0.01% HCl + 70% acetone. The coloured coating development was performed on the glass slides and cotton wools, which then preceded with the salt and heat tests carried out on the glass slides with different parameter of times, whereas the colour degradation of pigments were observed from cotton wools.

4.3.2.1 Natural Colourant from the Leaves – Chlorophylls

For the study of salt and heat tests from ethanolic (95% ethanol) and methanolic (0.15% HCl in 99.9% methanol) extracts of leaves, the absorbance values were recorded at 662 and 430 nm which were the regions that absorbed strongly by the pigment of chlorophyll. Figure 4.99 showed the glass slides coated with ethanolic and methanolic leaf extracts of *Allamanda cathartica* L. (mixed with PVA in the ratio of 1:1) before salt and heat tests. According to Figure 4.95 and 4.96, the absorbance values for salt test were decreased gradually from 0 to 60 minutes (at 662 and 430 nm) when the glass slides were

coated with ethanolic extract of leaves (green in colour) and immersed in the salt solutions (NaCl) at concentrations of 0.5, 1.0 and 1.5% (Figure 4.100(A)). Similar trend was observed for methanolic extract of leaves, in which the absorbance values at 662 (Figure 4.97) and 430 (Figure 4.98) nm declined gradually from 0 to 60 minutes because the pigment slowly disappeared from the glass slide after it was immersed in NaCl solutions at concentrations of 0.5, 1.0 and 1.5% from 0 to 60 minutes (Figure 4.100(B)). For both ethanolic and methanolic extracts of leaves, there were no significant differences on the absorbance values (at 662 and 430 nm) observed for all concentrations of salt solution.

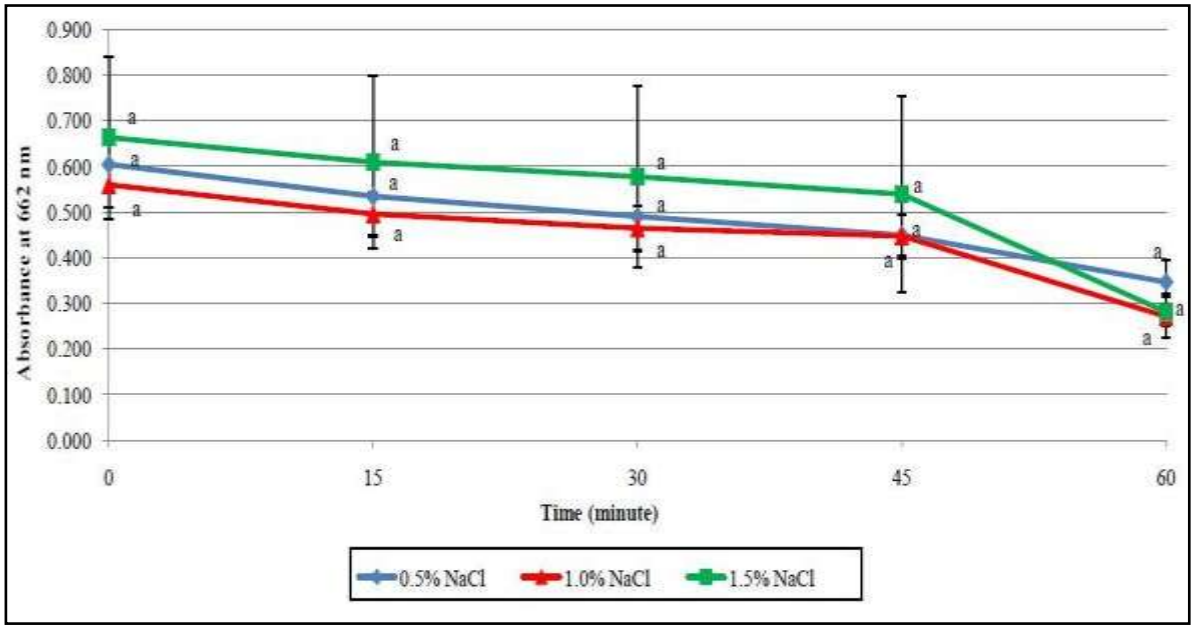

Figure 4.95: Graph of absorbance at 662 nm versus time (minute) for ethanolic leaf extract of *Allamanda cathartica* L. at different concentrations of salt solutions. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

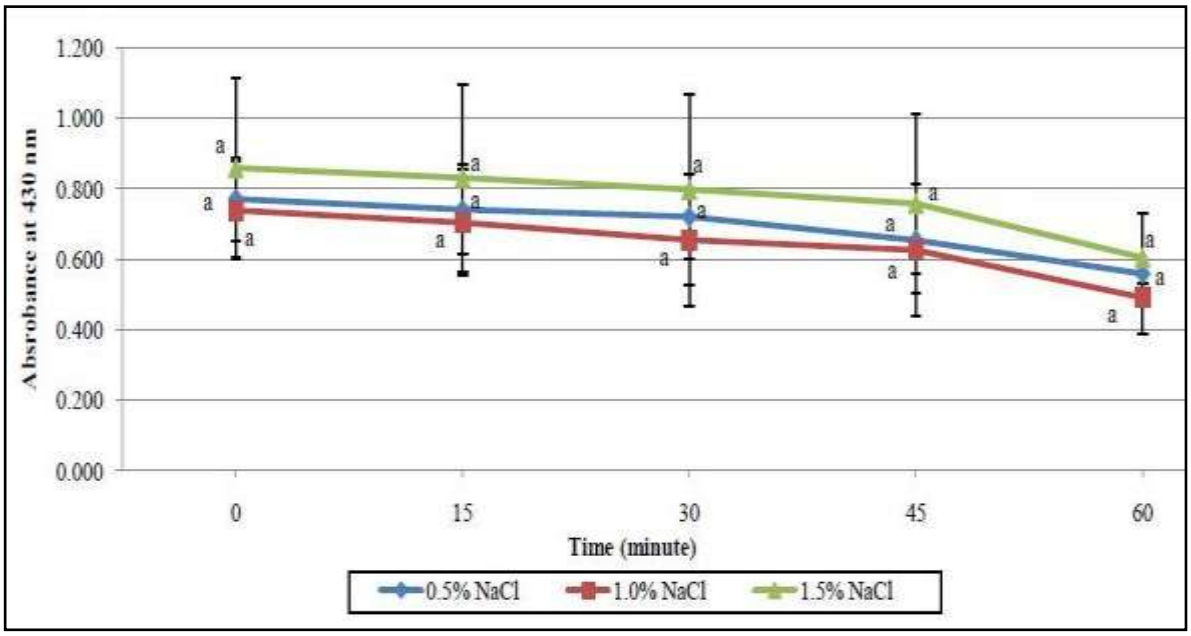

Figure 4.96: Graph of absorbance at 430 nm versus time (minute) for ethanolic leaf extract of *Allamanda cathartica* L. at different concentrations of salt solutions. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

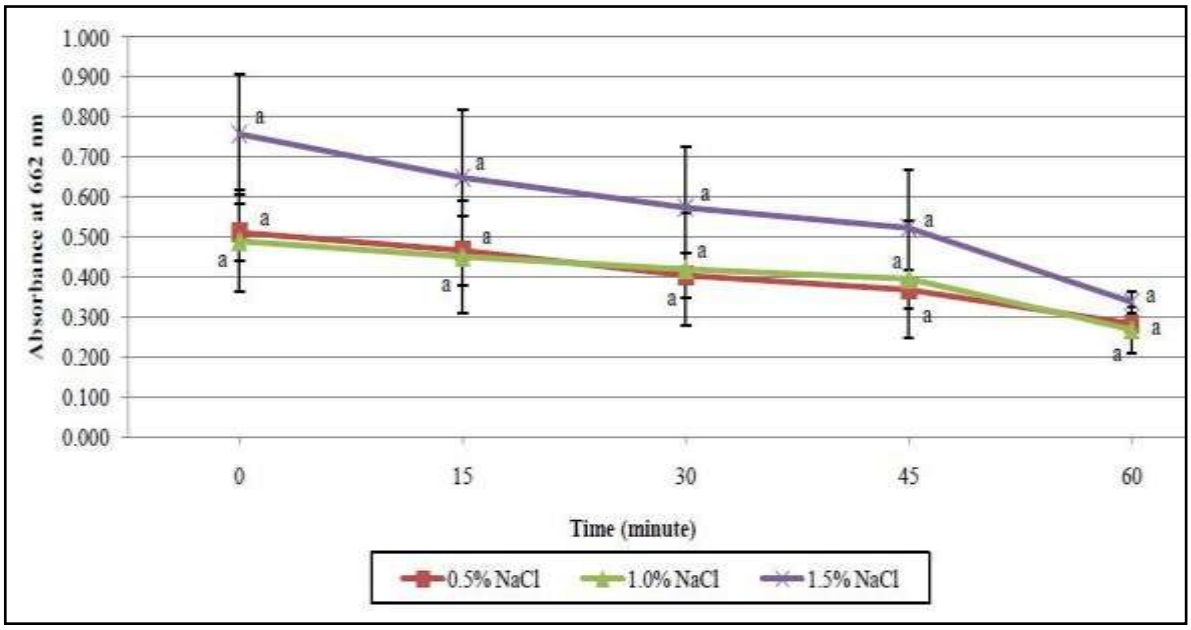

Figure 4.97: Graph of absorbance at 662 nm versus time (minute) for methanolic leaf extract of *Allamanda cathartica* L. at different concentrations of salt solutions. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

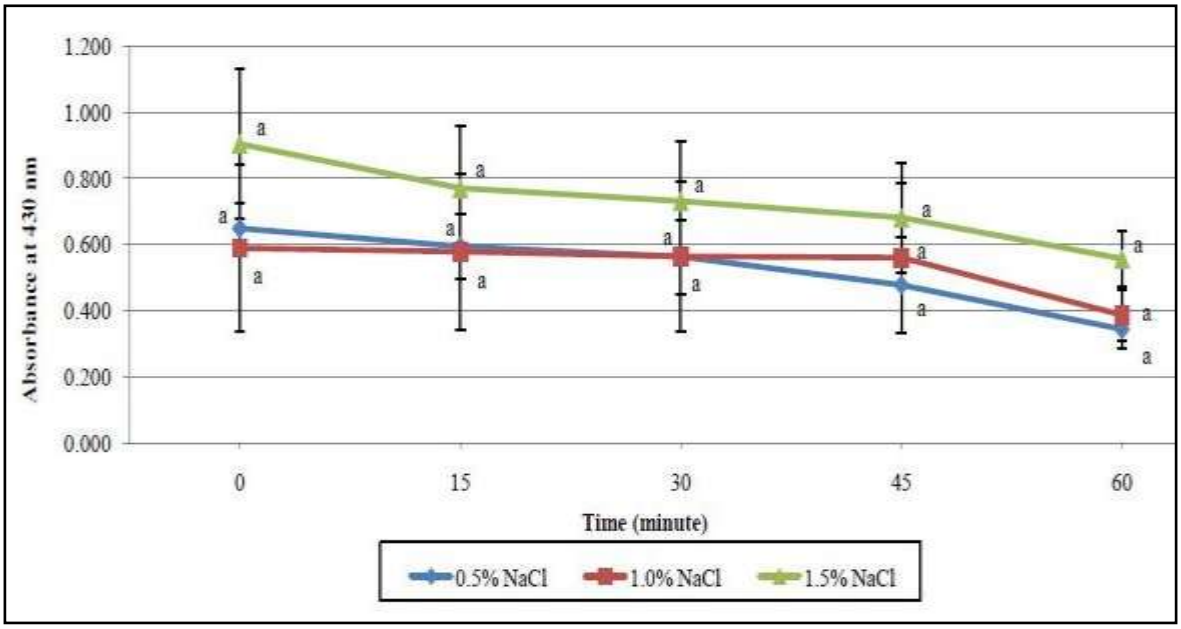

Figure 4.98: Graph of absorbance at 430 nm versus time (minute) for methanolic leaf extract of *Allamanda cathartica* L. at different concentrations of salt solutions. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

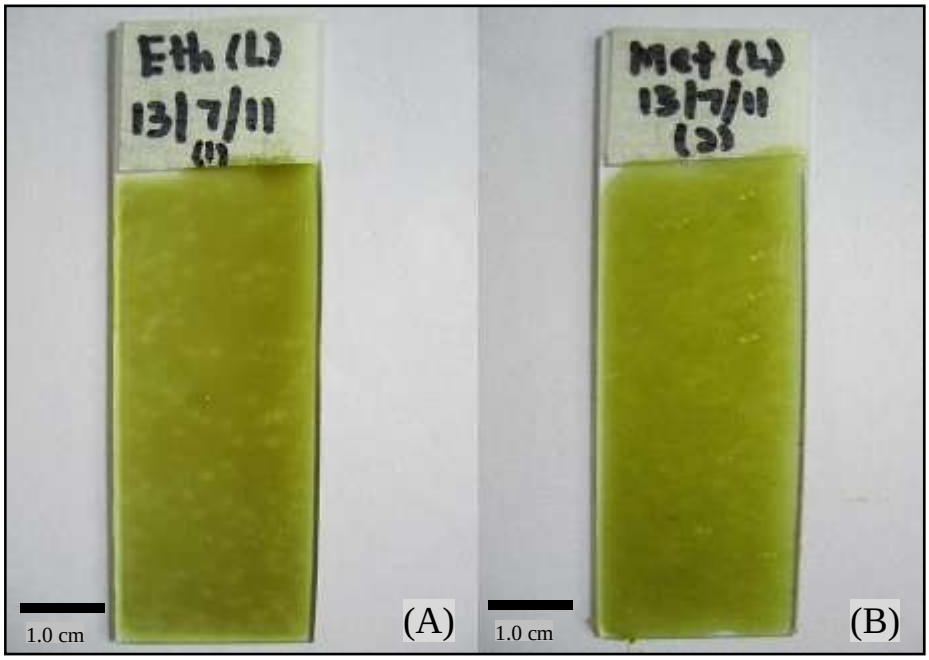

Figure 4.99: Glass slides coated with leaf extracts of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) before salt and heat tests. (A) Ethanolic and (B) Methanolic.

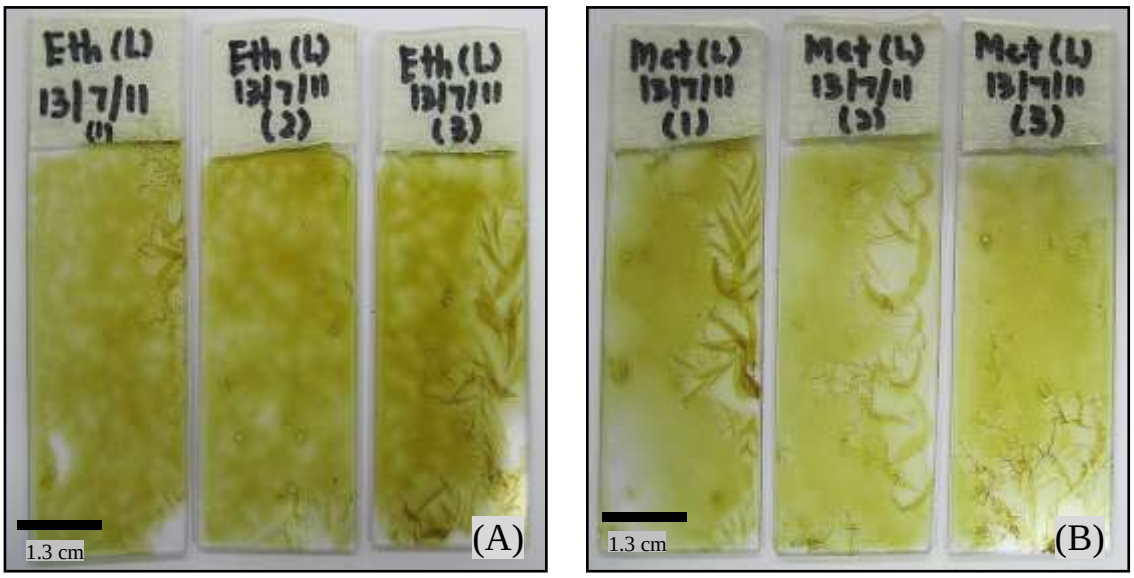

Figure 4.100: Glass slides coated with leaf extracts of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) after salt test. (A) Ethanolic and (B) Methanolic.

The heat tests on the glass slides that coated with ethanolic and methanolic leaf extracts (mixed with PVA in the ratio of 1:1) were carried out in the oven at temperature of 40ºC, the absorbance values at 662 and 430 nm were recorded for at different times, i.e. 24, 48, 72, 96, 120, 144 and 168 hours. According to Figure 4.101 and 4.102, the absorbances values at 662 and 430 nm for ethanolic leaf extract declined gradually from 0 to 168 hours. For methanolic leaf extract, the absorbance values at 662 and 430 nm were inversely proportional to the time, i.e. the absorbance values decreased as the times increased from 0 to 168 hours (Figure 4.101 and 4.102). For both ethanolic and methanolic extracts of leaves, no significant differences on the absorbance values (at 662 and 430 nm) observed for heat test from 0 to 168 hours. In the aspect of colouration, the green colour on the glass slides became paler and paler from 0 to 168 hours which was in accordance with the decreasing of absorbance values mentioned above (Figure 4.103 and 4.104).

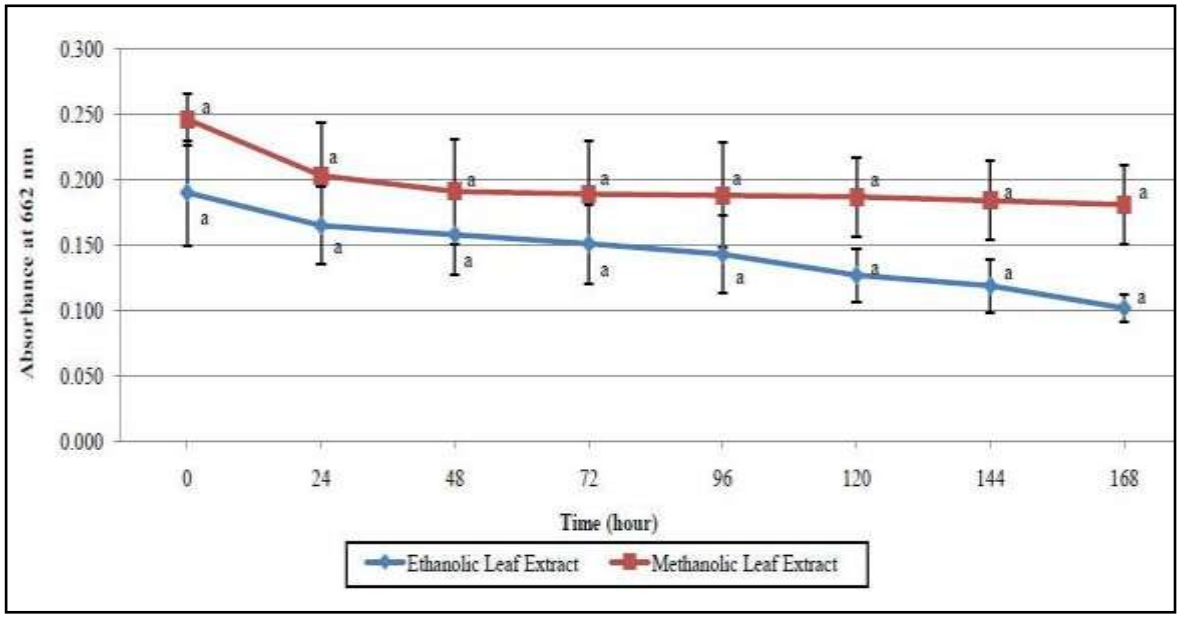

Figure 4.101: Graph of absorbance at 662 nm versus time (hour) for ethanolic and methanolic leaf extracts of *Allamanda cathartica* L. for heat test from 0 to 168 hours. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

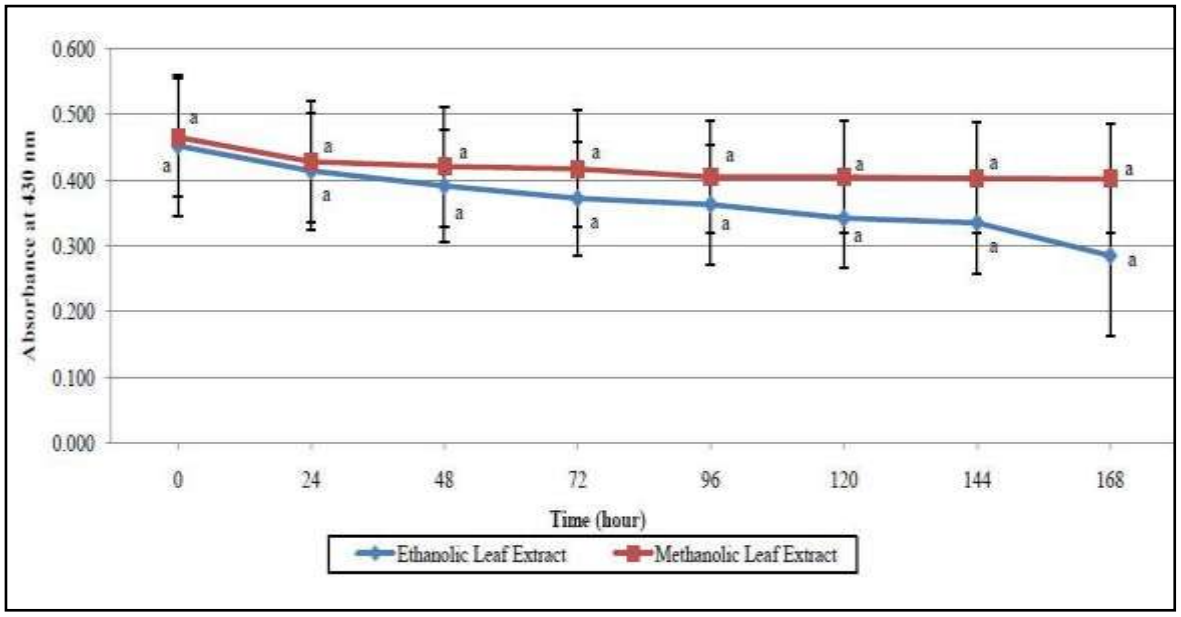

Figure 4.102: Graph of absorbance at 430 nm versus time (hour) for ethanolic and methanolic leaf extracts of *Allamanda cathartica* L. for heat test from 0 to 168 hours. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tykey's HSD test.

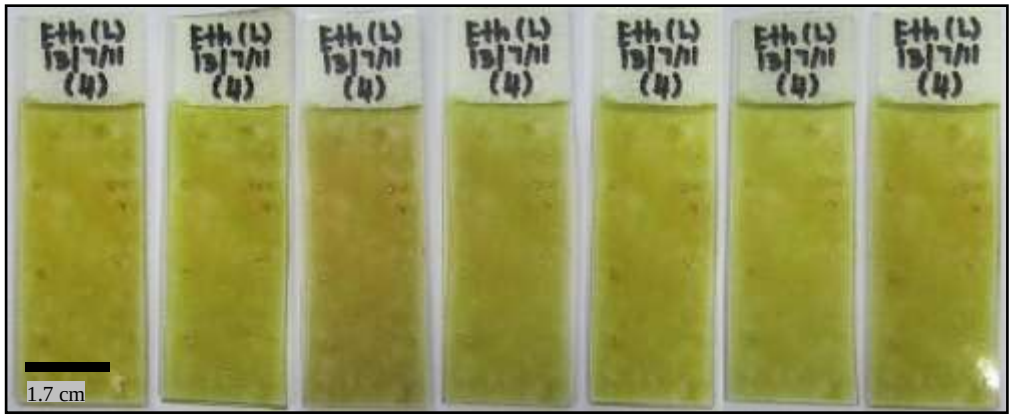

Figure 4.103: Glass slides coated with ethanolic leaf extract of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) after heat test from 24, 48, 72, 96, 120, 144 and 168 hours, respectively.

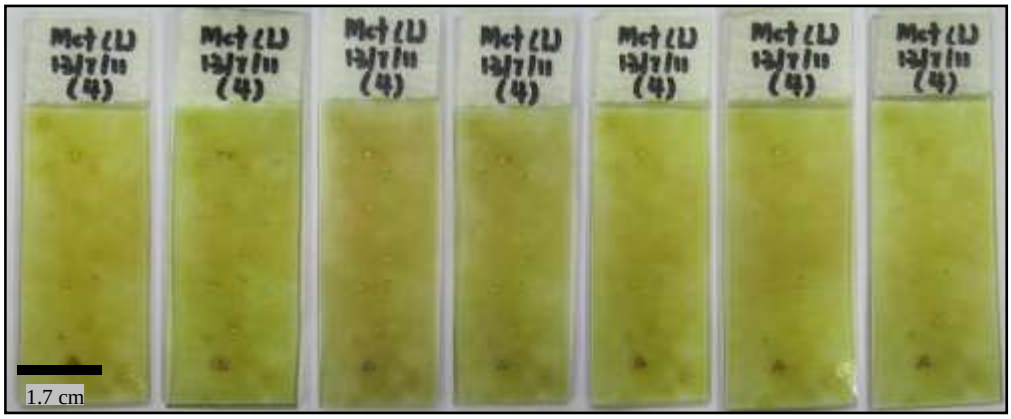

Figure 4.104: Glass slides coated with methanolic leaf extract of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) after heat test from 24, 48, 72, 96, 120, 144 and 168 hours, respectively.

Between the ethanolic and methanolic leaf extracts, the maintenance of green colour in methanolic extract was better than that in the ethanolic leaf extract when examined in the salt solution and heat test, i.e. the absorbance values (at 662 and 430 nm) for the glass slides coated with methanolic leaf extract were noticed to decrease constantly and more stable from 0 to 60 minutes (Figure 4.97 and 4.98) and 0 to 168 hours (Figure 4.101 and 4.102), respectively compared to ethanolic petal extract (Figure 4.95, 4.96, 4.101 and 4.102). This was in accordance with the results of pigment extraction, whereby 0.15% HCl in 99.9% methanol gave the most intense absorption spectral characteristic for the extraction of chlorophylls among all the extracting solvents used.

Apart from the salt and heat tests performed above, the test on natural colourant, i.e. the green chlorophylls extracted from the leaves of *A. cathartica* L. was also performed on the cotton wools by observing the degradation of colour over 1 week interval for 8 weeks. Figure 4.105 showed the colour degradation for natural colourant (green chlorophylls) present in the leaves of *A. cathartica* L. For chlorophyll pigment extracted from both ethanolic and methanolic leaves extracts, it can be observed that the colour tone of green on cotton wools was reduced gradually from a bright green into olive green from Week 0 to Week 8 (Figure 4.105).

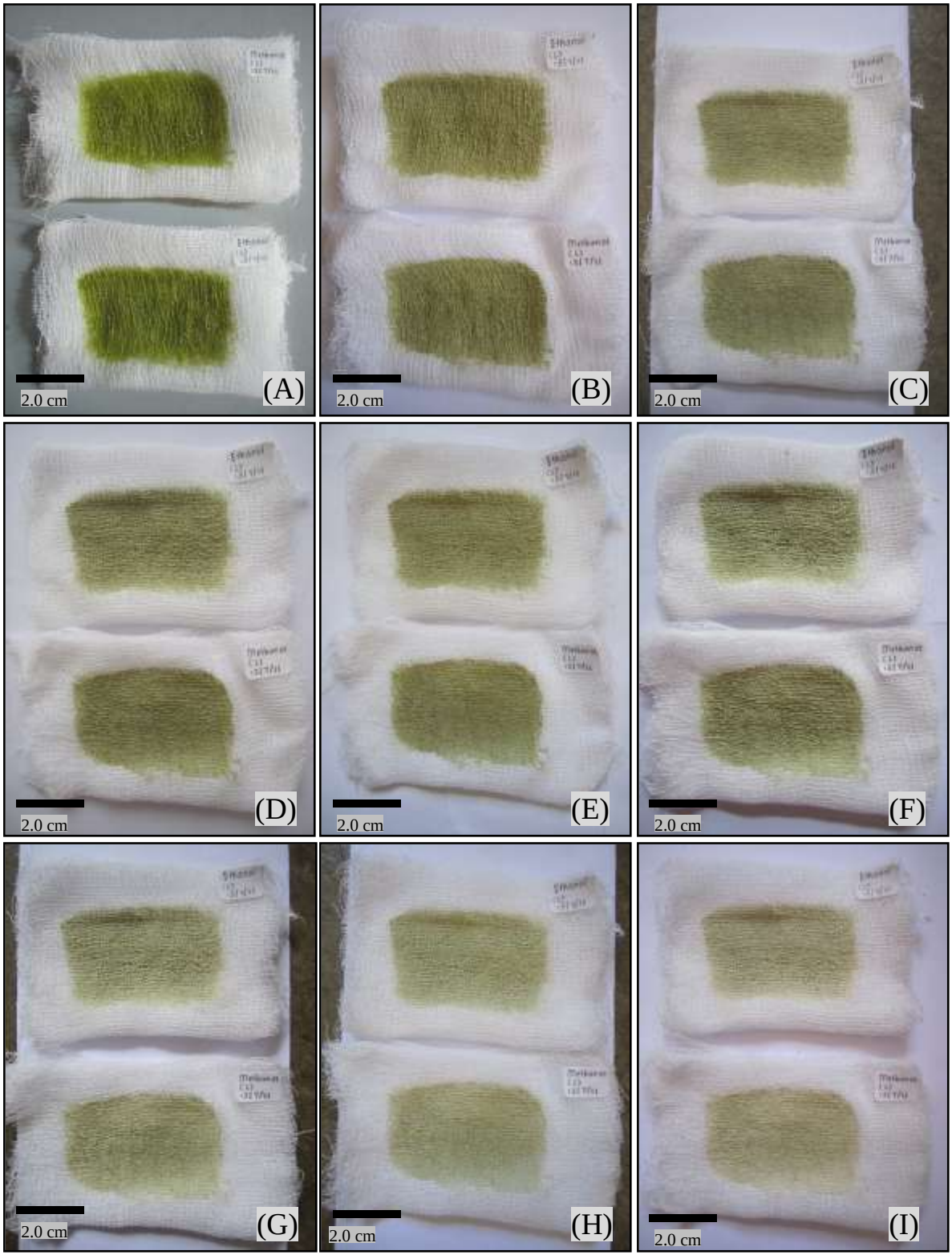

Figure 4.105:Observation for colour degradation on cotton wools coated with ethanolic (up) and methanolic (down) leaf extracts of *Allamanda cathartica* L. with PVA mixing ratio of 1:1. (A) Week 0; (B) Week 1; (C) Week 2; (D) Week 3; (E) Week 4; (F) Week 5; (G) Week 6; (H) Week 7 and (I) Week 8.

4.3.2.2 Natural Colourant from the Petals – Carotenoids

For the study of salt and heat tests from ethanolic (95% ethanol) and methanolic (0.15% HCl in 99.9% methanol) extracts of petals, the absorbance values were recorded at 450 nm which was the region absorbed strongly by the pigment of carotenoid. The image of glass slides coated with ethanolic and methanolic petal extracts of *A. cathartica* L. before salt and heat test presented on Figure 4.108. Figure 4.106 and 4.107 showed the absorbance values of salt test for ethanolic and methanolic petal extracts of *A. cathartica* L. recorded at 450 nm from 0 to 60 minutes. By referring to the figures, the absorbance values at 450 nm can be seen to reduce gradually when the glass slides coated with both ethanolic and methanolic petal extracts (mixed with PVA in the ratio of 1:1) were immersed in 0.5, 1.0 and 1.5% salt solution from 0 to 60 minutes (Figure 4.109). For ethanolic petal extract, there were no significant differences on the absorbance values from 0 to 60 minutes at 0.5% NaCl but a significant difference was observed on the absorbance values between 0 to 15 minutes and 30 to 45 minutes with 60 minutes at 1.0 and 1.5% NaCl. Meanwhile, for methanolic extract of petals, a significant difference was noticed on the absorbance values between 0 to15 minutes with 30 to 60 minutes at both 0.5 and 1.0% NaCl. For 1.5% NaCl, no significant difference on the absorbance values was observed between 0 to 15 minutes and 30 to 45 minutes but a significant difference on the absorbance values was observed between the two timings mentioned above with the time of 60 minutes.

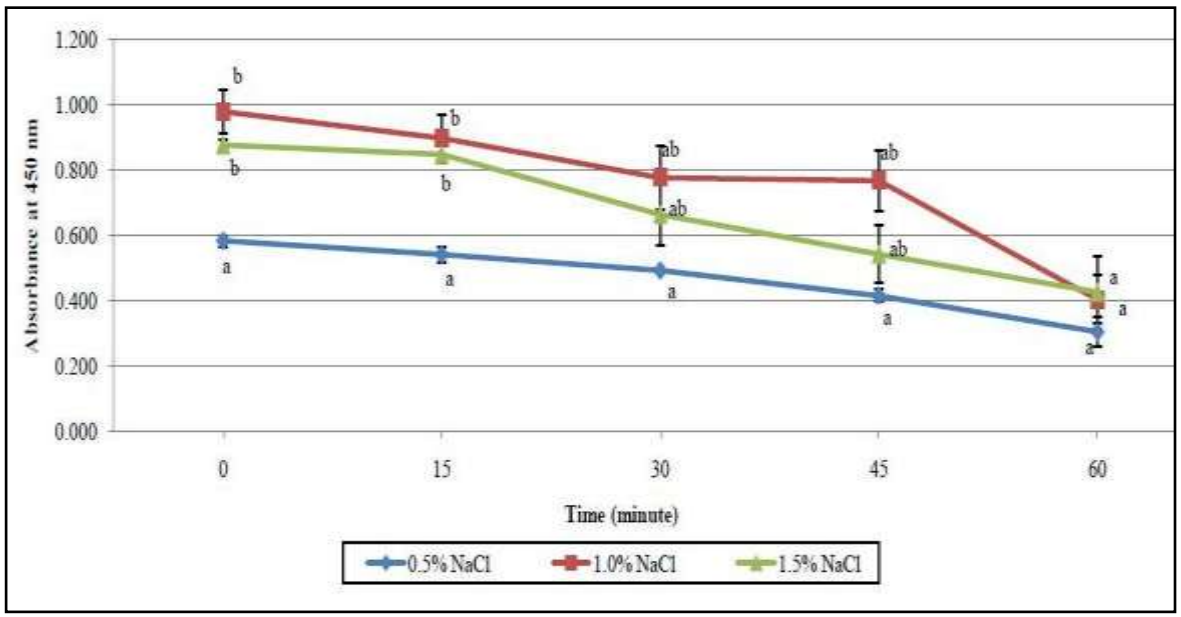

Figure 4.106: Graph of absorbance at 450 nm versus time (minute) for ethanolic petal extract of *Allamanda cathartica* L. at different concentrations of salt solutions. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

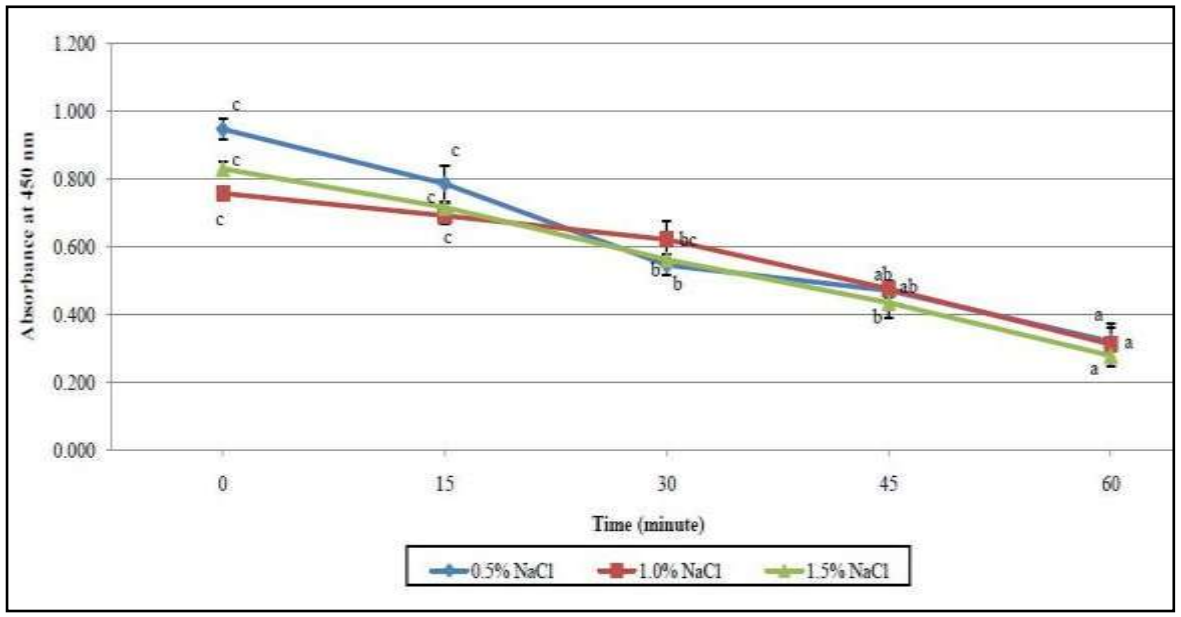

Figure 4.107: Graph of absorbance at 450 nm versus time (minute) for methanolic petal extract of *Allamanda cathartica* L. at different concentrations of salt solutions. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tukey's HSD test.

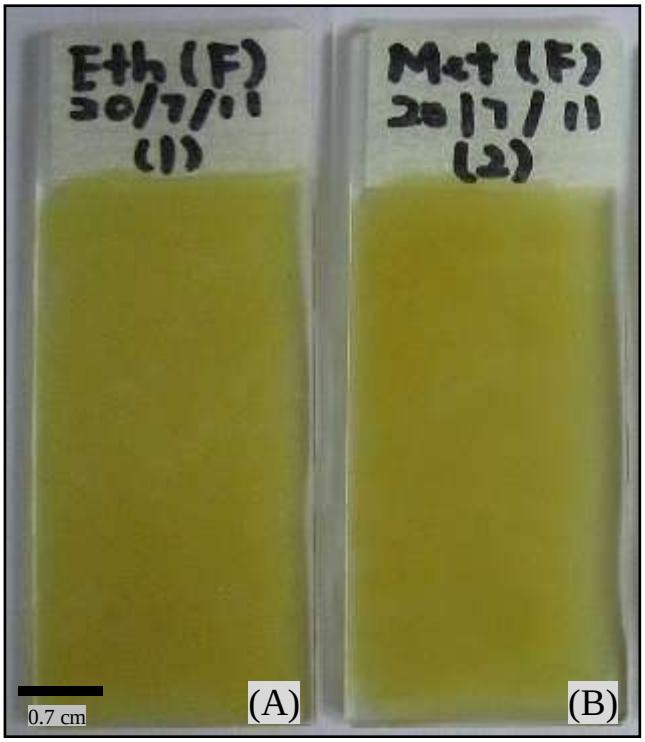

Figure 4.108: Glass slides coated with petal extracts of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) before salt and heat tests. (A) Ethanolic and (B) Methanolic.

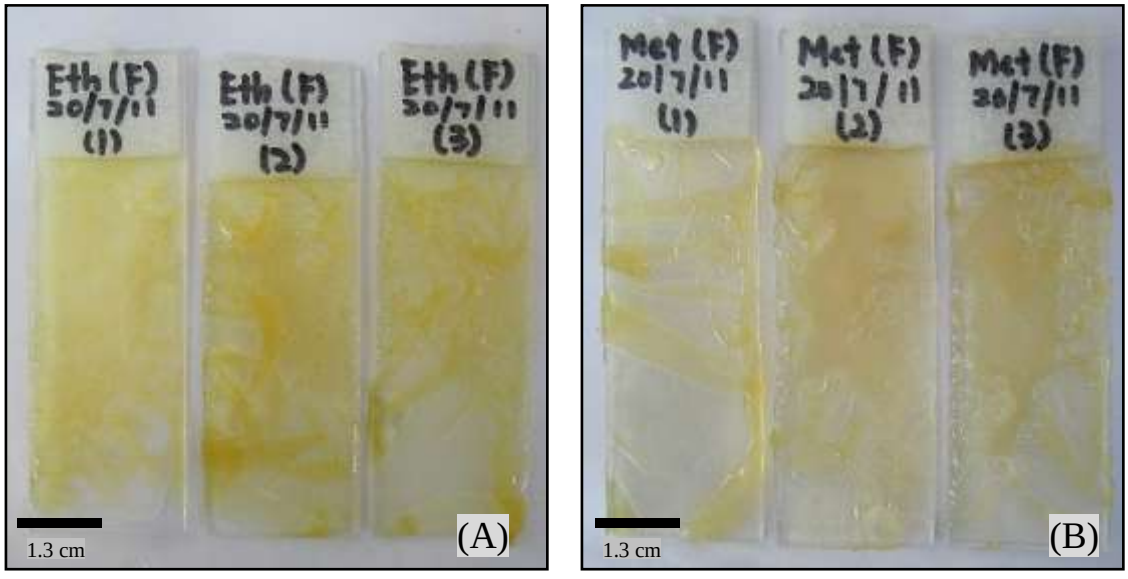

Figure 4.109: Glass slides coated with petal extracts of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) after salt test. (A) Ethanolic and (B) Methanolic.

The heat tests on the glass slides coated with ethanolic and methanolic petal extracts (mixed with PVA in the ratio of 1:1) were carried out in the oven at temperature of 40ºC, the absorbance values at 450 nm were recorded at different times, i.e. 24, 48, 72, 96, 120, 144 and 168 hours. According to Figure 4.110, the absorbances values at 450 nm for ethanolic petal extract declined gradually from 0 to 168 hours. For methanolic petal extract, the absorbance values at 450 nm were inversely proportional to the time, i.e. the absorbance values decreased as the times increased from 0 to 168 hours. For ethanolic extract of petals, no significant difference on the absorbance values (450 nm) were observed for heat test between 48 to 120 hours and 144 to 168 hours but a significant difference on the absorbance values was observed among the two timings mentioned above compared to 0 and 24 hours. For methanolic petal extract, a significant difference on the absorbance values was noticed between 0, 24, 144 and 168 hours compared with the timings of 48 and 72 hours, 96 and 120 hours. In the aspect of colouration, the orange yellowish colour on the glass slides became paler and paler from 0 to 168 hours which was in accordance with the decreasing of absorbance values (Figure 4.111 and 4.112).

Between the ethanolic and methanolic petal extracts, the maintenance of orange yellowish colour in ethanolic extract were better than that in the methanolic petal extract when examined with the salt solution and heat test, i.e. the absorbance values (at 450 nm) for the glass slides coated with ethanolic petal extract were noticed to decrease more stable from 0 to 60 minutes (Figure 4.106) and 0 to 168 hours (Figure 4.110), respectively compared to methanolic petal extract (Figure 4.107 and 4.110). This was in accordance with the results of pigment extraction, whereby 95% ethanol gave the most intense absorption spectral characteristic for the extraction of carotenoids among all the extracting

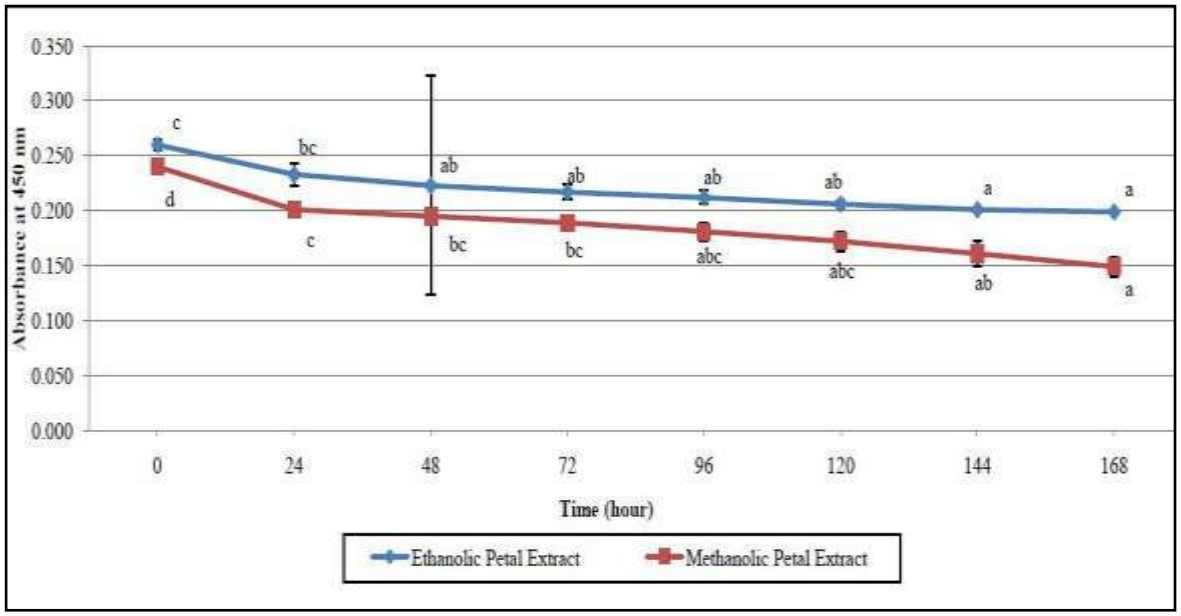

Figure 4.110: Graph of absorbance at 450 nm versus time (hour) for ethanolic and methanolic petal extracts of *Allamanda cathartica* L. for heat test from 0 to 168 hours. The error bars indicate the standard error. Same alphabet on the top indicate that the means of absorbance values do not differ significantly at $p<0.05$, according to the Tykey's HSD test.

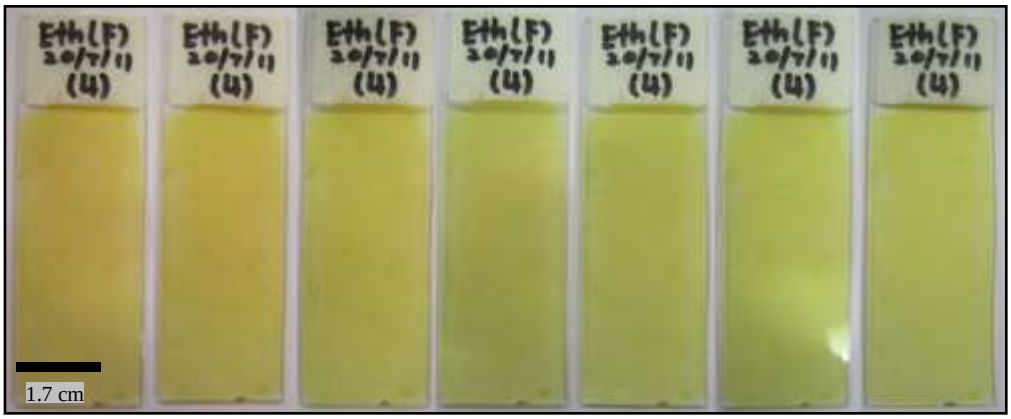

Figure 4.111: Glass slides coated with ethanolic petal extract of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) after heat test from 24, 48, 72, 96, 120, 144 and 168 hours, respectively.

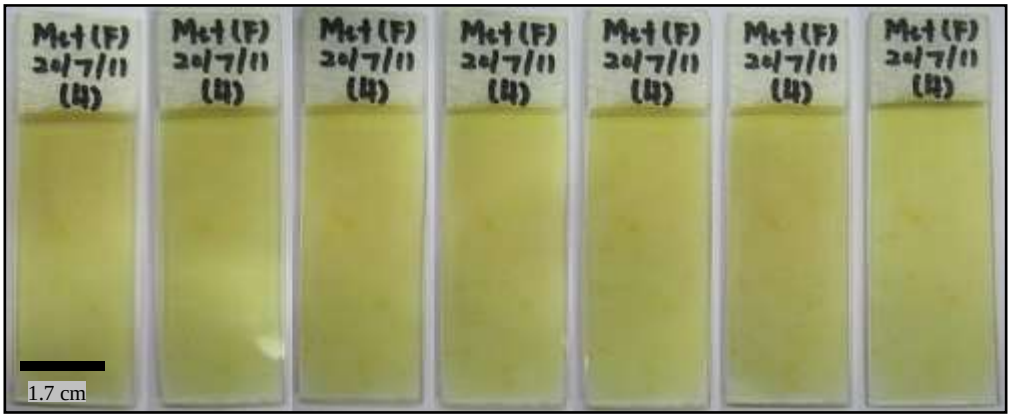

Figure 4.112: Glass slides coated with methanolic petal extract of *Allamanda cathartica* L. and PVA (mixing ratio of 1:1) after heat test from 24, 48, 72, 96, 120, 144 and 168 hours, respectively.

solvents used.

Apart from the salt and heat tests performed above, the test on natural colourant, i.e. orange yellowish carotenoids extracted from the petals of *A. cathartica* L. was also performed on the cotton wools by observing the degradation of colour over 1 week interval for 8 weeks. Figure 4.113 showed the observation of colour degradation for natural colourant (orange yellowish carotenoids) present in the petals of *A. cathartica* L. For carotenoid pigment extracted from both ethanolic and methanolic petal extracts, it can be observed that the colour tone of orange yellowish on cotton wool was reduced gradually from a bright orange yellowish into a pale colour from Week 0 to Week 8 (Figure 4.113).

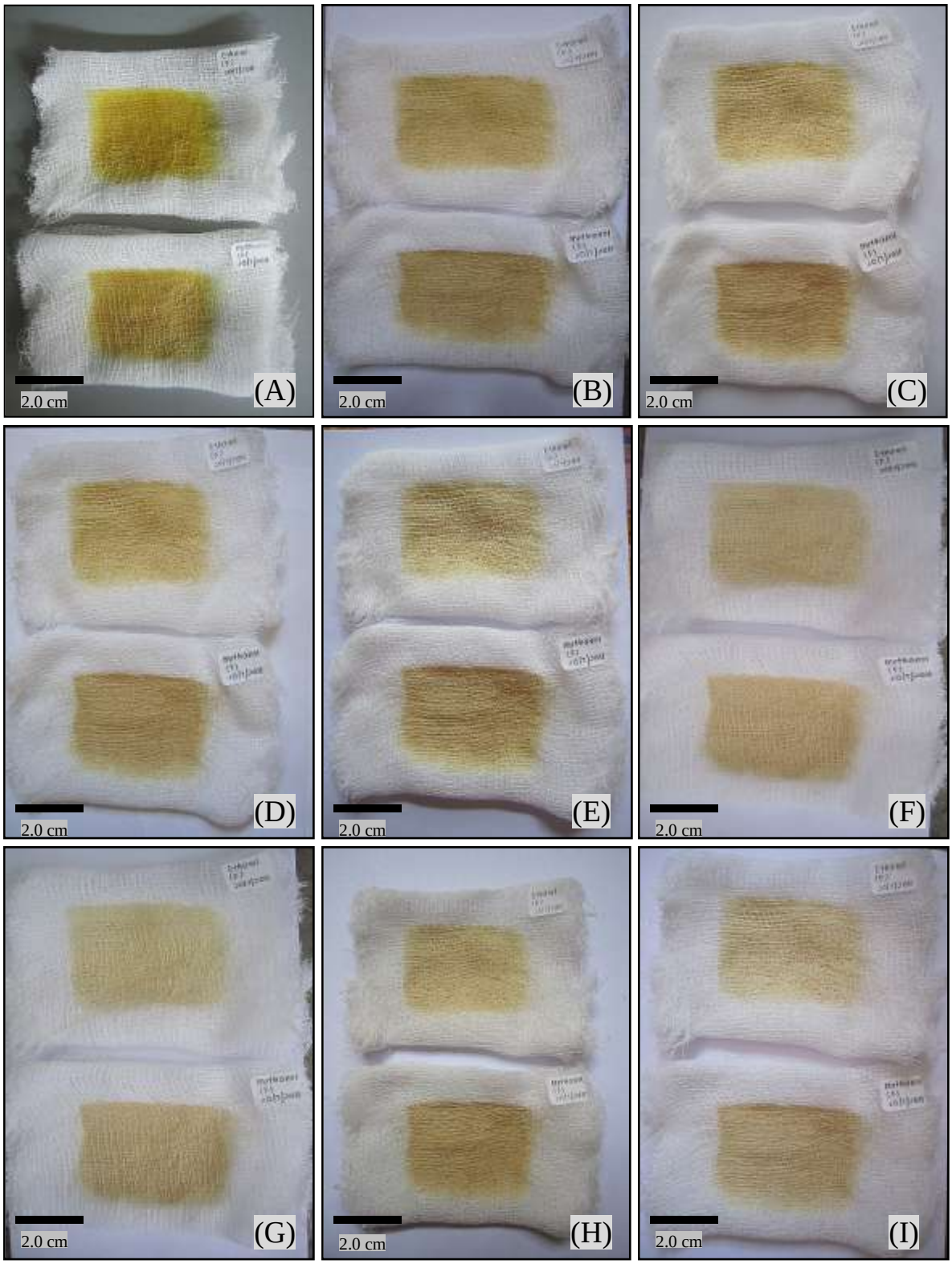

Figure 4.113: Observation for colour degradation on cotton wools coated with ethanolic (up) and methanolic (down) petal extracts of *Allamanda cathartica* L. with PVA mixing ratio of 1:1. (A) Week 0; (B) Week 1; (C) Week 2; (D) Week 3; (E) Week 4; (F) Week 5; (G) Week 6; (H) Week 7 and (I) Week 8.

CHAPTER 5

DISCUSSION

Tissue culture contamination frequently originates with the introduction into culture of explants contaminated with endophytic microorganisms or surface sterilization-resistant microorganisms. These include pathogens of the plant and common environmental microorganisms, both of which may become pathogenic in culture (Cassells, 2001). Many plants appear to have microbial contamination within the vascular system and other tissues (Smith, 2000). Furthermore, the use of field grown plants as a direct source of explants materials for the production of 'clean' *in vitro* plantlets, presents a major challenge (Webster *et al*., 2003).

Explant contamination depends on several plant and environmental related factors such as species, age, explants source and prevailing weather condition (Srivastava *et al*., 2010). The first experiment consists of finding the most suitable sterilizing agent. From the results obtained, leaf explants gave a higher percentage of contamination than stem and nodal explants in the Clorox treatments examined. This might be due to the ineffectiveness of Clorox solution and the amount of endogenous contaminants present in the leaf explants. In the treatments with $HgCl_2$, the percentages of contamination in leaf, stem and nodal explants were reduced compared to the Clorox treatments. This might be due to the suitability of type and concentration of chemical sterilant, i.e. $HgCl_2$ used during the process of surface sterilization. Although $HgCl_2$ was an effective sterilant to eliminate the microorganisms from the explants of *A. cathartica* L., one must be careful when preparing

the solution of $HgCl_2$ as it is an extremely toxic substance to both plants and human beings (Chawla, 2002).

During sterilization, the cells should not lose their biological activity and only the contaminants should be eliminated; thus explants are surface sterilized only by treatment with sterilant solution at suitable concentration for a specified period (Oyebanji *et al*., 2009). Depending on the type of explants, $HgCl_2$ at the concentration of 0.1% was the most suitable sterilant used to sterilize the leaf explants and 0.2% $HgCl_2$ was effective to sterilize both stem and nodal explants. Srivastava *et al*. (2010) reported that increasing time and concentration of sterilant significantly reduced contamination but showed adverse effect on explants. This was in accordance with the present study, i.e. the percentage of contamination on stem explants were reduced after sterilized with 0.2% $HgCl_2$ compared to 0.1% $HgCl_2$ but leaf explants did not survive after sterilized with 0.2% $HgCl_2$. In addition, only 0.01 to 1% $HgCl_2$ solution is recommended to sterilize the explants in plant tissue culture and rinsing must be very thorough in order to avoid its lethal on plant tissue (Chawla, 2002).

A. cathartica L. is a scrambling, perennial shrub or vine and it produces thick, milky sap when cut or wounded (Tropilab Inc., 2011). Explants with a thick lignin mantle were difficult to disinfect because not all the surface parts could be penetrated by Clorox or $HgCl_2$ solution (Bridg, 2000). As the stems and nodes were lignified, the milky sap inside these explants may be difficult to be eliminated even though the explants were cut into smaller pieces (1-2 cm) before surface sterilization. Therefore, this can be observed from

the percentages of contamination on stem and nodal explants, which were still higher than the leaf explants although they have been sterilized by a toxic sterilant, i.e. $HgCl_2$.

Smith (2000) stated that plant materials from the field were often more contaminated as compared to greenhouse or growth chamber-grown plant materials. Therefore, the protocol for surface sterilization of explants was modified several times with the manipulation of the concentration of Clorox and $HgCl_2$ solutions. The types of contaminants of plant tissue culture were bacteria and fungi, including spores (Stephens, 1995). Similar to the present study, the types of contaminants were fungus and bacteria where fungus contamination occurred more frequently than bacteria contamination. Both types of contaminants may survive in the plant material for several subculture cycles and over extended periods of time without expressing symptoms in the tissue or visible signs in the medium (Strosse *et al*., 2004).

Other sterilants can also be used to sterilize the explants such as hydrogen peroxide and alcohol. Hydrogen peroxide, a powerful oxidant can be used at 3-10% (v/v) for 1-30 minutes prior to sterile distilled water rinse in combination with other sterilants or alone. Ethyl or isopropyl alcohol at 70% (v/v) can be used to swab the plant material prior to disinfestations and it can be used to dip the plant material for 1-5 minutes before or after sodium or calcium hypochlorite treatment (Smith, 2000). A wetting agent such as Tween 20 is often included to improve contact between the sterilant and plant tissue (Fay, 1992). Antibiotics such as penicillin, streptomycin, amphotericin, erythromycin, gentamicin and ampicillin may be beneficial to reduce explants contamination after disinfestations using ethanol and bleach. However, they are only effective against bacteria and not fungi.

Chemical biocides have been tested for their ability to inhibit or prevent fungus contamination, but this treatment does not serve to inhibit bacterial contamination. Thus, combination of antibiotics and chemical biocides might have the ability to inhibit microbial contamination in plant tissue culture (Guri and Patel, 1998).

Callus is basically a more or less non-organized tumor like tissue, which usually developed on wounds of differentiated tissues and organs (Sarin and Bansal, 2011). One of the most important factors affecting culture initiation and plant regeneration of immature embryos is the composition of the media, including PGRs. PGRs are crucial in establishing an optimal culture conditions since they play a pivotal role in producing relatively undifferentiated callus tissue from the differentiated tissues (Serhantova *et al*., 2004) which involved three developmental stages, i.e. induction, cell division and proliferation (Sarin and Bansal, 2011).

Generally, a large portion of plant species required an auxin for dedifferentiation and profuse callus induction (Nirmalakumari, 2006). For this study, callus failed to produce in media free auxins. This showed that the presence of auxins capable of inducing callus formation (Abdellatef and Khalafallah, 2008) and *in vitro* callus formation is attributed to the presence of growth regulators in the medium (Fatima *et al*., 2011). In contrast, the leaf and stem explants of *Artemisia absinthium* L. were found to have callus formation on PGR-free basal medium with 75% callogenic reponse from the leaf explants and 50% stem explants produced calli (Muhammad *et al*., 2007). Besides, most of the herbaceous plants required only one type of auxin to be incorporated into the culture medium for the induction of callus from explants (Chan *et al*., 2008). A common feature of auxins is the

property of inducing cell division. In nature, auxins are involved in as rooting, abscission, elongation of stem, internodes and tropism (Razdan, 2003).

For callus induction from the leaf and stem explants of *A. cathartica* L., auxins such as 2,4-D, NAA and picloram, in two different concentrations were used, i.e. in 0.5 and 1.0 mg/L. The results indicated that different types of auxins with different concentrations showed different induction rates of callus and the morphology of callus obtained.

The auxin, 2,4-D, by itself or in combination with cytokinins, has been widely used to enhance callus induction and maintenance (Castillo *et al*., 1998). Both are required for cell division and during the *in vitro* culture, the balance between the two hormones determines what developmental tissue types are formed from the cell divisions (Serhantova *et al*., 2004). The callus was formed on the basal part of the explants which contained the meristematic tissue (Saensouk, 2011). Among different concentrations of 2,4-D treatments, MS medium supplemented with 1 mg/L of 2,4-D gave better response (16.7 ± 6.7%) for callus induction from the leaf explants of *A. cathartica* L. and produced more friable yellow-white and greenish calli compared to 0.5 mg/L of 2.4-D. The result was in accordance with the results obtained by Saensouk (2011) and Gopitha *et al*. (2010), in which callus induction from leaf explants of *Cornukaempferia aurantiflora* and *Saccharum officinarum*, respectively on MS supplemented with 1.0 mg/L of 2,4-D showed better response compared to 0.5 mg/L of 2,4-D. Bett *et al*. (2006) reported that an increase of 2,4-D levels increased the percentage of callus induction and the highest callus induction rate from *Lolium perenne* with the best callus quality assurance was obtained on MS medium containing 5 mg/L 2,4-D + 0.05 mg/L BAP (Liu *et al*., 2006). Callus was also induced from

the explants of *Cardiospermum halicacabum* (Thomas and Maseen, 2006) and *Salsola* species by Stefaniak *et al*. (2003) in the treatments of 2,4-D.

For the stem explants of *A. cathartica* L., no sign of callogenesis was observed for all concentrations of 2,4-D and the explants turned from green to brown after 4 weeks of culture. The result was in accordance to the result obtained by Bett *et al*. (2006) where no callus formed from the stem explants of sweet potato variety of SPK 013 on MS media supplemented with 0.5 and 1.0 mg/L of 2,4-D. No sign of callogenesis was also reported from the stem explants of *Vanda coerulea* Griff at MS medium supplemented with 1.0 mg/L of 2.4-D (Lang and Hang, 2006). However, the results differed from those of Dharmendra *et al*. (2010) who obtained 65% of callus formation from the stem explants of *Oxystelma secamone* (L) Karst at the lower concentrations of 2,4-D (0.5-1.0 mg/L) and those calli were formed at the proximal ends of the stem explants which suggested that callus formation may be due to the action of accumulated auxin at the basal cut ends, which stimulates cell proliferation.

NAA is a synthetic auxin that is an analogue of IAA which is commonly used either as a sole source of auxin or in combination with 2,4-D. NAA caused the formation of callus, but its morphogenic capacity was determined by its concentration, i.e. 0.015-0.03 mg/L NAA induced shoot-forming callus and continuous exposure to a higher concentrations of NAA caused callus to be produced which mainly give rise to root (George *et al*. 2007). In this study, no sign of callogenesis was observed from both leaf and stem explants of *A. cathartica* L. for all the concentrations of MS medium supplemented with NAA tested. The result was in accordance to the result obtained by Amoo *et al*. (2005) where no callus

formed from the explants of *Parkia biglobosa* throughout the culture period for all treatments with NAA. This might be due to the juvenility of the explants in response to the treatment of NAA (Kumar and Kanwar, 2006). In contrast to the studies reported by Savita *et al*. (2010) and Molina (2004), callus formation was observed from the leaf and stem explants of *Citrus jambhiri* Lush and *Salvia canariensis*, respectively when cultured on MS medium supplemented with NAA alone. In addition, NAA was also found to be the best auxin for producing creamish-green, fast growing and friable callus in the case of leaf explants of *Naringi crenulata* (Roxb) (Singh *et al*., 2011).

Commonly, NAA is often used as a rooting agent in plant tissue culture and its concentrations used in callus induction might be one of the factors that caused rooting from the explants (Razdan, 2003). Dabski and Parzymies (2007) reported that good results of rooting from *Hebe buchanii* and *H. canterburiensis* were obtained on MS medium supplemented with 1 mg/L of NAA. However, no rooting was observed from both leaf and stem explants of *A.cathartica* L. and the explants turned from green to brown after 4 weeks of culture. This might be due to the age of plant from which the explants which taken and microcuttings taken from actively growing shoot cultures generally root much more quickly and with greater uniformity than those harvested from quiescent or senescent cultures (Pierik *et al*., 1997). Besides, Pierik *et al*. (1997) also reported that no callus formation and rooting were observed in the older material but adventitious rooting and callus formation were noticed in the young explants of *Quercus robur 'Fastigiata'*.

Picloram is a potent auxin useful for the initiation and maintenance of callus and suspension cultures from certain plant tissues (Hagen *et al*., 1991). In the callus induction

of *A. cathartica* L., although the leaf explants showed poor growth of callus compared to the combination of 2,4-D and BAP, but its percentage of callus formation was relatively high among all the auxins used. Meanwhile, callus induction from stem explants showed a better result compared to that of leaf explants at 0.5 and 1.0 mg/L of picloram. From the results obtained, it was clear that the callogenesis response could be enhanced in both leaf and stem explants with the manipulation of picloram concentrations in a culture medium. Different explants were showed differences in callus induction based on the rate of proliferation and time as reported by Aftab *et al*. (2008). For both leaf and stem explants of *A. cathartica* L., a gradual increase in the percentage of callus formation and callus fresh and dry weights was observed with increasing concentrations of picloram from 0.5 to 1.0 mg/L which was similar with the results reported by Kaur and Kothari (2004).

Cytokinins are collection of naturally occurring N^6-substituted adenine derivatives with an isoprenoid side chain that promote plant cell division and affect most of the physiological responses in plants. Cytokinins are also able to promote cell expansion and shoot induction (Te-chato *et al*., 2008). Other major effects of cytokinins on plant growth include the promotion of lateral branching, inhibition of root growth, delaying of senescence and abscission of leaves, petioles and flowers (Srivastave, 2002). Cytokinins can be added together with an auxin to stimulate growth and development. At a higher concentration of cytokinins (1 to 10 mg/L), adventitious shoot formation is induced but root formation is inhibited. They also promote axillary shoot formation by decreasing apical dominance (Chawla, 2002).

In callus induction from the leaf and stem explants of *A. cathartica* L., cytokinin such as BAP in two different concentrations were used, i.e. in 0.5 and 1.0 mg/L. Medium free of cytokinins failed to induce callus formation from the leaf and stem explants of *A. cathartica* L., the explants slowly turned into brown colour and eventually died after 4 weeks of culture. Hence, callus induction was greatly influenced by PGRs, namely auxin or cytokinin in the nutrient media. Among the different factors influencing callus induction, genotype of the plant used, incubation condition and composition of nutrient media are the major factors that affect the *in vitro* raised cultures (Vijayakumar *et al*., 2010).

BAP is a synthetic cytokinin which can be used for the induction of callus formation. According to Te-chato *et al*. (1995), BAP is also used to micropropagate a wide range of plant species, including woody plants due to its great ability to stimulate shoot proliferation and regeneration. In the study of *A. cathartica* L., callus was unable to induce from both leaf and stem explants on MS media supplemented with different concentrations of BAP (0.5 and 1.0 mg/L). This basically could be due to plant specificity towards the concentration of PGR, whereby the amount of callus formation declined or no callus formation in the less appropriate medium, i.e. in an unsuitable medium of BAP (Ling *et al*., 2008).

The result was in accordance with the results obtained by Te-chato *et al*. (1995) and Molina (2004), whereby cytokinin in the presence of BAP alone showed no sign of callogenesis from the leaf explants of mangosteen and *Salvia canariensis*, respectively during culture *in vitro*. Meanwhile, the result obtained by Mohammad *et al*. (2003) did not coincide with our results, i.e. the treatment with BAP alone gave a good response on callus

induction and a good callus proliferation. Besides, by increasing the concentration of BAP (1.0-5.0 mg/L), a gradual increase in percentage of culture forming callus was also noted (12-41%) for callus induction from the leaf explants of *Pentanema indicum* Ling (Sivanesan and Jeong, 2007).

Regarding the applications and effect of PGRs to the plant cultures, great differences in culture response exist between species and cultivars and even between those of the same plants grown under different conditions. The most widely used types of PGRs *in vitro* are the auxins and cytokinins. They are frequently employed together, although the concentrations and specific chemicals are varied depending on the desired effects (Tyagi and Yadav, 2006). Callus formation from the explants involved three developmental stages, i.e. induction, cell division and proliferation which is regulated by the joint action of auxins and cytokinins, each of which appears to influence different phases of the cell cycle. Auxins might be considered as 'inducers' in the cell cycle which exert an effect on DNA replication, while cytokinins might behave as 'promoters' which seem to exert some control over the events leading to mitosis (George *et al*., 2007).

Generally, auxin and cytokinin are known to promote callus formation in tissue culture (Fatima *et al*., 2011). In the present study of callus induction from the leaf and stem explants of *A. cathartica* L., the combinations of auxins, i.e. 2,4-D or NAA with cytokinin, i.e. BAP at various concentrations were studied. The effects of auxin (especially 2,4-D) at different concentrations in combination with BAP induced significant increase in callusing percentage when compared to auxin alone. Moreover, the effect of auxin and cytokinin combination towards the induction of callus differs greatly according to the kind of plant

being cultured, the cultural conditions and the compounds used (George *et al*., 2007). For callus induction, the calli first formed at excised edges of explants and then expanded throughout the explants.

In the present study, nine combinations of 2,4-D and BAP were used to examine their effects on callus induction from the leaf and stem explants of *A. cathartica* L., i.e. 0.5 mg/L 2,4-D + 0.5 mg/L BAP, 0.5 mg/L 2,4-D + 1.0 mg/L BAP, 0.5 mg/L 2,4-D + 1.5 mg/L BAP, 1.0 mg/L 2,4-D + 0.5 mg/L BAP, 1.0 mg/L 2,4-D + 1.0 mg/L BAP, 1.0 mg/L 2,4-D + 1.5 mg/L BAP, 1.5 mg/L 2,4-D + 0.5 mg/L BAP, 1.5 mg/L 2,4-D + 1.0 mg/L BAP and 1.5 mg/L 2,4-D + 1.5 mg/L BAP. Among the combinations stated above, MS medium supplemented with 1.0 mg/L 2,4-D + 1.0 mg/L BAP showed the best response on callus induction from both leaf and stem explants of *A. cathartica* L., due to callusing from the explants was readily induced by an approximately equal amount of both auxin and cytokinin (Tyagi and Yadav, 2006).

MS medium containing 1.0 mg/L 2,4-D + 1.0 mg/L BAP gave the best response ofor callus induction from both leaf and stem explants of *A. cathartica* L. with the highest percentage of callus formation, callus fresh and dry weights. For callus induction from the leaf explants of *A. cathartica* L., the result was in accordance with the result reported by Isikalan *et al*. (2010) and Chowdhury *et al*. (2011), i.e. a friable callus was obtained from the leaf explants of *Amygdalus communis* L. cv. Yaltinski and *Vitex negundo*, respectively on MS medium supplemented with 1.0 mg/L 2,4-D + 1.0 mg/L BAP with the highest percentage of callus formation among the other combinations of 2,4-D and BAP. Besides, callus induction from the stem explants of *Amygdalus communis* L. cv. Yaltinski on 1.0

mg/L 2,4-D + 1.0 mg/L BAP also showed to have the highest percentage of callus responsive (80%) which was in accordance to the present study (Isikalan *et al*., 2010).

In addition, the percentage of callus formation from leaf and stem explants of *A. cathartica* L. for the treatment of 1.0 mg/L 2,4-D with BAP was increased from 1.0 mg/L 2,4-D + 0.5 mg/L BAP to 1.0 mg/L 2,4-D + 1.0 mg/L BAP but decreased once the concentration of BAP increased to 1.5 mg/L, callus fresh and dry weights appeared to have the similar feature as percentage of callus formation mentioned above. This was due to high concentration of auxins and low cytokinins in the medium which promote abundant cell proliferation with the formation of callus and vice versa (Mohammad *et al*., 2003). The results of present study was in accordance with the result reported by Anjaneyulu *et al*. (2011), i.e. percentage of explants induced callus formation from shoot tips of *Finger millet* increased initially but decreased once the concentrations of 2,4-D and BAP combination increased.

Combination of different concentrations of BAP (0.5, 1.0 and 1.5 mg/L) with 0.5 mg/L of 2,4-D was also examined in the callus induction from both leaf and stem explants of *A. cathartica* L. From the results, MS medium fortified with 0.5 mg/L 2,4-D + 1.0 mg/L BAP showed the lowest callus fresh and dry weights but the highest percentage of callus formation from the leaf explants compared to other combination of 0.5 mg/L 2,4-D and BAP. The result contradicted the result obtained by Lin and Wagner (1993), whereby MS medium containing 0.5 mg/L 2,4-D + 1.0 mg/L BAP (29%) did not show the best response on callus formation from *Ponderosa pine* among the four different combinations of 2,4-D and BAP used. Whereas, for the stem explants, the percentage of callus formation increased

with the concentration of BAP from 0.5 to 1.5 mg/L in combination with 0.5 mg/L 2,4-D which was in accordance with the study reported by Sivanesan and Jeong (2007), i.e. the percentage of callus induction from the stem explants of *Pentanema indicum* was increased with the concentrations of BAP. In addition, for 0.5 mg/L 2,4-D + 0.5 mg/L BAP and 0.5 mg/L 2,4-D and 1.0 mg/L BAP, similar percentage of callus formation was observed from the stem explants but in terms of callus fresh and dry weights, medium supplemented with 0.5 mg/L 2,4-D + 0.5 mg/L BAP was more outstanding compared to 0.5 mg/L 2,4-D + 1.0 mg/L BAP. This was contradicted with the result obtained by Elaleeme *et al*. (2009), i.e. the fresh weight of callus increased from 0.5 mg/L 2,4-D + 0.5 mg/L BAP to 1.0 mg/L BAP with the same percentage of callus formation (100%) from the explants of tuber segment of *Solanum tuberosum* L.

For last group of 2,4-D and BAP combination, i.e. 1.5 mg/L 2,4-D combined with BAP at concentrations of 0.5, 1.0 or 1.5 mg/L produced a similar percentage of callus formation from the leaf explants of *A. cathartica* L. This might be due to the same concentration of 2,4-D used in the medium that caused the explants to induce similar percentage of callus formation. A 2,4-D was a growth regulator essentially needed for callus initiation and growth in most of the *in vitro* culture studies (Mohammad and Mohammad, 2009). As for the stem explants, the percentage of callus formation decreased from 1.5 mg/L 2,4-D + 0.5 mg/L BAP to 1.5 mg/L 2,4-D + 1.0 mg/L BAP but increased to 93.3 ± 3.3% on 1.5 mg/L 2,4-D + 1.5 mg/L BAP. This was due to the media with balanced hormonal combinations had the highest percentage of calli production and the differences in the response of callus induction would be dependent on the different potential of explants for internal hormones metabolism (Mohammad and Mohammad, 2009).

Apart from the combination of 2,4-D and BAP, NAA and BAP at various concentrations was also a famous combination in the studies of *in vitro* culture and have been used on callus induction from both leaf and stem explants of *A. cathartica* L. Generally, high levels of NAA are necessary for callus formation and using BAP can accelerates this process (Safdari and Kazemitabar, 2010). From the results of *A. cathartica* L., there were no sign of callogenesis observed for the leaf and stem explants cultured on MS media fortified with 0.5 mg/L NAA + 0.5 mg/L BAP and 1.0 mg/L NAA + 0.5 mg/L BAP. All the explants turned from green to brown in colour after 4 weeks of culture and eventually died. The results of the present study were supported by Lima-Brito *et al*. (2011), i.e. no response of callogenesis from both leaf and stem explants of *Syngonanthus mucugensis* Giul. subsp. *mucugensis* on MS medium supplemented with 1.0 mg/L NAA + 0.5 mg/L BAP. Moreover, the results contradicted with result obtained by Borzabad *et al*. (2010), i.e. there was a callus formation from the stem explants of *Artemisia vulgaris* L. on MS medium supplemented with 0.5 mg/L NAA + 0.5 mg/L BAP (31%) and 1.0 mg/L NAA + 0.5 mg/L BAP (38%). Callus formation from the explants of *Helianthus annuus* L., *Aristolochia bracteata* Retz. and *Solanum nigrum* L. were also reported on MS medium supplemented with NAA and BAP (Muhammad *et al*., 1999; Sahaya *et al*., 2011; Sridhar and Naidu, 2011).

Plant tissue culture involves asexual methods of propagation. In recent years, plant regeneration using tissue culture is an alternative method used to multiply mainly vegetatively propagated plants (Ramanathan *et al*., 2011). Plant regeneration can be achieved by culturing tissue sections either lacking a preformed meristem (adventitious origin) or from callus and cell cultures (*de novo* origin) (Chawla, 2002). In the present

study of plant regeneration of *A. cathartica* L., shoot multiplication from nodal explants (MS medium supplemented with 1, 3 and 5 mg/L BAP) and shoot elongation (MS PGR-free medium) were studied.

BAP is the most effective hormone in stimulating and enhancing shoot multiplication (George *et al*., 2007) and nodal segment are mostly preferred as the explants choice (Jeyakumar and Jayabalan, 2002). Gopi *et al*. (2006) stated that in preliminary studies, a remarkable response was shown in the nodal explants cultured on MS medium individually supplemented with BAP. The shoots emerged from nodal part of explants in the form of small leaf clusters, proliferated rapidly and formed dense cluster of leaves. From the results, PGR-free medium showed no sign of shoot multiplication and this was supported by the study of shoot multiplication from the nodal explants of *Withania somnifera* L. Dunal and *Catharanthus roseus* (Kumar *et al*., 2011; Faheem *et al*., 2011) but it was contradicted by the result obtained by Genkov and Ivanova (1995), i.e. 1.05 ± 0.15 shoots were formed from the explants of *Dianthus caryophyllus* cv. White Sim on the control medium.

When the shoots were multiplied from the nodal part of *A. cathartica* L., MS medium supplemented with 5 mg/L of BAP showed the best response with more shoots formation compared to 1 and 3 mg/L BAP. The result obtained from the study of *A. cathartica* L. was supported by Ndoye *et al*. (2003), i.e. as the BAP concentration increased from 1 mg/L BAP to 5 mg/L BAP, the percentage of shoot formation increased. But it was contradicted by the results obtained by Aftab *et al*. (2008), whereby the explants of *Gladiolus hybridus* gave better multiplication response on MS medium containing 1 mg/L

BAP compared to other treatments. Hassan *et al*. (2011) also reported that the lowest mean number of shoots from nodal explants of *Aquilaria hirta* was obtained in 5.0 mg/L BAP and the number of shoots produced decreased as the BAP concentration in the medium increased. Furthermore, Sivanesan *et al*. (2008) reported that an increase in BAP concentration from 0.5 to 2.0 mg/L resulted in a significant increase in the frequency of shoot induction and average number of shoots produced per explants, however, further increase (3 mg/L) led to decrease in shoot number and browning or death (5 mg/L) of the explants from *Scrophularia takesimensis*.

According to Prematilake *et al*. (2006), multiplied shoots development and elongation can be obtained on MS PGR-free medium. In the study of *A. cathartica* L., the length of shoot increased gradually within 4 weeks of observation period on MS medium without any supplementation of PGR and each of the node gave rise to one plantlet which can serve as an important plant material for micropropagation. The result obtained from the study of *A. cathartica* L. contradicted the result obtained by Karuppusamy and Kalimuthu (2010), whereby the multiple shoots generated from the nodal explants of *Andrographis neesiana* failed to elongate after transferring to hormone-free MS medium.

Moreover, PGR-free MS medium can also be used to stimulate the production of root (Santos *et al*., 1990). This is consistent with the result of *A. cathartica* L., i.e. 2 out of 15 explants were observed to have root formation and it was in accordance with the result reported by Santos *et al*. (1990), whereby the production of roots by shoots and even leaves of *Datura insignis* was observed on MS PGR-free medium. Apart from that, rooting of elongated shoots of *Capsicum annuum* L. and *Amomun krervanh* was also carried out on

MS medium in the studies of Qin *et al*. (2005) and Tefera and Wannakrairoj (2004), respectively.

Since the primary use of SEM is to examine surface structures, the original topography of specimen must be well-preserved (Krause, 1983). Before the viewing of specimen under SEM, the specimen should be stabilized immediately after collection. The two most common used methods of stabilization are freezing and chemical fixation such as 4% glutaraldehyde with Sorensen's phosphate buffer and 1% osmium in the present study of *A. cathartica* L. The purposes of specimen fixation were to prevent natural changes in the specimen and to increase the specimen's resistance to deformation by subsequent preparatory manipulations (Dunlap and Adaskaveg, 1997). Substitution of water with solvent (dehydration) on the specimen was then carried out using a series of ethanol solutions from 10% to 100%.

After the process of fixation, preservation of the specimen took place by critical point drying (CPD) in order to keep the specimen in the dry condition. With this method, the specimen was placed in a pressure chamber with a liquefied gas such as carbon dioxide (CO_2) in this case. The liquefied gas must be miscible with the intermediate solvent in the specimen (i.e. acetone) and it would replace the intermediate solvent in the specimen after sufficient equilibration time. The temperature of the pressure chamber was then raised above the critical point of the gas and the liquefied gas returns to the gaseous state without a change in volume or density and without surface tension forces (Dunlap and Adaskaveg, 1997).

After the process of drying, the specimen was mounted on stubs with carbon adhesive tape and then specimen surface is coated with gold by sputter coater for signal localization to the surface of the specimen. The sputter coater is done under partial vacuum, where molecules of argon gas are ionized in the high voltage field between the cathode and the anode. The positively charged argon ions are accelerated to the cathode that is made of the coating metal. The ions etch the cathode target and the negatively charged metal atoms accelerate to the anode upon which the specimen has been placed, thus, it is possible to put a thin coating on the underside of a structure. The advantages of using sputter coater are the usage of gold or other metal is comparatively small and no high vacuums are required, but the greatest potential disadvantage of sputtering is heat damage to the specimen (Dunlap and Adaskaveg, 1997). Anyway, the specimen is ready to examine through SEM after the processes of mounting and coating.

Microscopic studies of the structures of *in vitro* and *in vivo* leaves of *A. cathartica* L. were carried out using the SEM. When compared between the abaxial (lower) surfaces of both *in vitro* and *in vivo* leaves, closely packed irregular cell arrangement with densely distributed anomocytic stomata were found on the *in vitro* leaf specimen (Figure 4.19), whereas numerous anomocytic stomata with larger epidermal cells were observed on the *in vivo* leaf specimen (Figure 4.21). According to the SEM micrographs, those epidermal cells and stomata from abaxial surface of *in vivo* leaf specimen were covered by the thread-like structure of fungus and bacteria, this might be due to the examined specimen which was excised directly from the field grown plant (without undergo surface sterilization) that contained high contamination rate compared to the *in vitro* leaf specimen (Taski-Ajdukovic and Vasic, 2005).

For the adaxial (upper) surface, *in vitro* leaf was densely covered with the wax (thread-like structure) giving spongy appearance and noticed to have fewer anomocytic stomata which were raised above the epidermal surface (Figure 4.20). Whereas, no stoma was found on the adaxial surface of *in vivo* leaf with regular epidermal cell arrangement (Figure 4.22). Furthermore, due to the controlled conditions available in the culture room, the thread-like structure of wax can be generated from the adaxial surface of *in vitro* leaf specimen, while, for the *in vivo* leaf specimen, it often influenced by the uncontrollable environmental conditions such as humidity and temperature, thus, wax was not available on *in vivo* leaf specimen due to the development of wax can be influenced by those environmental conditions stated above (Baker, 1974). In addition, anomocytic stomata are also called irregular-celled type of stomata in which the subsidiary cells are indistinguishable from other epidermal cells (Syeda and Muhammad, 2009).

The functions of stomata are important on the physiology, adaptation and productivity of plants (Ozeker and Misirli, 2001) and allow gases to pass in and out of the leaf (Roberts and Mitchelmore, 2000). In the present study, the number of stomata on the abaxial surface was more abundant compared to the adaxial surface for both *in vitro* and *in vivo* leaves. This can be supported by the study of AbdulRahaman and Oladele (2003), whereby the leaves of *Sesamum indicum*, *Amaranthus cruentus*, *Talinum trangulare* and *Celosia argentea* showed to have more stomata on the abaxial surface than on adaxial surface and this feature was called hypoamphistomatic leaves. However, all cultivars of wheat (*Triticum aestivum* L.) had more stomata on the adaxial compared to abaxial surface, i.e. epiamphistomatic leaf in the study of Maghsoudi and Moud (2008).

Apart from that, stomata are microscopic pores bounded by two crescent shaped guard cells and the stomatal opening of *in vitro* leaf specimen was observed to be smaller (Figure 4.23) and not obvious (Figure 4.24) compared to *in vivo* leaf specimen (Figure 4.25), the reason might be due to the special environmental conditions inside the culture vessels and heterotrophic or mixotrophic nutrition may generate anomalies of micropropagated plants with malformed and malfunctioning stomata (Saez *et al*., 2012). Moreover, the difference of stomata structures between *in vitro* and *in vivo* leaves might be also caused by the limited rate of photosynthesis on *in vitro* plantlet caused by two major ambient factors, i.e. low carbon dioxide (CO_2) concentration in the headspace of the culture vessel and low light intensity (During and Harst, 1996). Maseda *et al*. (2004) reported that *in vitro* leaves of *Lycium chilense* had lower stomatal density and their stomata were less functional when compared to acclimated leaves.

The stem is largely a supporting structure. Microscopic studies of the structures of *in vitro* and *in vivo* stems of *A. cathartica* L. were performed using the SEM. As compared between the surfaces of both stem specimens, the surface of *in vitro* stem (Figure 4.26) was found to be cleaner and no contamination compared to *in vivo* stem (Figure 4.27) due to the specimen has been sterilized properly with specific sterilant through the aseptic techniques (Smith, 2000).

Moreover, *in vitro* cultured plant grow in conditions different from those growing outside the culture vessel and frequently exhibit peculiarities in shoot development compared to those grow *in vivo* (Robinson *et al*., 2009). Therefore, no stoma was observed from the surface of *in vitro* stem but fewer anomocytic stomata were noticed on *in vivo*

stem specimen (Figure 4.28). However, the amount of stomata observed from the stem specimens was lesser than that on the leaf specimens of *A. cathartica* L. for both *in vitro* and *in vivo* specimens due to the primary functions of stem, i.e. to support and orient the leaves in a manner that they are exposed to maximum sunlight and for efficient gaseous exchange during photosynthesis and respiration, to conduct water and minerals from roots to leaves and manufactured food from leaves to different parts of the plant and to bear flowers and fruits (Hopkins and Huner, 2004).

Figure 4.29 and 4.30 showed the cross sections of *in vitro* and *in vivo* stem specimens of *A. cathartica* L., respectively. In the *in vitro* stem, it displayed more or less uniform size of cells for the regions of cortex, interfascicular region and pith throughout the cross section of stem. Whereas in the *in vivo* stem, the stem is thick and surrounded with a thin waterproof layer of cuticle (outer layer of epidermis) which is secreted by a thin layer of epidermis in order to keep water inside the plant. Due to the high humidity in the culture vessel (90-100%), the cuticle layer is often poorly develop in the *in vitro* plant, thus, no cuticle layer was observed in the *in vitro* stem specimen of *A. cathartica* L. and this resulted in extra water loss through cuticular evaporation (Pierik, 1998). Epidermis is a protective layer which acts as a barrier between the internal organization of the plant and the environment. Cortex serves to strengthen the young stem and the chloroplasts inside are responsible for the synthesis of organic food during photosynthesis. The interfascicular region consists of the vascular bundles and the pith occupies the large central part of the stem for water and starch storing and allows for the exchange of gases through intercellular air spaces (Mishra, 2009).

Phytochemical screening or analysis of plant extract is necessary to identify the class of secondary metabolites presence in the plant extract that could be responsible for different biological activities (Jamil, 2010) and treatment of diseases (Chitravadivu *et al*., 2009). Besides, phytochemical screening is of paramount importance in identifying new source of therapeutically and industrially valuable compound having medicinal significance, to make the best and judicious use of available natural wealth (Mungole and Chaturvedi, 2011). Before the plant sample being screened for the presence of certain phytochemicals, extraction of the plant sample must be carried out which is a process that separated the medicinally active portions of plant tissue using selective solvents and these solvents are diffused into the solid plant material and solubilize compounds with similar polarity (Tiwari *et al*., 2011).

Phytochemical screening on ethanolic extracts of both dried and fresh plant materials (i.e. leaves, stems, petals and roots) of *A. cathartica* L. was performed in order to identify the class of secondary metabolites such as reducing sugars, anthraquinones, terpenoids and steroids, saponins, tannins, glycosides, essential oils and flavonoids that presence in the extracts of *A. cathartica* L. In the present study, ethanol was chosen to be the extracting solvent because it was easier to penetrate the plant cellular membrane to extract the intracellular ingredients from the plant material and non-toxic compared to the methanol (Tiwari *et al*., 2011). Moreover, dried or fresh plant materials can be used as a source for the extraction of secondary plant components since plants are usually used in the dry form (or as an aqueous extract) by traditional healers and fresh plant materials are often used as ethno medicinal among the traditional and tribal people (Tiwari *et al*., 2011).

Reducing sugars from the group of carbohydrates served as a source of available energy, as reserve food and as structural materials in all the plants. Reducing sugars are also one of the main groups of food substances to be synthesized in the plant from simple organic substances (Lee *et al*., 1970). Fehling's test is based on the reducing property of monosaccharides (glucose, galactose and fructose) and disaccharides (lactose and maltose), which in turn depends to the available of free keto or an aldehyde group. This test is carried out by adding few drops of Fehling's mixture, i.e. Fehling's A solution ($CuSO_4$) and Fehling's B solution (KOH and Na-K-tartrate) into the plant extract. With the presence of reducing sugar, heated alkaline solution of cupric hydroxide (blue colour) gets reduced to insoluble yellow or red coloured cuprous oxide and it gets precipitated. Hence, formation of a brick red precipitate indicates the present of reducing sugars in the plant extract (Nigam and Ayyagari, 2007).

Since reducing sugars are widely prevalent in the plant kingdom, all the ethanolic extracts of leaves, stems, petals and roots for both dried and fresh material of *A. cathartica* L. revealed to have the positive response when tested with Fehling's solutions, i.e. formation of a brick red precipitation at the basal end of the test tubes. The present study of was in accordance with the results reported by Ayoola *et al*. (2008), whereby the ethanolic extracts of the leaves of *Carica papaya* L., stem bark of *Mangifera indica* L., leaves of *Psidium guajava* L. and the leaves of *Vernonia amygdalina* Del. showed the presence of reducing sugars when examined with Fehling's solutions. Rajesh *et al*. (2009) also reported the presence of reducing sugars on the aqueous and ethanol leaf extracts of *Capparis sepiaria* L. However, reducing sugars were not detected in the ethanolic extracts of

Ocimum sanctum, *Zanthoxylum aromaticum*, *Rhododendron setosum*, *Azadirachta indica*, *Colquhounia coccinea*, *Eltsholtzia fructicosa* and *Curcuma longa* (Chhetri *et al*., 2008).

Anthraquinones are phenolic compounds that typically form strongly coloured pigments covering the entire visible spectrum and are the agents of antibacterial, antifungal and cytotoxic agents (Jamil, 2010). Generally, anthraquinones are soluble in hot water or diluted alcohol and can be detected by Bontrager's test (Odugbemi, 2008). Initially, the plant extract is boiled with H_2SO_4 and filter while hot, after the filtrate shaken with chloroform, dilute ammonia is added and a rose to pink colouration in the ammonical layer denotes the present of anthraquinones (Ayoola *et al*., 2008).

The properties of antibacterial (Mohammad *et al*.,2010), antifungal (Mohammad *et al*.,2010), and cytotoxic (Kupchan *et al*., 1976) in *A. cathartica* L. can be correlated due to the presence of various bioactive chemical constituents such as anthraquinones which were detected on all the ethanolic extracts of leaves, stems, petals and roots (both dried and fresh plant materials) of *A. cathartica* L. with a rose pink to red colouration in the ammonical layer. Based on the preliminary phytochemical screening obtained by Deshmukh *et al*. (2011), anthraquinones were reported in the leaf, fruit and root derived suspension cultures of Indian Mulberry (*Morinda citrifolia*). Anthraquinones were also identified in the chloroform, ethyl acetate and methanol extracts of *Diospyros lotus* in the study of Uddin *et al*. (2011b), however, no anthraquinones were detected in the extracts of hexane. In addition, anthraquinones were also detected in the methanolic and aqueous extracts of *Lasienthera africanum* which have antibacterial properties (Adegoke and Adebayo-tayo, 2009).

Terpenoids constitute the largest family of natural products in plants such as steroids, essential oils and flavonoids and all natural compounds built up from isoprene subunits are denoted as terpenes (McGarvey and Croteau, 1995). The biological and ecochemical functions of terpenoids have not yet been fully investigated but some plants may produce volatile terpenoids in order to attract specific insects for pollination and those less volatile terpenoids with strongly bitter-tasting or toxic are involved in the protection of plants from being eaten by animals. Besides, terpenoids also play an important role as signal compounds and growth regulators of plants (Breitmaier, 2006). It should be noted that steroidal compounds are of importance and of interest in pharmacy due to their relationship with sex hormones (Santhi *et al*., 2011). In Salkowski test, chloroform and H_2SO_4 are added to the plant extract to form a layer. A red violet and green bluish colourations formed at the interface indicates the present of terpenoids and steroids, respectively (Chhetri *et al*., 2008).

With the presence of triterpene esters such as plumieride, ursolic acid, β-amyrin, β-sitosterol, lupeol and fluvoplumeirin (categorized under the group of terpernoids or steroids) in the *A. cathartica* L. (refer to Chapter 2), a matched result was obtained in the present study, i.e. all the ethanolic extracts of *A. cathartica* L. for different parts were showed positive response in the Salkowski's test. For both dried and fresh materials, the ethanolic leaf extract was showed the presence of steroids with the formation of green bluish colouration at the interface, which was in accordance with the results obtained by Chhetri *et al*. (2008), i.e. steroids were present in the ethanolic leaf extract of *Ocimum sanctum*, however, it contradicted with the ethanolic leaf extract of *Rhododendron setosum*. Moreover, only terpenoids were found in the ethanolic stem and root extracts of *A.*

cathartica L. this was in accordance with the results obtained by Kamba and Hassan (2010) but in contrast with the study of Oshomoh and Idu (2011).

In addition, both terpenoids and steroids were present in the petal extract of *A. cathartica* L. which was in accordance with the study reported by Devmurari *et al*. (2010), i.e. both terpenoids and steroids were found in the ethanolic petal extract of *Artemisia nilagirica*. Egwaikhide and Gimba (2007) also reported the presence of terpenoids and absence of steroids in the ethanolic extract of *Plectranthus glandulosis* whole plant (leaves, stems and root).

Saponins are high molecular weight glycosylated plant secondary metabolites under the group of glycosides which showed the characteristics of detergent and can be found in different plant parts such as roots, shoots, flowers and seeds. Saponin-containing plants have been used as soaps, as folk medicines especially in Asia and intensively used in food, veterinary and medical industries (Wiesman and Chapagain, 2003). Since saponins have a typical characteristic of detergent properties, a stable persistent froth will be observed from the plant extract when shaken with water vigorously in a test tube. Formation of an emulsion can also be observed after mixing with 3 drops of olive oil (Ayoola *et al*., 2008).

The present study showed that *A. cathartica* L. ethanolic extracts of stem, petal and root of dried and fresh plant materials have saponins with mild emulsion formed on top of the mixture between water and extract. No saponin was present in the ethanolic leaf extract of *A. cathartica* L. (dried and fresh materials) and this can be supported by the results of Santhi *et al*. (2011). Kulip *et al*. (2010) also reported no saponin present in the leaf extract

of *Alstonia angustiloba* from the family Apocynaceae. By referring to the review reported by Tiwari *et al*. (2011), ethanol was not a suitable extracting solvent for saponins and this might be one of the reasons that caused saponins to be absent in the ethanolic leaf extract of *A. cathartica* L. However, the study of Majaw and Moirangthem (2009) and Kasolo *et al*. (2010) were in contrast with the present study, i.e. saponins were present in the leaf extract of *Clerodendron colebrookianum* Walp. and *Moringa oleifera*, respectively. Olajuyigbe *et al*. (2011) also reported the presence of saponins in the ethanolic stem, petal and root extracts of *Waltheria indica* L.

Natural plant tannins represent a group of high molecular weight polyphenolic materials which are widely distributed throughout the plant kingdom. Generally, condensed tannins (catecholic tannins) are more resistant to microbial decomposition or polymerization compared to hydrolyzed tannins (gallic tannins) (Mallika and Dhar, 1980). Applications of tannins help to drain out all irritants from the skin, useful as an anti-inflammatory agent in burn and wound treatments based on their anti hemorrhagic and antiseptic potentials and many others (Ukoha *et al*., 2011). Besides, the presence of tannins and flavonoids in all the plants is likely to be responsible for the free radical scavenging effects observed (Ayoola *et al*. 2008). In the test of tannins with $FeCl_3$, a colour reaction occurred between the plant extracts and $FeCl_3$, whereby blue colour is observed for gallic tannins and green black for catecholic tannins (Chhetri *et al*., 2008).

In the present study, only catecholic tannins were observed in dried materials of leaf, stem and petal extracts and fresh materials of leaf, petal and root extracts. Natural products vary in both type and concentration in different parts of the plant and extraction is the

process used to withdraw the desired constituents from the plant material by using solvents of selected polarity, in which the desired substances are soluble (Jagessar and Cox, 2010). From the results obtained, tannins were not extracted from dried root and fresh stem, this might be caused by the unsuitability methods of plant materials preparation or due to the extracting solvent used (Tiwari *et al*., 2011). Chitravadivu *et al*. (2009) reported the presence of tannins in extracts from the root and leaf samples of *Cassia auriculata*, *Eclipta alba* and *Phyllanthus niruri*. Tannins were also detected in the ethanolic root extract of *Musa paradisiaca* Lam and *Morinda citrifolia* which were reported by the studies of Biswas *et al*. (2011) and Pal *et al*. (2012), respectively. In addition, Igbinosa *et al*. (2009) and Hosseinzadeh and Younesi (2002) revealed the presence of tannins in the ethanol extracts of *Jatropha curcas* L. stem bark and *Crocus sativus* petal, respectively.

Glycosides are usually compounds of plant origin made up of one or more sugars combined with an alcohol, a phenol or a complex molecule such as a steroid nucleus (Hollman, 1985). One of the popular glycosides present in many plants is cyanogenic glycosides which can release highly toxic HCN or prussic acid when the plant enzymes hydrolyze the glycosides to cyanide due to the damaged plant cells when chewed, crushed wilted or frozen (Knight and Walter, 2002). The presence of glycosides can be examined by hydrolyzing the plant extract with HCl and neutralizing with NaOH. A red precipitate will be formed after few drops of Fehling's solution is added to the plant extract (Uddin *et al*., 2011a).

Plumieride coumarate and plumieride coumarate glocoside were the iridoid glycosides isolated from *A. cathartica* L. (Coppen, 2001). Therefore, according to the

results obtain in the present study, all the ethanolic extracts of leaves, stems, petals and roots, no matter dried or fresh materials of *A. cathartica* L. showed a positive response with glycosides test with the formation of red precipitate at the basal end of the test tubes. However, the results obtained by Uddin *et al*. (2011a) were contradicted with the present study, i.e. no glycosides were detected in the extracts of galls, leaves, barks and roots of *Pastacia integerrima* but glycosides were detected in the leaf extracts of *Acalypha wilkesiana*, *Vernonia amygdalina*, *Mangifera indica*, *Carica papaya* and *Ficus glomerata* (Gotep *et al*., 2010; Suleiman, 2011; Poongothai *et al*., 2011). Furthermore, study of Mohammad *et al*. (2011) also showed the presence of glycosides in stem and root extracts and absence in flower, leaf and bark extracts of *Rhododendron arboreum*.

The most important usage of essential oil are in urology, dermatology, sleep and nervous disorders, laxatives, erosive gastritis, cardiac and vascular systems, immunomodulating drugs, colds and coughs (Svoboda and Hampson, 1999). Essential oils are among the most complex substances synthesized by aromatic plants at the peak of their development. They consist of huge number of medicinal substances, which is the reason why therapeutic applications is so wide. Essential oils have extraordinary diffuse and penetrating functions, thus they can be used to disinfect and strengthen the immune system such as antiseptic, antibiotic, antiviral, bacteriostatic and bactericidal, antimycotical and fungicidal and wound healing, to stimulate and promote functional equilibrium and to regulate the neuro-endocrine functions (Balz, 1996). Phytochemical screening of essential oils can be carried out using NaOH followed by adding HCl. White precipitate indicates the present of essential oils (Ahumuza and Kirimuhuzya, 2011).

Since *A. cathartica* L. has the properties of relieving cough, wound healing and antimicrobial as stated in Chapter 2, certain constituent such as essential oils existed in all the ethanolic extracts of leaves, stems, petals and roots (both dried and fresh materials). Kubmarawa *et al*. (2007) reported the presence of essential oils in the ethanolic leaf extracts of *Boswellia dalzielii* Hutch, *Callotropis procera*, *Ximenia americana* L, ethanolic stem extracts of *Acacia albida* Del., *Acadia nitolica* (Gull & Par) Kuntze, *Acacia senegal* L., *Acacia tortilis*, *Combretum molle*, *Commiphora kerstingii* Engl and ethanolic root extracts of *Balanites aegyptiaca, Terminalia avicenoides*. Moreover, Shanthi *et al*. (2010) and Idu *et al*. (2007) also reported the presence of essential oils in the root extract of *Hemidesmus indicus* L. and flower extract of *Senna alata*, respectively. However, ethanolic stem extracts of *Afromosia laxiflora*, *Amblygonocarpus andogenesis*, *Anogeissus leiocarpus* DG, *Butyrospermum paradoxum* and ethanolic root extracts of *Acacia sebriana*, *Afzelia africana*, *Aristolochia albida* Ducha, *Jatropha curcas* L. showed to have negative response on the screening of essential oils (Kubmarawa *et al*., 2007).

Flavonoids are class of plant secondary metabolites often present in plant vacuoles derived from the condensation of a cinnamic acid with three malonyl-CoA groups catalyzed by the chalcone synthase enzyme (Packer, 2001). The class of flavonoids contains more than 8000 known compounds and this number is constantly growing because of the great structural diversity arising from the various hydroxylation, methoxylation, glycosylation and acylation patterns (Rice-Evans and Packer, 2003). There are several health beneficial properties of dietary flavonoids recognized in human for their antioxidant and antiproliferative effects which may protect the body from various diseases, such as cancer, cardiovascular disease and inflammatory (Yang *et al*., 2008). Flavonoids in the plants can

be detected using two methods, i.e. concentrated H_2SO_4 (Method I) and dilute NaOH (Method II), a yellow colouration that disappear on standing (Ayoola *et al*., 2008) and formation of golden reddish precipitate (Kadiri and Ajayi, 2009) indicates the present of flavonoids, respectively.

As stated in Chapter 2, quercetin, kaempferol, naringenin and glabridin were the examples of flavonoids isolated from *A. cathartica* L. By using Method I, flavonoids were detected in the ethanolic extracts of stems, petals and roots of *A. cathartica* L. for both dried and fresh materials. The results of the present study were in accordance with study of Govindappa *et al*. (2011) and Pal *et al*. (2012), i.e. flavonoids existed in the ethanolic stem and petal extracts of *Crotalaria pallid*a and root extract of *Morinda citrifolia*, repectively. Meanwhile, a golden reddish precipitate was formed in the ethanolic extracts of petals and roots (dried and fresh materials) when tested with NaOH. No flavonoids were observed in the ethanolic leaf extract of *A. cathartica* L. which was in accordance with the results obtained by Santhi *et al*. (2011), whereby flavonoids were absent in the ethanolic leaf extract of *Momordica charantia* and *Nerium oleander*. However, flavonoids were present in the ethanolic leaf extract of *Cardiospermum halicacabum* L. which was contradicted with the present study of *A. cathartica* L. (Viji and Murugesan, 2010).

Plants are serving several purposes in health, nutrition, beauty and medicine. Since time immemorial, man has been using plant extracts to protect against several diseases and also to improve health and lifestyle. Plants are susceptible to damage caused by active oxygen and thus develop numerous antioxidant defense systems resulting in formation of numerous potent antioxidants (Mishra *et al*., 2007). An antioxidant is any substance that

significantly delays or prevents oxidation of cell content like proteins, lipids, carbohydrates and DNA which can be classified into first line defence antioxidants, second line defence antioxidants and third line defence oxidants (Gupta and Sharma, 2006).

In the present study, the antioxidant activities of ethanolic leaf, stem, petal and root extracts of *A. cathartica* L., for both dried and fresh plant materials were determined by DPPH free radical scavenging assay which is popularly used in antioxidant screening due to its simple and rapid way of measuring the ability of antioxidants present in the plant extracts to trap the free radicals (Mythili *et al*., 2011). The DPPH$\bullet$ radical is one of the few stable organic nitrogen radicals, which bears a deep purple colour. This assay is based on the measurement of the reducing ability of antioxidants toward DPPH$\bullet$ which can be evaluated by measuring the decrease of its absorbance at 517 nm using UV-VIS spectrophotometer (Prior *et al*., 2005). The phenolic compounds presence in the plant extracts can quench DPPH free radicals by providing hydrogen atom or by electron transfer , conceivably via a free radical attack on the DPPH molecule, which in turn convert the purple colour of DPPH to α,α -diphenyl-1- β -picrylhydrazine (yellow coloured) (Maizura *et al*., 2011). Hence, the more rapidly absorbance decreases, the more potent antioxidant activity of the extract in terms of hydrogen atom donating capacity or electron transfer ability (Gowri and Vasantha, 2010).

The radical scavenging activity between the antioxidants and DPPH$\bullet$ can be calculated using the formula of $([A_b\text{-}A_a]/A_b)$ X 100%, where A_b is the absorbance value of the control sample and A_a is the absorbance value of the extract (Ayoola *et al*., 2008). IC_{50} was defined as the concentration of plant extract that causes 50% loss of the DPPH activity

which can be determined from the graph of percentage scavenging activity of DPPH versus extract concentration (Prior *et al*., 2005). A lower IC_{50} value would reflect greater antioxidant activity of the extract and vice versa. In addition, the stock solution of DPPH was prepared in 95% methanol in the present study of *A.cathartica* L. due to its ability to generate stable free radicals in methanolic solution (Haripyaree *et al*., 2010).

Flavonoids and tannins are phenolic compounds and plant phenolics are a major group of compounds that act as primary antioxidants or free radical scavengers (Ayoola *et al*, 2008). In the present study of phytochemical screening, both flavonoids and tannins were found to appear in the extracts of *A. cathartica* L., thus, the antioxidant activities were also detected in all the leaf, stem, petal and root extracts of *A. cathartica* L. via the DPPH assay. From the results obtained, the power of scavenging activity of all the leaf, stem, petal and root extracts increased with increasing concentrations from 0 to 100 μg/ml. As compared between the dried and fresh plant materials examined, i.e. leaves, stems, petals and roots of *A. cathartica* L., all the dried plant materials showed a better percentage scavenging activity of DPPH and IC_{50} compared to the fresh plant material due to differences in water content within different plant tissues in the fresh plant materials that might have affected the readings in this study (Tiwari *et al*., 2011).

Among the crude ethanolic extracts tested, root extract from *A. cathartica* L. appeared to be as potent as ascorbic acid (88.40 ± 1.02%) with a maximum inhibition of 89.21 ± 0.65% (IC_{50}= 2.40 μg/ml) and 83.60 ± 1.00% at 100 μg/ml (IC_{50}= 3.49 μg/ml) at 100 μg/ml for both dried and fresh materials, respectively. This indicated that the highest antioxidant activity occurred in the root extract. The results of the present study of were

supported by the study of Chew *et al*. (2012) and Jamaludin *et al*. (2011), whereby the root extract of *Leucas aspera* and *Donax grandis*, respectively exhibited the greatest free radical scavenging and IC_{50} value among the plant extracts. However, it was in contrast with the study reported by Cai *et al*. (2012), i.e. the root extract of *Houttuynia cordata* showed the weakest antioxidant activity among the different plant parts tested. Apart from that, Ayoola *et al*. (2008) revealed that an increase in total antioxidant status in the plant extract shown to be important in recovery from malaria because antioxidant activity possess in the plants can counteract the oxidative damage induced by the malaria parasite, therefore, the root tea from *A. cathartica* L. has been used in various formulations for the treatment of malaria symptoms (Nellis, 1997) which has high antioxidant capacity.

Flowers are the important reproductive parts of the plants which contain some active compounds that exhibit medicinal properties and used to cure various kinds of health problems in the living organism (Britto and Gracelin, 2011). In the present study of phytochemical screening, the petal extract was observed to have all the phytochemical constituents, i.e. reducing sugars, anthraquinones, terpenoids and steroids, saponins, tannins, glycosides, essential oils and flavonoids. With the contribution of these phytochemical constituents stated above, the ethanolic dried petal extracts of *A. cathartica* L. was comparable with ascorbic acid and was the second potent free radical scavenger with maximum inhibition of 88.41 ± 1.58% (IC_{50}= 2.59) at 100 μg/ml compared to other dried materials (i.e. leaves and stems). Due to the influenced of water content in fresh petal extract of *Allamanda cathartica* L., its percentage scavenging activity of DPPH was lower (64.73 ± 11.21% with IC_{50} of 4.83) compared to the dried petal extract at the same concentration. The results of the present study of were in accordance with the studies of

Guo *et al*. (2001) and Cai *et al*. (2012). Besides, ethanolic petal extract of *Eupatorium odoratum* L. also showed the second potent free radical scavenger with IC_{50} of 50.90 µg/ml in the study of Amatya and Tuladhar (2011).

As for the ethanolic stem extract of *A. cathartica* L., it was the third (dried material) (85.22 ± 2.16% with IC_{50} of 4.05 µg/ml at 100 µg/ml) and second (fresh material) (73.20 ± 1.76% with IC_{50} of 4.14 µg/ml at 100 µg/ml) potent free radical scavengers among the extracts of different plant parts and it was less potent compared to ascorbic acid. The moderate antioxidant activity of the stem extract of *A. cathartica* L. was in accordance with the studies of Sunil *et al*. (2010), Amin *et al*. (2012) and Govindappa *et al*. (2011). Meanwhile, the study of Diwani *et al*. (2009) contradicted the present study, whereby the stem extract of *Jatropha curcas* was less potent than the extracts of leaves, nodes and roots. Ripa *et al*. (2009) also reported that the stem extract of *Passiflora edulis* demonstrated the strongest antioxidant activity compared to the leaf extract.

For both dried and fresh leaf materials, ethanolic leaf extract of *Allamanda cathartica* L. was the least potent (less potent than ascorbic acid) showing a maximum inhibition of 70.10 ± 12.55% (IC_{50}= 4.58 µg/ml) and 62.84 ± 12.70% (IC_{50}= 5.04 µg/ml) at 100 µg/ml, respectively. This might be due to less bioactive compounds appeared at the above ground part (i.e. leaves) compared to the underground part (i.e. roots) (Tiwari *et al*., 2011). The present study of *A. cathartica* L. was in accordance with the results obtained by Gowri and Vasantha (2010), Gowri *et al*. (2011) and Patil *et al*. (2011), i.e. ethanol leaf extract of *Sesbania grandiflora* L. Pers., *Acacia nilotica* L. Del. and *Erythrina indica* expressed low percentage of free radical scavenging activity than the rest of the plant

extracts. In contrast, the leaf extract of *Spinacia oleracea* L. showed high radical scavenging activity than the other extracts (Evanjelene and Natarajan, 2011).

The major photosynthetic pigments of higher plants can be divided into two groups, the chlorophylls and the carotenoids (Boyer, 1990). Plants possess pigments that can absorb light in specific regions of the visible light spectrum, such as chlorophyll *a* readily absorbs violet/blue and red light, chlorophyll *b* absorbs more in the blue and orange-red ranges and carotenoid absorbs light from violet into the greenish-blue range (Nishio, 2000). Usually, the plant material can be directly extracted without any pretreatment. But, in the case of plant sample with extremely high water content, the sample should be in the dry condition before extraction; otherwise, the lipid-soluble pigments cannot be adequately extracted and the absorption maxima of pigments in organic solvents are shifted towards longer wavelengths when water is present (Lichtenthaler, 1987).

Extraction of photosynthetic pigments can be performed using a mortar and pestle, a motor-driven grinder or by shaking the plant material with glass or steel balls. Compared to a motor-driven grinder, a mortar and pestle are safer and easier to clean. During pigment extraction, it is advisable to start with a small volume of solvent and add more solvent to give a final defined volume of the extract solution when the plant sample is well homogenized (Lichtenthaler and Buschmann, 2001). The samples have to be homogenized in order to improve the extraction efficiency as a result of cell wall disruption (Wasmund *et al*., 2006). After centrifugation for 5 minutes, the clear extract solution is placed into a cuvette for quantitative determination in the UV-VIS spectrophotometer (Lichtenthaler and Buschmann, 2001).

The selection of the solvent to promote the extraction is a very important issue since it determines the degree of affinity to the chemical composition of the substances to be extracted. Apart from the dissolution ability towards the compounds to be extracted and quantified, the solvent also plays an important role in cell lysis, whereby more aggressive solvents can increase the extraction yields in plant cell with strong cell walls (Henriques *et al*., 2007).

In the present study, the major plant pigments such as chlorophylls (chlorophyll *a*, chlorophyll *b*) and carotenoids were extracted from *in vivo* leaves, stems, petals and calli (*in vitro*) using three different extracting solvents, i.e. 0.15% HCl in 99.9% methanol, 0.01% HCl in 70% acetone and 95% ethanol at difference volumes (5.0, 12.5, 20.0 and 25.0 ml). The extracting solvents used are categorized as polar solvents which can mix up with water in the extraction of chlorophylls and carotenoids from water-containing plant samples (Lichtenthaler and Buschmann, 2001) and the purpose of adding HCl into the solvent is to maintain the pH stability (Krstic *et al*., 1996). The most suitable extracting solvent at specific volume that revealed maximum absorption for the pigments for each plant sample was determined. The extraction was carried out in the dim light condition to avoid the destruction of plant pigments (Wasmund *et al*., 2006).

Absorption in the red and blue maxima is highest in freshly isolated chlorophyll and the absorption maxima of extracted pigments strongly rely on the type of solvent and the type of spectrophotometer used (Lichtenthaler and Buschmann, 2001). Methanol was the first solvent to be used to extract chlorophylls (Henriques *et al*., 2007). In pigment extraction from the leaves of *A. cathartica* L., 0.15% HCl in 99.9% methanol with a

volume of 20 ml was the most suitable extracting solvent among the other two solvents examined. By using 20 ml of 0.15% HCl in 99.9% methanol, maximum absorption for both chlorophyll *a* at the wavelengths of 663 (Absorbance of 2.443) and 437 (Absorbance of 2.914) nm and chlorophyll *b* at the wavelength of 468 (Absorbance of 2.914) nm from the leaf extract can be observed. Other extracting solvents (i.e. .01% HCl in 70% acetone and 95% ethanol) showed lower absorbance values of chlorophyll *a* and chlorophyll *b* at the red and blue regions of visible spectrum. This might be due to the extracting solvents of acetone and ethanol which have lower permeability to the plant cell wall (leaves) compared to 0.15% HCl in 99.9% methanol (Henriques *et al*., 2007). The results reported by Dere *et al*. (1998) was in accordance with the present study. However, it was contradicted by the study of Dunn *et al*. (2004), whereby acetone solvent was more preferred for the extraction of chlorophylls from leaves of higher plants compared to methanol solvent.

Chlorophyll *a* and chlorophyll *b* were also extracted from the stems of *A. cathartica* L. using three different extracting solvents. Chlorophylls are bound to thylakoid membranes in the chloroplasts and the difference between these two chlorophylls is a methyl moiety in chlorophyll *a* replaced by a formyl group in chlorophyll *b* (Kamffer *et al*., 2010). In comparison between methanol, acetone and ethanol at different volumes tested, 95% ethanol at the volume of 20 ml was the most suitable extracting solvent and intense absorption can be observed from the absorption spectrum at 664 (Absorbance of 0.864) and 435 (Absorbance of 1.339) nm for chlorophyll *a* and 467 (Absorbance of 0.943) nm for chlorophyll *b* using this solvent. By using acetone as the extracting solvent, chlorophyll *b* was unable to extract out from the stem samples at most of the volumes tested, i.e. 5.0, 12.5 and 25.0 ml. This might be due to the destruction of chlorophylls by light photochemically

during the measurement of pigments using UV- spectrophotometer as the absorption of chlorophylls decrease with time due to the formation of allomeric chlorophyll forms. It might also due to too much solvent was utilized, i.e. at 25.0 ml of methanol and acetone, the pigments will be too diluted and absorbance readings will no longer be possible to obtain (Lichtenthaler and Buschmann, 2001).

There are several different classes of pigments responsible for colouration but in many yellow/orange/red flowers, the pigments synthesized by the plant are carotenoids. Carotenoids are natural pigments found in all parts (root, leaf, flower, fruit and seed) but they are usually most noticeable in the flowers (Tinoi *et al*., 2006), comprising a class of hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). For extraction, water content is considered an important factor and working with low-moisture samples simplifies the extraction process (Rodriguez, 2001). In the present study, the most suitable extracting solvent that successfully extracted the pigment of carotenoid from the petal was 95% ethanol at the volume of 5 ml with maximum absorption at 439 (Absorbance of 1.940) and 469 (Absorbance of 1.679) nm. The solvent used for extraction must be chosen according to the polarity of the pigments presumably present (Rodriguez, 2001) and the solvent must be pure (without oxygen, acids or halogens) to avoid degradation for the extraction of carotenids (Vargas *et al*., 2000). Therefore, ethanol was the correct solvent for the extraction of carotenoids which was in accordance with the study reported by Bunghez and Ion (2011). However, carotenoids extraction by ethanol method was not well documented from *Citrus grandis* Osbeck (Tao *et al*., 2010), although it was successfully extracted in other plants (Howard *et al*., 2000; Moros *et al*., 2002). Besides, if the plant

tissue is previously dried, the water-immiscible solvents are used such as petroleum or ethyl ether; with fresh materials acetone or ethanol are used.

Additionally, the synthesis of photosynthetic pigments in callus cells relies on many factors, i.e. part of plant from which explants are obtained, conditions of callus subculturing, mainly substrate characteristics and plant species (Krstic *et al*., 1996). From the results obtained, none or less chlorophyll *a*, chlorophyll *b* and carotenoid pigments were present in the methanol, acetone and ethanol extracts of calli generated from the explants of *A. cathartica* L. due to the weekly differentiated of plastid system (Dumitrescu, 2002). It was supported by the study of Stobart *et al*. (1967), whereby the chlorophyll content of green callus cultures and carotenoids in colourless callus of *Kalanchoe crenata* were low in comparison with *K. crenata* leaves. Moreover, chlorophyll was not detected in dark-grown callus of *K.e crenata* but appeared in callus which had been exposed to light in the study of Stobart and Thomas (1968). Among the three different extracting solvents tested, 0.01% HCl + 70% acetone at the volume of 20 ml gave the best absorption characteristic on the visible spectrum for pigment extraction from the calli. Birchem *et al*. (1981) reported that acetone extracts of pigments from *Pinus palustris* callus contain quantifitable levels of chlorophylls and carotenoids, but the amount of pigment contained in comparable extracts of *P. elliottii* callus is practically nil.

The plant-based colourants offer an ecologically friendly alternative to synthetic dyes because they come from plants, which can be renewable nontoxic resources and are biodegradable (Duerr, 2010). In the present study, PVA was prepared with distilled water under the hot condition because it is soluble in hot water but insoluble in cold water and

common organic solvents (Saxena, 2004). PVA can be used as a thickener, stabilizer and binder which provide excellent adhesion to porous, water absorbent surfaces (Katz, 2005). On evaporation of water, transparent films were formed which have high tensile strength and tear resistance and if left in the environment, it will be broken down rather than persist as some plastics do requiring clean-up (Saxena, 2004).

All paints contain a film forming component (i.e. PVA), a solvent thinner (i.e. extracting solvent) and a pigment (i.e. chlorophylls and carotenoids from the leaves and petals). In conventional paint, the film forming component and pigment are deposited on the surface as the solvent evaporates. In order to develop and provide the new potential of natural colourants for the industries of paint and textile, the natural colourants present in the pigments of leaves (green) and petals (orange yellowish) of *A. cathartica* L. were extracted using the best solvent that has been identified in the pigment extraction, i.e. 95% ethanol and 0.15% HCl in 99.9% methanol to obtain the better colouration. The plant extracts (supernatants) were then mixed with PVA in the mixing ratio of 1:1 and the mixture was painted on the glass slides and cotton wools. The experiment was further conducted with the salt and heat tests at different parameters on the glass slides and the pigments colour degradation was observed from cotton wools for both leaves and petals. Dry powdered plant materials were more concentrated and gave better colouration compared to the fresh plant materials (Buchanan, 1999).

In continuing replacement of synthetic dyes, there is a growing interest on natural colourants from plant sources for food, paint and textile manufacturers imposed by the consumers. Replacing synthetic dyes with natural colourants posed a challenge since the

colour and stability of plant pigments are influenced by several factors, such as structure and concentration of the pigment, light intensity, pH, presence of metallic ions, temperature, sugars, enzymes, ascorbic acid, oxygen and their degradation products, among others (Tang and Norziah, 2007). By knowing the limitations of each pigment means that a specific pigment can be prevented for certain applications, in which the conditions are unfavorable for the pigment and that alternatives can be find or that attempts can be made to increase the stability of the pigments by formulation (Mortensen, 2006), such as by adding a suitable stabilizer.

Salt test using NaCl at different concentrations, i.e. 0.5, 1.0 and 1.5% was denoting the natural rainwater which was slightly acidic due to its interaction with carbon dioxide (CO_2) in the atmosphere (Nelson, 2007). Meanwhile, the heat test at temperature of 40ºC (at different ranges of times) indicated the extreme air temperature around the environment. The effects of both tests on the pigments were studied which provided an idea for the paint and textile manufacturers regarding to the stability of the pigments of *A. cathartica* L. toward the environmental conditions.

Chlorophylls are the source of green colour and their structure may be modified by the enzymatic or chemical reactions (Scipioni *et al*., 2010). From the results obtained, the absorbance values for the chlorophyll pigments at 662 and 430 nm were gradually reduced with time (from 0 to 60 minutes) when the glass slides coated with ethanolic and methanolic leaf extracts immersed in the salt solutions (NaCl) at the concentrations of 0.5, 1.0 and 1.5%. Similar trend was observed for the heat test of both ethanolic and methanolic

leaf extracts due to the continuous heating in the oven which inactivated chlorophyllase and enzymes responsible for senescence and rapid loss of green colour (Koca *et al*., 2007).

Besides, due to the decreasing of absorbance values on the chlorophyll pigments, the appearance of green colouration on the glass slides also discoloured due to the degradation of chlorophylls to pheophytins by the replacement of magnesium in the chlorophyll with hydrogen under the influence of salt solutions and temperature in the oven (Koca *et al*., 2007). Also, the colour tone of green was reduced gradually from a bright green into olive green for the study of colour degradation on cotton wools. Maintenance of green colour in methanolic extract was better than that in the ethanolic extract for the chlorophyll pigments of leaves since chlorophylls were more thoroughly extracted with methanol (Bahceci *et al*., 2005).

Apart from that, the most characteristic feature of the carotenoid structure is the presence of several conjugated double bonds in the chain. The polyene chain is responsible for the light absorption properties and also for the susceptibility of carotenoids to degradation under some extreme factors (Landrum, 2009). In *A. cathartica* L., the flowers with bright yellow colour are a mark of quality for the manufacturers. From the results obtained, the absorbance values at 450 nm for carotenoid pigments extracted in ethanol and methanol solvents decreased slowly with time for both salt and heat tests due to molecular structure destabilization of the carotenoid pigments and hence caused the decolorized of its natural colour (Laleh *et al*., 2006), i.e. the appearance of orange yellowish colouration from the petal extract of *A. cathartica* L. on the glass slides became paler with the decreasing of the absorbance values.

On the other hand, isomerization of *trans*-carotenoids to the *cis*-isomers and oxidation reactions would occur particularly during the heat test, whereby their biological activity will be altered and caused colour degradation (Rodriguez-Amaya and Kimura, 2004). In addition, the visual orange yellowish colour was lost gradually from a bright orange yellowish into a pale colour for the study of colour degradation on cotton wools. In addition, between ethanolic and methanolic petal extracts of *A. cathartica* L., the maintenance and stability of orange yellowish colour in ethanolic extract were better than that in the methanolic extract and the orange yellowish colour might be due to the presence of xanthophylls in the petals of *A. cathartica* L. (Mlodzinska, 2009).

CHAPTER 6

SUMMARY AND CONCLUSION

Throughout this study, only fungus and bacteria contaminations were observed in leaf, stem and nodal explants of *Allamanda cathartica* L., where fungus contamination occurred more frequently than bacteria contamination. The results obtained in this study showed that 0.1% of $HgCl_2$ was the most suitable sterilant which could sterilize the leaf explants with total percentage of contamination of 2.5 ± 1.4%, i.e. no bacteria contamination and the percentage of fungus contamination was 2.5 ± 1.4%. Meanwhile, 0.2% of $HgCl_2$ was the most suitable sterilant which could sterilize the stem explants with total contamination percentage of 5.0 ± 2.9% (i.e. no bacteria contamination and fungus contamination of 5.0 ± 2.9%) and nodal explants with total contamination percentage of 8.3 ± 4.4% (i.e. fungus contamination of 5.0 ± 2.9% and bacteria contamination of 3.3 ± 1.7%).

The results obtained in the present study showed that the MS basal medium which acted as a control medium was not suitable for callus induction from both the leaf and stem explants. The results also revealed that the most suitable medium for callus formation was MS medium supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L BAP for both the leaf (100 ± 0%) and stem (100 ± 0%) explants. A significant difference on the percentage of leaf and stem's callus formation was observed between the treatment combinations of 1.0 mg/L 2,4-D and BAP at concentrations of 0.5, 1.0 and 1.5 mg/L. For leaf explants that were cultured on MS supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L BAP, it showed the shortest time for initiation of callus formation (6.0 ± 0 days) with the heaviest fresh and dry weights

(0.3857 ± 0.0939 g and 0.0707 ± 0.0549 g) of yellow-white, greenish friable callus. For stem explants that were cultured on MS medium fortified with 1.0 mg/L 2,4-D and 1.0 mg/L BAP, the callus was initiated after 5.0 ± 0.3 days with brown-white, greenish friable morphology with fresh and dry weights of 0.4177 ± 0.0108 g and 0.0207 ± 0.0009 g, respectively.

Plant regeneration of *A. cathartica* L. involved the shoot multiplication from the nodal explants and shoot elongation. In the study of shoot multiplication, no shoot was formed on MS basal medium, while MS medium supplemented with 5 mg/L of BAP showed the best response (100 ± 0%) for shoot multiplication compared to MS medium supplemented with 1 (90.0 ± 5.7%) and 3 (96.7 ± 3.3%) mg/L of BAP. Multiple shoots (6.0 ± 0.6 shoots per explant) formed from the nodal explants using greenish leaves. Meanwhile, for the study of shoot elongation, the elongated shoot was obtained (1.01 ± 0.07 cm) and the formation of white hairy root was started to be observed on Week 4.

For microscopic studies using the scanning electron microscope (SEM), the differences between the leaf and stem specimens from *in vitro* and *in vivo* (intact) shoots were distinguished. From the SEM micrographs obtained, the number of stomata on the abaxial surface was more abundant compared to the adaxial surface for both *in vitro* and *in vivo* leaves. No stoma found on *in vitro* stem but few stomata were observed from *in vivo* stem. Several structures such as cuticle, epidermis, cortex, interfascicular region and pith were also identified from the cross sections of *in vitro* and *in vivo* stem specimens. Moreover, the surfaces of *in vitro* specimens were found to be less contaminated compared to the *in vivo* specimens of leaves and stems.

In the study of phytochemical screening, all the ethanolic extracts of leaves, stems, petals and roots of *A. cathartica* L. (for both dried and fresh plant materials) were screened to detect photochemical constituents, which included reducing sugars, anthraquinones, terpenoids and steroids, glycosides and essential oils that gave positive responses when tested with specific phytochemical tests. For both dried and fresh plant materials, saponins and flavonoids were absent in the leaf extract but present in the stem, petal and root extracts. Meanwhile, tannins were not found in the dried plant material of root extract and fresh plant material of stem extract compared with other ethanolic extracts of *A. cathartica* L.

In the study of antioxidant activities, the ethanolic extracts from dried and fresh plant materials, i.e. leaves, stems, petals and roots of *A. cathartica* L. were screened for their free radical scavenging properties using ascorbic acid as standard antioxidant. Free radical scavenging activity was evaluated using DPPH free radical. For dried plant materials, the overall antioxidant activity of ethanolic extract of roots (IC_{50} of 2.40 μg/ml) was found to be the strongest with percentage of DPPH scavenging of 89.21 ± 0.65%, at 100μg/ml, followed in descending order by the extracts of petals (IC_{50} of 2.59 μg/ml), stems (IC_{50} of 4.05 μg/ml) and leaves (IC_{50} of 4.58 μg/ml) which showed the percentage scavenging activities of 88.41 ± 1.58%, 85.22 ± 2.16% and 70.10 ± 12.55%, respectively. For fresh plant materias, ethanolic extract of roots (IC_{50} of 3.49 μg/ml) showed the best scavenging activity of 83.60 ± 1.00% at 100 μg/ml, followed by the extracts of stems (IC_{50} of 4.14 μg/ml), petals (IC_{50} of 4.83 μg/ml) and leaves (IC_{50} of 5.04 μg/ml) which gave the percentage scavenging activities of 73.20 ± 1.76%, 64.73 ± 11.21% and 62.84 ± 12.70%, respectively. As a reference standard, ascorbic acid showed the IC_{50} value of 3.28 μg/ml with percentage scavenging activities of 88.40 ± 1.02% at 100 μg/ml.

Pigment extraction was also carried out for the extraction of major photosynthetic pigments present in the leaves, stems, petals and calli of *A. cathartica* L., i.e. chlorophyll *a*, chlorophyll *b* and carotenoids. For pigment extraction from the leaves, the pigments of chlorophyll *a* (Absorbance of 2.443 at 663 nm and Absorbance of 2.914 at 437 nm) and chlorophyll *b* (Absorbance of 2.914 at 468 nm) showed the maximum absorption in 20.0 ml of 0.15% HCl in 99.9% methanol. For pigment extraction from the stems, 95% ethanol in 20 ml gave the best absorption characteristic among the other extracting solvents, i.e. chlorophyll *a* was absorbed strongly at 664 (Absorbance of 0.864) and 435 (Absorbance of 1.399) nm and chlorophyll *b* was absorbed strongly at 467 (Absorbance of 0.943) nm. For pigment extraction from the petals, 95% ethanol in 5.0 ml was the most suitable extracting solvent which gave the maximum absorption of carotenoid pigment at 439 and 469 nm with absorbance values of 1.940 and 1.679, respectively. For pigment extraction from the calli, less or none chlorophyll *a*, chlorophyll *b* and carotenoid pigments were noticed and 0.01% HCl in 70% acetone (20 ml) gave better absorption characteristic for chlorophyll *a* at 441 nm with the absorbance value of 0.678.

The coloured coating was developed from the natural colours of leaves and petals of *A. cathartica* L. The green (chlorophylls) and orange yellowish (carotenoids) colours from leaves and petals, respectively were painted on the glass slides which then subjected to the salt and heat tests. The absorbance values were measured at 662 and 430 nm for chlorophylls and 450 nm for carotenoids and compared. For both ethanolic and methanolic extracts, the absorbance values were observed to decrease gradually with time when the glass slides coated with chlorophyll and carotenoid pigments examined in the salt (from 0 to 60 mins) and heat (from 24 to 168 hours) tests. For the study of pigment's colour

degradation on cotton wools, the colour tones of the chlorophyll and carotenoid pigments extracted from ethanol and methanol solvents disappeared slowly from bright to pale colour, i.e. bright green to olive green for chlorophylls and bright orange yellowish to pale orange yellowish for carotenoids from Week 0 to Week 8. However, the methanol and ethanol extracts showed better colour maintenance and pigment stability for leaves and petals, respectively according to the measured absorbance values.

The present work on tissue culture studies, phytochemical screening, determination of antioxidant activities, pigment extraction and coloured coating development from natural pigments of *A. cathartica* L. were recent and innovative. Compare with other *Allamanda* species such as *A. schottii* and *A. blanchetti* (Sousa *et al*., 2009; Schmidt *et al*., 2006), works on these studies from *A. cathartica* L. by other researchers were very limited. Therefore, the interesting findings on these studies could add to the present knowledge and information can be shared with others in similar field.

In the future, regeneration from callus can be obtained by culturing the callus into medium containing a high concentration of cytokinin and a low concentration of auxin for shoot induction. After induction of shoot, subsequently cultures can be placed into a rooting medium with a high concentration of auxin for root induction. The studies of direct and indirect somatic embryogenesis can also be done on *A. cathartica* L. using different types of explants with different types of PGRs. Moreover, production of synthetic seeds of *A. cathartica* L. can be tried out for the future because it is an alternative method for mass propagation that enables multiplication of seedless plants or plants with problem in seed propagation to ensure the continuous supply of desired plant species. Synthetic seeds can be

produced through the encapsulation of micro shoots from shoot multiplication or somatic embryos using the beads formed from sodium alginate ($NaC_6H_7O_6$) and calcium chloride dehydrate solution ($CaCl_2 \bullet 2H_2O$).

Furthermore, the total contents of phenolic and flavonoid determination can also be performed on different plant parts for the future study of *A. cathartica* L. via spectrophotometric analysis, in which the total phenolic content is determined by using Folin-Cicocalteu reagent and expressed as gallic acid equivalents (GAE) per gram of dry weight (DW), while total flavonoid content is determined using aluminium chloride colorimetric assay and expressed as rutin equivalents (RE) per gram DW. In addition, the carotenoid constituents that are present in the petal of *A. cathartica* L. can be further determined by HPLC method, i.e. by comparing their retention times in HPLC and their UV-VIS absorption spectra with the reference standard of carotenoids such as β-cryptoxanthin, canthaxanthin, fucoxanthin, lutein, neoxanthin, violaxanthin, zeaxanthin, α-carotene and β-carotene.

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APPENDICES

APPENDIX A

Table A (I): Nutrient composition of MS medium (Murashige and Skoog, 1962)

Nutrient	Concentration (mg/L)
(a) Macronutrient	
$MgSO_4 \bullet 7H_2O$	370
KH_2PO_4	170
KNO_3	1900
NH_4NO_3	1650
$CaCl_2 \bullet 2H_2O$	440
(b) Micronutrient	
H_3BO_4	6.2
$MnSO_4 \bullet H_2O$	15.6
$ZnSO_4 \bullet 7H_2O$	8.6
$NaMoO_4 \bullet 2H_2O$	0.25
$CuSO_4 \bullet 5H_2O$	0.025
$CoCl \bullet 6H_2O$	0.025
KI	0.83
(c) Iron	
$FeSO_4 \bullet 7H_2O$	27.8
Na_2EDTA	37.3
(d) Vitamin	
Thiamine HCl	0.5
Pyridoxine HCl	0.5
Nicotinic acid	0.05
Myo-inositol	100
(e) Sucrose	30 000
(f) Agar	8 000
(g) pH	5.7 ± 0.1

APPENDIX B

RESEARCH ARTICLE PUBLISHED

1. **Wong, K. F.** and Taha, R. M. (2012). The effect of 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine on callus induction and plant regeneration of *Allamanda cathartica* – a valuable medicinal plant. *Research Journal of Biotechnology*. **7(3)**: 75-80.

The effect of 2,4-dichlorophenoxyacetic Acid and 6-benzylaminopurine on Callus Induction and Plant Regeneration of *Allamanda cathartica* – A Valuable Medicinal Plant

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Abstract
Allamanda cathartica is a potential medicinal plant which starts to achieve awareness in society due to its value to treat various types of diseases, especially in the treatment of jaundice, malaria and cancer. In the present study, the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) on the callus induction from the leaf and stem explants were investigated. Plant regeneration from the nodal explants was achieved. Surface sterilization by mercuric chloride ($HgCl_2$) was utilized in order to surface sterilize the leaf (0.1%), stem and nodal (0.2%) explants. The leaf and stem explants were cultured on full-strength Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-D alone (0.5 and 1.0 mg/L) or in combinations of 2,4-D (0.5, 1.0 and 1.5 mg/L) with BAP (0.5, 1.0 and 1.5 mg/L). In the study of plant regeneration, the nodal explants were cultured on MS medium supplemented with BAP at 1.0, 3.0 or 5.0 mg/L for shoot multiplication.

MS basal medium was used as a control and also used for shoot elongation. All the cultures were incubated under a photoperiod of 16 hours light and 8 hours darkness. For callus induction, the leaf and stem explants cultured on 1.0 mg/L 2,4-D and 1.0 mg/L BAP gave the best callus response (100%) with yellow-white, greenish friable callus (0.0707 ± 0.0549 g with callus initiated after 6 days) and brown-white, greenish friable callus (0.0207 ± 0.0009 g with callus initiated after 5 days), respectively. For shoot multiplication, MS supplemented with 5 mg/L BAP gave the best response (100%) with multiple shoots formed (6 ± 0.6 shoots per explant) from the nodal explants. In the plant growth regulator (PGR)-free medium, the elongated shoots were developed (1.01 ± 0.07 cm) with white hairy roots.

Keywords: Callus induction, plant regeneration, *Allmanada cathartica*, Malaysia.

Introduction

Allamanda cathartica is one of the medicinal plants in Malaysia and is apparently native to northern Brazil, Guyana, Surinam and probably French Guiana. It is commonly known as 'Alamanda Kuning', 'Golden Trumpet', 'Yellow Bell' or 'Buttercup Flower' from the family of *Apocynaceae*. In general, *Allamanda cathartica* can be used to relieve coughs in which the leaves are boiled and the vapor is inhaled to clear the nasal passages. Besides, the leaves can also be made into decoctions for use as a purgative purpose[1]. *Allamanda cathartica* is believed to have the properties of anti-bacterial and anti-cancerous[2], it has also been used in the treatment of jaundice and malaria as well as wound healing[3]. To meet its great demand in pharmaceutical industry, it is necessary for applying non-conventional methods of propagating for the conservation of this important medicinal plant.

Currently, interest in the medicinal use of plants has increased dramatically. In Malaysia, traditional medicine is accepted as one of the various treatment systems and is being practiced widely in the society[4]. An advanced biotechnological method of culturing plant cells and tissues in plant tissue culture can provide new means of conserving and rapidly propagating the valuable medicinal plants[5]. Plant tissue culture can also provide potential impact on production and improvement of medicinal plants, as well as *in vitro* manufacturing of crude drugs and active compounds[6]. Despite being a valuable medicinal plant, plant tissue culture studies of *Allamanda cathartica* are limited and rare. In this study, we examined the effect of plant growth regulators (BAP and 2,4-D) at various concentrations on the callus induction from the leaf and stem explants and established an efficient regeneration protocol using nodal explants of *Allamanda cathartica*.

Material and Methods

The intact plant of *Allamanda cathartica* (2-month-old) was purchased from Bukit Jalil Nursery Sdn Bhd, Malaysia. The healthy shoots were collected and the plant parts were separated into leaves, stems and nodes. Throughout the process, the explants were sterilized separately. First, the explants were washed thoroughly in running tap water for 2 hours with few drops of Tween-20, then, the leaves, stems and nodes were washed with Dettol for 3 minutes and 6 minutes respectively. In the laminar flow hood, the explants were rinsed with sterile distilled water for 5 times. The leaf explants were soaked in 0.1% $HgCl_2$ for 1 minute, stem and nodal explants in 0.2% $HgCl_2$ for 2 minutes. Finally, the explants were rinsed with sterile distilled water for 5 times.

Full strength of MS medium was variously supplemented with PGRs for callus induction and shoot multiplication. For callus induction, the leaf (0.5 cm X 0.5 cm in squares) and stem (1-2 cm in length) explants were cultured on the medium supplemented with different concentrations of 2,4-D alone (0.5 and 1.0 mg/L) or in combinations with 2,4-D (0.5, 1.0 and 1.5 mg/L) and BAP (0.5, 1.0 and 1.5 mg/L). After 4 weeks of culture, the callus formed was excised from the explants and dried in the oven at 34°C for 1 week (to exclude the water content) in order to determine the callus weight. For shoot multiplication, the nodal explants (1-2 cm in length) were cultured vertically by inserting about 0.4cm of the basal end into semi-solid MS medium containing 1.0, 3.0 and 5.0 mg/L BAP. After 4 weeks of culture, a total of 15 selected new shoots (0.50 cm in length) that formed from the nodal part were transferred to a PGR-free medium for shoot elongation. MS medium containing 30 g (w/v) sucrose and gelled with 8 g (w/v) agar technical was employed throughout the experiments. The pH of medium was adjusted to 5.7 ± 0.1 with NaOH or HCl before autoclaving at $121\,°C$, 15psi for 15 minutes. MS medium without any supplementation of PGR was used as the control.

All the cultures were incubated in the culture room under the conditions of $25 \pm 1\,°C$ with a 16 hours light and 8 hours darkness provided by cool white fluorescent tubes. Each treatment contained 5 replicates and the experiments were repeated 3 times for callus induction and shoot multiplication. One way analysis of variance (One-Way ANOVA) and Post Hoc multiple comparisons Tukey's Honestly Significant Difference (HSD) at 95% of confidence interval ($p<0.05$) was used to analyze the result of each experiment by using SPSS version 15.0 for Window®.

Results and Discussion

One of the most important factors affecting culture initiation and plant regeneration of immature embryos is the composition of the media, including plant growth regulators (PGRs). PGRs are crucial in establishing an optimal culture conditions since they play a pivotal role in producing relatively undifferentiated callus tissue from the differentiated tissues[7].

Generally, a large portion of plant species required an auxin or cytokinin for dedifferentiation and profuse callus induction[8]. For this study, leaf explants failed to produce callus in media free auxins or cytokinins and the leaf turned from green to brown after 4 weeks and eventually died. This showed that growth of callus cannot be induced without supplemented with auxins or cytokinins[9] and most of the plants required at least one type of auxin or cytokinin to be incorporated into the culture medium for the induction of callus from explants[10]. When the leaves were cultured on MS medium supplemented with various concentrations of 2,4-D alone (0.5-1.0 mg/L) and combinations of 2,4-D (0.5-1.5 mg/L) and BAP (0.5-1.5 mg/L), callus growth was initiated in about 6 days and rapid growth followed for majority of cultures. The calli were friable, yellow-white and greenish in all the treatments (Figure 1).

Of the various concentrations of 2,4-D and BAP tested, the maximum response was observed on MS medium supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L BAP where percentage of callus formation from the leaf explants was 100% with the highest callus weight of 0.0707 ± 0.0549 g (Table 1). This was in contrary with the results obtained for *Gladiolus hybridus*, whereby no callus was induced from the leaf explants cultured on 1.0 mg/L 2,4-D and 1.0 mg/L BAP[11]. In addition, combination between auxin and cytokinin has been widely used to enhance callus induction[12] and was proven to show better callogenic response compared to either auxin or cytokinin alone[13]. Therefore, the leaves cultured on MS medium supplemented with 2,4-D alone showed the poor response compared to the combinations of 2,4-D and BAP at different concentrations which only produced 16.7% callus formation with callus weight of 0.0002±0.0001 g and 0.0018±0.0001 g at 0.5 and 1.0 mg/L 2,4-D respectively (Table 1). This was in accordance with the results obtained from callus induction of *Gymnema sylvestris*[14].

For callus induction from the stem explants, stems cultured on MS medium supplemented with 0.5 and 1.0 mg/L 2,4-D were exhibited no sign of callogenesis (Table 1). Perhaps this was due to the plant specificity towards PGRs i.e. initiation of the callus could be seen faster in suitable medium whereas induction rate was slower or no callus was induced in the less appropriate medium[15]. The brownish of the stem explants was normally due to the phenolic compound which occurs as a secondary metabolite in all plant species[16] and can be stimulated or induced by mechanical injury of plant tissue[17]. When the explants were cut and cultured on any media, the cytoplasm and vacuoles contents would come out from the explants and phenolic compounds could readily become oxidized by air. These oxidized phenolic compounds inhibited enzyme activity and caused darkening of the culture medium which in turn results in lethal browning of explants[18]. Furthermore, among the different concentrations of 2,4-D and BAP tested, 1.0 mg/L 2,4-D and 1.0 mg/L BAP gave the best response on the formation of callus (100%) from the stem explants with the shortest time for initiation of callus formation i.e. 5±0.3 days (Table 1). The calli formed were friable, brown-white and greenish in colour (Figure 1) with the highest callus weight of 0.0207±0.0009 g (Table 1).

In the study of plant regeneration from the nodal parts of *Allamanda cathartica*, shoot multiplication on MS medium supplemented with 1, 3 and 5 mg/L of BAP and shoot elongation on MS medium without any supplementation of PGR was investigated. BAP is the most effective hormone in stimulating and enhancing shoot multiplication[19] and nodal segments are mostly preferred as the explants choice[20]. Based on the results in table 2, PGR-free medium

showed no sign of shoot multiplication. The highest percentage of shoot formation (100%) and number of shoots formation (6±0.6 shoots per explants) were observed on MS medium supplemented with 5 mg/L BAP (Table 2). Meanwhile, the lowest percentage of shoot formation (90.0±5.8%) and number of shoots formation (1±0.3 shoots per explant) were observed on MS medium supplemented with 1.0 mg/L of BAP (Table 2).

The results obtained from this study are supported by the results of shoot multiplication from *Balanites aegyptiaca*[21], i.e. as the BAP concentration increased from 1.0 mg/L BAP to 5.0 mg/L BAP, the percentage and number of shoot formation were increased. However, the explants of *Gladiolus hybridus* gave better multiplication response on MS medium containing 1 mg/L BAP compared to other treatments[11]. For all concentrations of BAP, multiple shoots were formed from the explants with greenish leaves (Figure 2).

Multiplied shoots development and elongation can be obtained on MS PGR-free medium[22]. In the present study, the lengths of the shoots were gradually increased from week 0 to week 4, i.e. 0.50±0 cm, 0.80±0.01 cm, 0.92±0.02 cm, 0.98±0.05 cm and 1.01±0.07 cm respectively with greenish leaves (Figure 3). Moreover, PGR-free MS medium can also be used to stimulate the production of roots[23] and this is consistent with the results of *Allamanda cathartica*, i.e. in average 2 out of 15 explants were observed to have root formation on Week 4 in the PGR-free medium (Figure 4).

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Table 1
Effect of MS medium supplemented with 2,4-D and combinations of 2,4-D with BAP at different concentrations on callus induction from leaf and stem explants of *Allamanda cathartica* after 4 weeks of culture

Plant Growth Regulator (mg/L)		Day of initial callus formation (Day ± SE[a])		Percentage of callus formation (%) ± SE[a]		Callus weight (g) ± SE[a]		Callus morphology	
2,4-D	BAP	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
0	0	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	Green leaf turned brown. No callus was formed	Green stem turned brown. No callus was formed
0.5	0	16 ± 1.8^{c}	0^{a}	16.7 ± 12.0^{a}	0^{a}	0.0002 ± 0.0001^{a}	0^{a}	Yellow-white, greenish friable callus	
1.0	0	17 ± 1.5^{c}	0^{a}	16.7 ± 6.7^{a}	0^{a}	0.0018 ± 0.0001^{a}	0^{a}		
0.5	0.5	8 ± 0.3^{b}	6 ± 0.3^{bc}	90.0 ± 10.0^{b}	80.0 ± 5.8^{bc}	0.0325 ± 0.0141^{a}	0.0194 ± 0.0001^{de}		Brown-white, greenish friable callus
0.5	1.0	6 ± 0.3^{b}	8 ± 0.3^{c}	93.3 ± 6.7^{b}	80.0 ± 5.8^{bc}	0.0158 ± 0.0016^{a}	0.0131 ± 0.0004^{b}		
0.5	1.5	9 ± 1.2^{b}	7 ± 0.6^{bc}	86.7 ± 6.7^{b}	83.3 ± 3.3^{bc}	0.0419 ± 0.0146^{a}	0.0134 ± 0.0002^{b}		
1.0	0.5	10 ± 1.7^{b}	8 ± 0.9^{c}	80.0 ± 10.0^{b}	93.3 ± 3.3^{bc}	0.0251 ± 0.0049^{a}	0.0175 ± 0.0015^{cd}		
1.0	1.0	6 ± 0^{b}	5 ± 0.3^{b}	100.0 ± 0^{b}	100.0 ± 0^{c}	0.0707 ± 0.0549^{a}	0.0207 ± 0.0009^{e}		
1.0	1.5	9 ± 0.6^{b}	8 ± 0.3^{c}	93.3 ± 6.7^{b}	83.3 ± 8.8^{bc}	0.0217 ± 0.0050^{a}	0.0181 ± 0.0010^{cde}		
1.5	0.5	9 ± 1.2^{b}	7 ± 0.3^{bc}	96.7 ± 3.3^{b}	86.7 ± 6.7^{bc}	0.0147 ± 0.0026^{a}	0.0194 ± 0.0001^{de}		
1.5	1.0	6 ± 0.3^{b}	8 ± 0.3c	96.7 ± 3.3^{b}	73.3 ± 3.3^{b}	0.0176 ± 0.0017^{a}	0.0153 ± 0.0005^{bc}		
1.5	1.5	6 ± 0.3^{b}	6 ± 0.3^{bc}	96.7 ± 3.3^{b}	93.3 ± 3.3^{bc}	0.0523 ± 0.0206^{a}	0.0206 ± 0.0003^{de}		

SE[a] = Standard Error. The value for the same alphabet is not significantly different (Tukey's multiple range test, $p<0.05$).

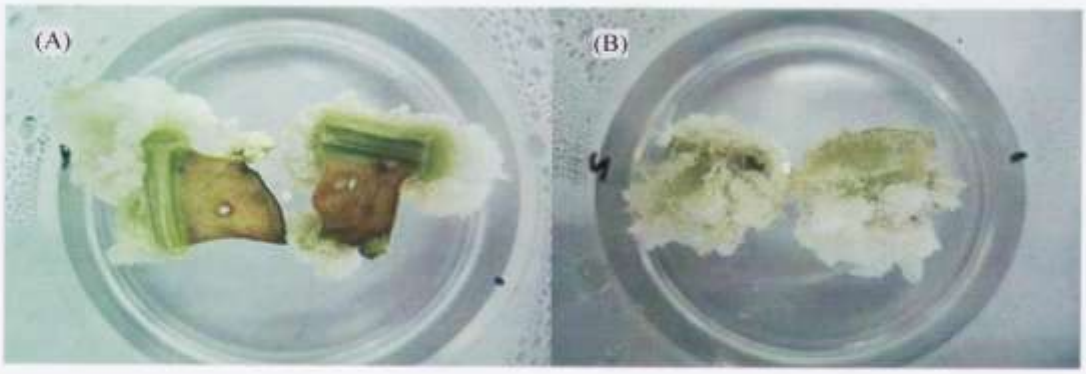

Figure 1: Induction of callus from (A) leaf and (B) stem explants of *Allamanda cathartica* after four weeks of culture on MS medium supplemented with 1 mg/L 2,4-D and 1.0 mg/L BAP

Table 2
Shoot multiplication on MS medium supplemented with BAP at the concentrations of 1.0, 3.0 and 5.0 mg/L from nodal explants of *Allamanda cathartica* after 4 weeks of culture

Plant growth regulator	Concentration (mg/L)	Percentage of shoot formation (%)± SE[a]	Number of shoots formation (Number of shoots ± SE[a])	Shoot morphology
Control	0.0	0^{a}	0^{a}	No shoot formation was observed
BAP	1.0	90.0 ± 5.8^{b}	1 ± 0.3^{a}	Multiple shoots were formed from the explants with greenish leaves
	3.0	96.7 ± 3.3^{b}	4 ± 0.3^{b}	
	5.0	100.0 ± 0^{b}	6 ± 0.6^{c}	

SE[a] = Standard Error. The value for the same alphabet is not significantly different (Tukey's multiple range test, $p<0.05$).

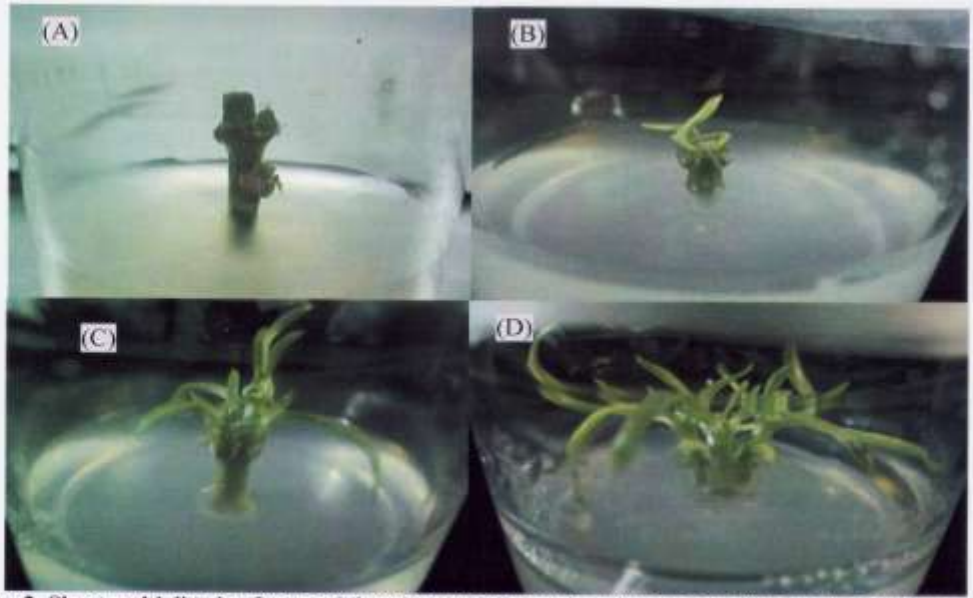

Figure 2: Shoot multiplication from nodal explants of *Allamanda cathartica* after four weeks of culture on MS supplemented with BAP. (A) control medium (B) 1.0 mg/L BAP (C) 3.0 mg/L BAP and (D) 5.0 mg/L BAP

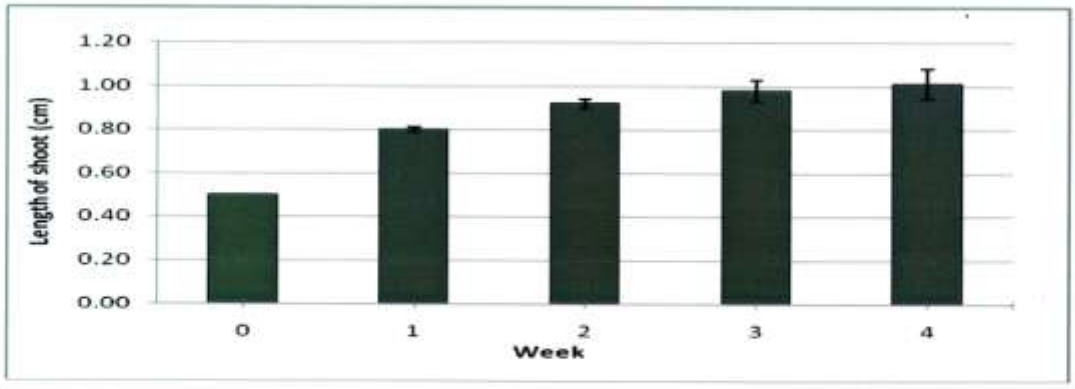

Figure 3: Shoot elongation on MS medium from nodal explants of *Allamanda cathartica* from week 0 to 4.

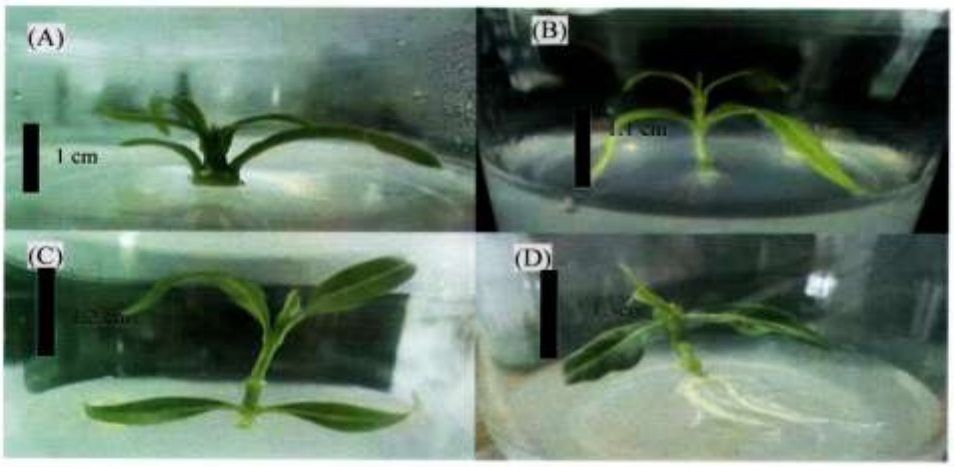

Figure 4: Shoot elongation on MS medium from nodal explants of Allamanda cathartica from week 1 to 4. (A) Week 1 (B) Week 2 (C) Week 3 and (D) Week 4.

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APPENDIX C

PAPERS PRESENTED

1. **Wong, K. F.** and Taha, R. M. (2011). The Effect of 2,4-dichlorophenoxyacetic Acid and 6-benzylaminopurine on Callus Induction and Plant Regeneration of *Allamanda cathartica* – A Valuable Medicinal Plant. 16th Biological Sciences Graduate Congress at Department of Biological Sciences, Faculty of Science, National University of Singapore (NUS), Singapore. 12-14 December 2011.

2. **Wong, K. F.** and Taha, R. M. (2012). Plant Regeneration and Pigment Identification of *Allamanda cathartica*. The International Symposium on Orchids and Ornamental Plants at Imperial Mae Ping Hotel, Chiang Mai, Thailand. 9-12 January 2012. Organized by Department of Agriculture, Horticultural Science Society of Thailand, International Society for Horticultural Science.

Figure C (I): Certificate of participation for 16th Biological Sciences Graduate Congress.

Figure C (II): Certificate of participation for The International Symposium on Orchids and Ornamental Plants.