In vitro PROPAGATION AND PHYTOCHEMICAL SCREENING FROM *Peperomia pellucida* L. Kunth.

TEOH LYDIA

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

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TEOH LYDIA

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FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
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In vitro PROPAGATION AND PHYTOCHEMICAL SCREENING FROM *Peperomia pellucida* L. Kunth.

ABSTRACT

In the present study, regeneration of *Peperomia pellucida* L. Kunth. has been successfully developed through tissue culture technique. The main focus of the study was to assess the effects of plant growth regulators on *in vitro* propagation of shoot and callus induction. Additional assessment concerning the medicinal property of the plant was also examined. The best medium for seed germination was Murashige & Skoog (MS) basal medium. The optimum regeneration medium for *Peperomia pellucida* L. Kunth. shoot multiplication and shoot elongation was MS supplemented with 0.5 mg/L kinetin (KN) which resulted in 6.2 number of shoots with 0.7 cm in height. In combination of PGRs, MS medium supplemented with 1.5 mg/L benzylaminopurine (BA) and 1.5 mg/L kinetin (KN) with 10.3 shoots and 0.73 cm height. In combination of cytokinin and auxin, MS medium supplemented with 1.0 mg/L benzylaminopurine (BA) and 1.0mg/L Indole-3-acetic acid (IAA) induced 38.7 shoots. Meanwhile, the highest shoot elongation was observed on MS supplemented with 1.0 mg/L KN and 1.0 mg/L IAA (0.81 cm). When tested with Cytokinin and auxin, MS supplemented with 1.0 mg/L BA and 1.0 mg/L IAA resulted in 38.7 number of shoots. The highest shoot elongation was observed on MS supplemented with 1.0 mg/l KN and 1.0 mg/L IAA (0.81 cm). When comparing the shoot induction in different temperature and charcoal concentrations, MS medium supplemented with 15% charcoal at 13ºC gave the highest shoot elongation (4.72 cm) and MS medium without charcoal showed the highest shoot multiplication with 2.60 number of shoots at 25ºC which was insignificant with those grown at 13ºC (2.53 shoots). For root induction, MS supplemented with 2.0 mg/l Indole-3-butric acid (IBA) gave the highest number (5.33) of root production. All the plantlets were successfully acclimatized with 85% survival

rate under natural environment. Callus was successfully produced from leaf explants in MS medium with (0.1-0.6 mg/L) 2,4-D and resulted in formation of embryogenic callus. *In vivo* and *in vitro* plant methanol and ethanol extracts of *Peperomia pellucida* L. Kunth. were investigated by GC-MS and the presence of chemical compound such as apiol, phytol, hhenol, 9-octadecenoic acid (z) and caryophyllene was detected. All the plant extracts of *Peperomia pellucida* L. Kunth were effective against *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Pseudomonas aeruginosa.* Methanol extract from *in vitro* plant showed the highest inhibition zone (12.5) against *Staphylococcus aureus* and followed by ethanol extract from *in vitro* plant shows (12 mm) inhibition zones against *Salmonella typhi* and *Pseudomonas aeruginosa.* Upon testing the extracts on human breast adenocarcinoma cell line (MCF-7) and human lung adenocarcinoma cell line (A549), *in vitro* ethanol extracts were toxic against proliferation of MCF-7 cells while *in vitro* methanol extracts were toxic for A549 cell. The reproducible protocol in this study has potential for establishment of selected and standardized plants suitable for the exploitation in various industries such pharmaceutical industries, agricultural, agrochemical industries and food industries.

Keywords: *Peperomia pellucida*, tissue culture, extracts, antimicrobial, anticancer.

PROPAGASI *in vitro* DAN PENGESANAN FITOKIMIA DARIPADA *Peperomia pellucida* L. Kunth.

ABSTRAK

Dalam kajian ini, regenerasi *Peperomia pellucida* L. Kunth. telah berjaya dihasilkan melalui teknik kultur tisu. Fokus utama kajian ini adalah menilai kesan penggalak tumbesaran tumbuhan serta propagasi *in vitro* pucuk dan induksi kalus. Penilaian sampingan yang dikaji adalah mengenai ciri ubatan tumbuhan ini. Media yang sesuai untuk percambahan biji benih ialah media Murashige dan Skoog (MS) tanpa hormon. Media regenerasi yang optimum bagi *Peperomia pellucida* L. Kunth untuk menghasilkan pucuk yang banyak serta panjang adalah media MS yang ditambah dengan 0.5mg/L kinetin, yang menghasilkan 6.2 pucuk dengan 0.7 cm ketinggian. Bagi gabungan penggalak tumbuhan, media MS ditambah dengan 1.5 mg/L benzylaminopurin (BA) dan 1.5 mg/L kinetin (KN) menghasilkan 10.3 pucuk dan 0.73 cm tinggi. Apabila dikaji dengan sitokinin dan auksin, media MS yang ditambah dengan 1.0 mg/L benzylaminopurin (BA) dan 1.0 mg/L asid indol-3-asetik (IAA) menghasilkan 38.7 pucuk. Sementara itu, pucuk yang terpanjang diperhatikan dalam media MS yang ditambah dengan 1.0 mg/L KN dan 1.0 mg/L IAA (0.81 cm). Perbandingan antara perbezaan suhu dan kepekatan arang untuk penghasilan pucuk, mendapati media MS yang ditambah dengan 15% arang menunjukkan kadar pemanjangan pucuk yang tertinggi (4.72 cm) pada suhu 13ºC manakala media MS tanpa arang menunjukkan penghasilan pucuk yang paling banyak (2.60 pucuk) pada suhu 25ºC. Walau bagaimanapun, keputusan ini tidak ketara pada suhu 13ºC (2.53 pucuk). Untuk induksi akar, media MS yang ditambah dengan 2.0 mg/L asid indol-3-butrik (IBA) menunjukkan penghasilan bilangan (5.33) akar yang paling banyak. Kesemua anak pokok telah berjaya menyesuaikan diri dengan persekitaran semulajadi dengan 85% kadar kemandirian. Kalus

berjaya dihasilkan dari daun dalam media MS yang mengandungi 2,4-D dan menunjukkan pembentukan kalus embriogenik. Ekstrak metanol dan etanol dari tumbuhan *in vivo* dan *in vitro Peperomia pellucida* L. Kunth, juga dikaji menggunakan GC-MS dan menunjukkan kehadiran beberapa kompaun kimia seperti apiol, phytol, phenol, 9-octadecenoic acid (z) dan caryophyllene. Semua ekstrak dari tumbuhan *Peperomia pellucida* L. Kunth adalah berkesan merencat pertumbuhan bacteria seperti *Staphylococcus aureus, Salmonella typhi, Escherichia coli* and *Pseudomonas aeruginosa*. Ekstrak metanol dari tumbuhan *in vitro* menujukan perencatan bakteria yang tertinggi (12.5mm) terhadap *Staphylococcus aureus* dan diikuti oleh ekstrak etanol dari tumbuhan *in vivo* merencatkan pertumbuhan *Salmonella typhi* dan *Pseudomonas aeruginosa* (12mm). Apabila ekstrak diuji terhadap sel-sel kanser seperti sel adenokarsinoma payudara manusia (MCF-7) dan sel adenokarsinom paru-paru manusia (A549), ekstrak *in vitro* etanol mempunyai kesan antikanser yang lebih baik terhadap MCF-7 dan ekstrak *in vitro* metanol mempunyai kandungan antikanser yang lebih tinggi terhadap A549. Penghasilan semula protokol bagi kajian ini mempunyai potensi untuk mendapatkan tumbuhan yang terpilih dan seragam serta sesuai untuk mengeksploitasi pelbagai industri seperti industri farmaseutikal, agrokultur, industri agrokimia dan industri pemakanan.

Kata kunci: *Peperomia pellucida*, kultur tisu, ekstrak, antimikrobial, antikanser.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
µ	:	Micro
µg	:	Microgram
µL	:	Microliter
2,4-D	:	2,4-dichlorophenoxy-acetic acid
A549	:	Human lung adenocarcinoma cell line
A549	:	Adenocarcinomic human alveolar basal epithelial cells
BA	:	Benzylaminopurine
cm	:	Centimeter
DMRT	:	Duncan's Multiple Range Test
g	:	Gram
GA^3	:	Gibberellin
GC-MS	:	Gas chromatography-mass spectrometer
h	:	Hour
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butric acid
kg	:	Kilogram
KN	:	Kinetin
L	:	Liter
m	:	Meter
$m^{-2}s^{-1}$	:	Meter squared per second
MCF-7	:	Human breast adenocarcinoma cell line
mg	:	Milligram
mL	:	Milliliter

mm	:	Millimeter
MS	:	Murashige & Skoog
NAA	:	Naphthalene-acetic acid
NaOH	:	Sodium hydroxide
nm	:	Nanometer
^{o}C	:	Degree Celsius
p-CPA	:	Picloram
PGRs	:	Plant growth regulator
pH	:	Power of hydrogen
SE	:	Somatic embryogenesis
WHO	:	World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction to Plant Tissue Culture

Plant tissue culture is a technique used for plant cells, tissues and organs to culture on synthetic media under controlled and aseptic conditions. Plant tissue culture is also carried out in controlled conditions of light, temperature and humidity (Dagla, 2012). A successful plant tissue culture is determined by the quality of the culture medium used. The culture medium contains essential vitamins, hormones, nutrients, iron sources, carbon sources and gelling agent to induce the plant growth (George et al., 2008). A healthy and actively growing plant will be used in tissue culture and any parts of the plants can be used for plant tissue culture such as leaves, stem, roots, flowers, shoots, nodes, fruits, seeds as well as undifferentiated cells (Mineo, 1990).

Proliferation of nodal shoots has much potential as supporting technology for micropropagation of ornamental and medicinal plants, especially. Availability to culture the shoots throughout the year and the avoidance of surface sterilizing procedures with greater assurance of non-contaminated cultures are the factors that have made them as perfect source for numerous purposes. Direct shoot proliferation using nodal explants is a major biotechnological approach for micropropagation of novel ornamental and medicinal plants. With the assurance, micropropagated plants can be tested or commercialised as they are genetically uniform. Most of the time, efficient plant regeneration was obtained using nodal explants (Jain & Bashir, 2010).

In vitro cultures depend on the medium composition and physiochemical characteristics for a successful initiation. An optimal environment condition for cell differentiation and organ growth should be provided to the medium. Apart from serving

as a regulatory compounds, it is also serve as a source of assimilable nitrogen, carbon and other minerals (Wiszniewska et al., 2015).

Great potential are being offered through *in vitro* micropropagation techniques not only for prompt multiplication of current stock of plant species but also for protecting of vital and selected herbal medicinal plants. Micropropagation is also an alternative method for multiplication of highly valuable cultivars at faster rates than conventional practice and producing uniform plants at larger scales while maintaining their genotypes. (Manan et al., 2016; Martin et al., 2003). To form adventitious shoots with significant application in genetic transformation and vegetative clonal propagation, reliable and efficient tissue culture approaches is required. (Al-Ahmad, 2015).

Many medicinal and ornamental plants have been commercially propagated through plant cell and tissue culture (Ahmadabadi & Bock, 2010; Sahai et al., 2010; Verma et al., 2011). Plant tissue culture loom as a sustainable biotechnological appliance for the production of bioactive compounds in sustainable conservation and rational use of biodiversity (Karuppusamy, 2009). Biotechnological method through plant tissue culture provide promising bio-production platform for desired natural products. Apart from that, by culturing either shoots or roots, undifferentiated metabolic characteristics are revealed as compared to their parent plant. Tissue culture offers an alternative potential for the fabrication of great value natural product in the plant (Yue et al*.,* 2016). The most studied natural product is phenolic compounds for their bioactive properties. Phenolic compound has immense structural and chemical diversity and produce naturally by plants. Due to this reason, phenolic compound from *in vitro* culture are chosen to provide required stress conditions for their production and increase the secretion of secondary metabolites through plant tissue culture activities (Dias et al., 2016).

1.2 Introduction to Medicinal Plants

Plants have been very important source of medicine for thousands of years. Many people have healed themselves with traditional herbal medicines since ancient times till this very day. Lately, many people are more interested in using herbal drugs for treatments against different diseases as they are non-toxic. By evaluating the effectiveness of plants, World Health Organization (WHO) has recommended herbal plants rather than modern drug due to its increased safety elements (Ayyanar et al., 2008). Natural products from plants have the basic treatment for many diseases. In many developing countries, more than 80% of people depending on traditional medicine from plants or animals to meet their basic health needs. Recently, the demand and popularity of herbal medicines are increasing day by day as they are more suitable for human health with the advantage of low cost and completely safe (Upadhayay et al., 2012; Verma & Singh, 2008). Some applications where herbal medicine become a major component and common element include homeopathic, Ayurveda, naturopathic and other medicine systems. Since herbals belongs to natural sources traditionally, it is considered as harmless. People are consuming the herbal medicines without the need of prescriptions. In herbal medicine, the seeds, stems, flowers, leaves, roots, barks and extracts of all of these have been used for medicinal purposes (Atmakuri & Dathi, 2010).

Currently, huge group of people from various parts of the world continue to use complementary practices such as medicinal plants for their health care regardless of advertisement and reassurance during the evolution of allopathic medicines from the pharmaceutical industry (Bonifácio et al., 2014). The effectiveness keeping the body healthy and free from diseases have been proven by using the plants as food and herbal products as the dietary supplements. In scientific communities, the researchers begin to focus and reveal the chemical composition and the uses of medicinal plants. In many

countries, plant based dietary supplements are now gaining greater importance. In developed countries, the uses of herbal supplements and medicines are increasing sharply, so much so the demand for herbal product is predicted to increase rapidly (Sen et al., 2011). About 21000 plants around the world were listed by WHO as plants with medicinal purposes (Upadhayay et al., 2012).

1.3 Introduction to *Peperomia pellucida* L. Kunth.

Peperomia pellucida L. Kunth. (Figure 1.1) is a heart shaped leaves plant belonging to the Piperaceae family. This plant can be found in various parts of the world and they generally grow in clumps and thrive in the loose humid soils (Rojas-Martínez et al., 2013). This plant is known to have medicinal value. In many countries, this plant is used to treat many diseases such as fever, headache, gout, fatigue, colic, acne, abscesses, skin diseases and eye inflammation (Hamzah et al., 2012; Roslida & Aini, 2009; Susilawati et al., 2015). *Peperomia pellucida* L. Kunth have been reported to have biological properties such as antibacterial, antifungal, antioxidant and anti-inflammatory activities (Verma & Singh, 2008). Many chemical compounds have been isolated from the essential oil of Piperaceae family. It is also known that *Peperomia pellucida* L. contains, flavonoids, steroids, saponin, tannin, triterpenoid and carbohydrate (Abere & Okpalaonyagu, 2015; Mappa et al., 2013).

Figure 1.1: Intact plant of *Peperomia pellucida* L. Kunth. growing in natural habitat.

1.4 Research Objective

Peperomia pellucida L. Kunth. is one of the medicinal plants found in Malaysia. This plant attracts unlimited attention to researchers due to its medicinal properties. Nevertheless, scientific investigation and plant tissue culture studies in order to determine the therapeutic potential and medicinal property of this plant is limited compared to other medicinal plants. Therefore, the research on *Peperomia pellucida* L. Kunth was carried out with the following objectives:

1. To optimize the culture conditions for plant regeneration and callus formation using different plant growth regulators.
2. To study the presence of bioactive compounds from *Peperomia pellucida* L. Kunth. via GC-MS.
3. To elucidate the antimicrobial properties of both *in vivo* and *in vitro* plantlets of *Peperomia pellucida* L. Kunth.
4. To study the anticancer activity of *Peperomia pellucida* L. Kunth. from leaf extracts obtained from *in vivo* and *in vitro* grown plants.

CHAPTER 2: LITERATURE REVIEW

2.1. *Peperomia pellucida* L. Kunth.

Peperomia pellucida L. Kunth. belongs to the family of Piperaceae. The family comprises of more than 12 genera and this flowering plant is one of the most widely distributed families (Akinnibosun et al., 2008). This family comprises of about 1500 to 1700 species distributed throughout tropical regions and subtropical regions (Ahmadabadi & Bock, 2010; Tchoumbougnang et al., 2009; Verma et al., 2014). Nearly half of the Piperaceae is represented by genus *Peperomia* (Akinnibosun et al., 2008). The Piperaceae family is closely related to the monocotyledonous boundary. They have certain unique features among dicotyledonous plants having both inner and outer rings of separate vascular bundles arranged variously in the axis (Jose & Sharma, 1985). Apart from the geophytic and epiphytic characteristics, *Peperomia pellucida* have contributed to their establishment in a wide variety of biomes. Some of this species have been popular and cultivated worldwide due to ornamental purposes.

2.1.1 History of *Peperomia pellucida* L. Kunth

The name Piperaceae was introduced in the year of 1815 by Rich in Humboldt, Bonpland and Kunth's in a book titled *Nova Genera et Species Plantarum.* This plant was widely distributed in tropics and subtropics with major distribution in Latin America and Malaya Peninsular. In 1753, a total of 17 species were recognized by Linnaeus comprising the genus *Piper*. In 1794, Ruiz and Pavon introduced *Peperomia* as the second genus. During the subsequent century, additional genera were included by various authors. The family was subdivided into two tribes *Peperomeae* with 5 genera and 209 species and *Pipereae* with 15 genera and 304 species by Miquel in the year 1843 to 1844. In the year of 1896, De Candolle recognized a little over 1000 species among the two genera

Peperomia and *Piper*. According to Rendle in 1956, there are two larger genera, 700 species in *Piper* and *Peperomia* with 7 smaller genera in addition more than 600 species. In the 1957, according to Lawrence, the family consist of 10-12 genera including with two larger ones, *Peperomia* and *Piper* (Jose & Sharma, 1985). According to a journal, in year 2000, *Piper* is the largest and single genus in the Family of Piperaceae with 1000 species worldwide (Ajith & Rao, 2000). In 1999, a journal reported that Piperaceae is the large pantropical family with over 3000 species in 8-13 genera of *Piper* L. and *Peperomia* Ruiz and Pav. as the major genera (Mathew et al., 1999).

2.1.2 Taxonomy of *Peperomia pellucida* L. Kunth.

Peperomia pellucida L. Kunth. belongs to the Piperaceae family. Even though this plant species is known as *Peperomia pellucida*, it still has different scientific names at different parts of the world. Its synonymous names are *Peperomia brittonii* C. DC., *Peperomia ephemera* Ekman, *Peperomia freireifolia* A.Rich., *Peperomia manabina* C. DC., *Peperomia molleri* C. DC., *Peperomia nana* C.DC., *Peperomia negotiosa* Trel., *Peperomia petiolaris* C.DC., *Peperomia translucens* Trel., *Peperomia Triadophylla* Peter, *Peperomia vogelii* Miq., *Peperomia yapemsis* C. DC., *Piper exigum* Blume, *Piper freireaefolium* Hochst., *Piper freireifolia* Hochst. Ex A Rich., *Piper pellucidum* L. and *Pier concinnum* Haw (Jstor; Global plants). Table 2.1 shows the scientific classification of *Peperomia pellucida* L. Kunth.

Table 2.1: Scientific classification of *Peperomia pellucida* L. Kunth. (Majumder, 2012; Majumder et al., 2011)

Kingdom	**Plantae – Plants**
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliophyta* - Dicotyledons
Subclass	*Magnoliidae*
Order	*Piperales*
Family	Piperaceae
Genus	*Peperomia*
Species	*Peperomia pellucida* L. Kunth.

2.1.3 Botanical Description

Peperomia pellucida L. Kunth. (Figure 2.1) is a common fleshy tropical annual shallow-rooted herb. It typically grows to a height of about 15 to 45 cm (Ooi et al., 2012). This plant can be found grow as a shrubby creeper epiphyte on rich habitat like plantations, on damp ground under the shade of trees and above 400 m sea level in shady areas near houses. Usually this plant can be found growing on humid soils in clumps, thriving in loose (Abere & Okpalaonyagu, 2015; Manokari et al., 2016; Zubair et al., 2015). It is an herbaceous plant characterized by tiny shiny heart-shaped fleshy leaves, succulent stems and fibrous roots. This herbs has dot like seeds (Figure 2.2a) attached on the cord-like spikes or inflorescence (Figure2.2b). When the seeds are crushed, it has a distinct mustard-like odour (Hartati et al., 2015; Igwe & Mgbemena, 2014; Majumder, 2011; Mensah et al., 2013; Ooi et al., 2012). *Peperomia pellucida* has round, delicate and glabrous and hairless fleshy stem. The stem (Figure 2.2d) of this plant are like angular

trailing waxy and thread like. Those plants growing in a healthy surroundings have fleshy and stout stems. Their stems are ascending, erect, or translucent pale green (Akinnibosun et al., 2008; Ibibia, 2012; Pappachen & Annam, 2013). The elongated stems have leaves rising up 6 to 9 cm above the surface, which look like a vine and their internodes are generally 3-8 cm long (Majumder, 2012).

The *Peperomia pellucida* leaves (Figure 2.2c) are blunt, heart shaped and surface of leaves covered with transparent and smooth as candle wax like. The plant grows as a long shrubby looking creeping cover or as an epiphyte. The leaves are arranged alternately on the stalks for about 1-2 cm long. The blades of the leaves are broadly-ovate and heart-shaped at the base (Majumder, 2012; Ooi et al., 2012;. Verma et al., 2014). The leaves are in green on upper surface and whitish green on lower surface. The leaves are fleshy even though thin, broadly ovate, drying papery with 1.5-4 cm long, 1-3.3 cm wide. The nerves are arranged in palmately 3 nerved or 5 nerved, base sub cordate to truncate with 0.5 – 2 cm long petioles. The foliage of the plant looks ornamental (Majumder, 2011; Majumder et al., 2011; Zubair et al., 2015). Flowers are very tiny and arranged unnoticeable. The rachis are 0.4-0.6mm in diameter with 3-6 cm long filiform,. They grow bi-sexually in the form of cord like spikes arising from the leas axils, 1 to several terminal and axillaries. The flowers are various without petals scattered on the spike but with only stamens and an ovary (Akinnibosun et al., 2008). The peduncles are 0.6-1.0 cm long, glabrous, ovary and stigmas terminal. Inflorescence comprises of compressed, erect spikes of minute creamy white flowers and maturing gradually from the base to the tip, turning brown when it ripe. The seeds are oval or round tiny, smooth dot like and ridged. During early stage, the seed are green in colour and later turn to black. The seeds are 0.5 mm long longitudinally sub-globose, apex beaked. These develop partially embedded in the spike with their hooked beaks swelling outside and where it mature gradually from

the base to the tip by turning brown when ripe (Kumar & Jena, 2012; Majumder, 2012; Majumder et al., 2011)

2.1.4 Geographical Distribution

Peperomia pellucida is an herbaceous plant which can be found in various parts of the world. This is a native plant widely distributed in many North and South American and Asian countries (Kurniawan et al., 2016; Oloyede et al., 2011; Sheikh et al., 2013). *Peperomia pellucida* is an annual weed native to tropical North America and South America (Ooi et al., 2012). Occasionally, this plant is cultivated and economically naturalized in Hawaii. In the country of Fiji, this plant can be found along roadsides, in plantations, along forest trails and also on damp ground in shady places at elevations of sea level to about 400m as a weed (Majumder et al., 2011). This diversely distributed plant can also be found in Africa grown at damp, gardens and other humid area (Kartika et al., 2016). This plant empirically used as medicine in Nigeria (Kurniawan et al., 2016). This plant is also found mostly in tropical and subtropical parts of India (Majumder et al., 2011). This plant grows wildly in the Amazon and Guyana (Oloyede et al., 2011). In several other regions across the globe like southern regions of Samoa, New Guinea and China, this species mostly found 700m above from sea level, in damp shady conditions and living on bare ground (Majumder, 2011). Now this plant abundantly available in Malaysia and distributed pantropically (Wei et al., 2011). Verma et al. (2014) & Ooi et al. (2012), have reported that this herb is also found in westerns countries such as Australia, New Zealand, Spain and France. Apart from that, in Asia this plant can be found in Philippines, Indonesia, Bangladesh, Thailand, Vietnam and Japan (Appapchen & Annam, 2013; Ooi et al., 2012; Sangsuwon et al., 2015; Zubair et al., 2015).

2.1.5 Common Names

In other parts of the world, *Peperomia pellucida* is known by various local names in different countries. In Malaysia, *Peperomia pellucida* is known as 'sirih Cina', 'ketumpangan air' or 'tumpang angin'. In Thailand it is known as "Phakkra sang" (Sangsuwon et al., 2015). This plant is known as 'ćang cua' in Vietnam and in Philippines it is known as 'pansit-pansitan' or 'ulasimang bato' (Ooi et al., 2012; Wei et al., 2011). In Indonesia, the plant known as 'sasaladaan', 'kaca-kaca' or 'surukan'. In India, it is known as 'mashitandu chedi', 'pononoa', 'toyakandha' or 'varshaboo'. In North America *Peperomia pellucida* is called as silverbush, man-to-man, pepper elder or rat ear, in Japan known as 'usuba sunakosho' and in Spain known as 'alumbre' or 'erva-de-vidro'. 'Pépéromie' or 'herbe á couleuvre' is known in France and known as 'luchi pata' in Bangladesh. In Brazil known as 'coraçãozinho' or little heart. In Africa this plant is known as 'rinrin' and in Sri Lanka, it is known as 'diya thippili' (Abere & Okpalaonyagu, 2015; Alves et al., 2018; Kartika et al., 2016; Ooi et al., 2012; Zubair et al., 2015).

Figure 2.1: The whole intact plant of *Peperomia pellucida* L. Kunth.

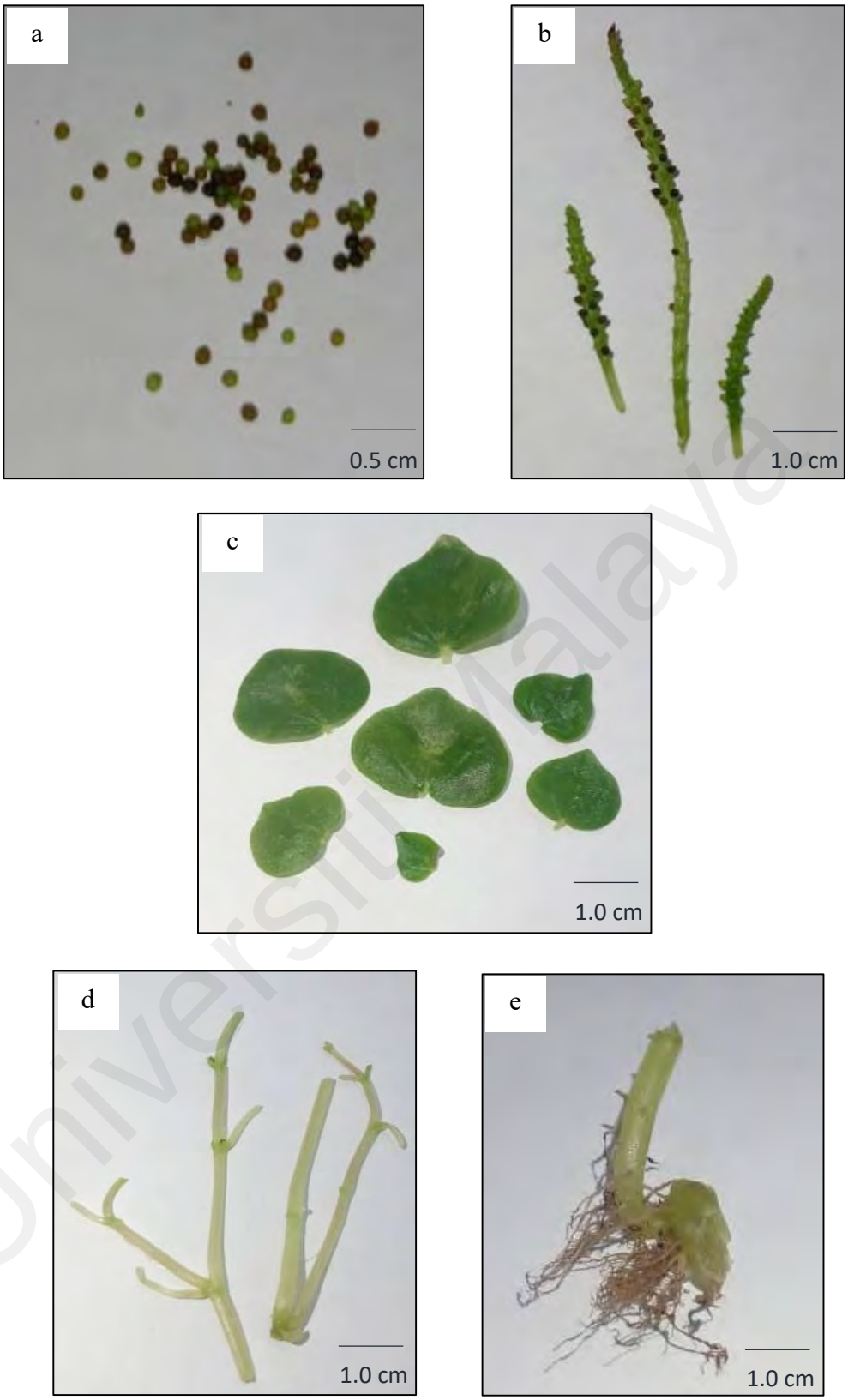

Figure 2.2: Morphology of *Peperomia pellucida* L. Kunth. a) Seed. b) Inflorescence. c) Leaf. d) Stem. e) Roots.

2.1.6 Traditional Uses

Ever since ancient time, nature has bestowed its benefits and the value of medicinal plants to the mankind is well proven. It provides food, shelter, medicine and further resources according to human need (Sen et al., 2011; Shanmugam et al., 2012). Through human evolution, the knowledge of plants as herbal medicines has occurred in several population. This was a beginning when man starts to learn to choose plants for food and to relieve ailments and illnesses. Most developing countries uses natural medicines (Bonifácio et al., 2014). Same as other natural medicines, *Peperomia pellucida* has been used as a traditional medicine worldwide. For thousands of years, plants have played a significant role in sustaining human health and improving the quality of human life for. Apart from that, plant served as valuable components of diet and medicines (Majumder et al., 2011; Razmavar et al., 2014). The whole plant parts of *Peperomia pellucida* are being used to treat different diseases (Awe et al., 2013). It is believed the whole plant comprises therapeutic value in treating fever, headaches, acne, fatigue, wounds, bleeding, gout, renal disorders, mental disorder, coughing and eczema (Oloyede et al., 2011; Ooi et al., 2012). *Peperomia pellucida* leaves and stems could also be eaten as vegetable (Majumder, 2011). Peoples in Africa cultivate *Peperomia pellucida* as vegetables for cuisine. In South Eastern Nigeria, *Peperomia pellucida* leaves are used topically to treat athlete foot and skin infections caused by bacteria and fungi, apart from that, they also use warmed leaves to apply on sore and boils (Igwe & Mgbemena, 2014; Kartika et al., 2016). Hamzah et al. (2012), reported Nigerians, consume this plant as a tonic for healthy well-being and to treat hypertension and diabetes. While in Southern Nigeria, the traditional practitioners without scientific basis uses this plant as traditional recipe in managing sickle cell anemia (Abere & Okpalaonyagu, 2015). In Ayurveda treatment, this plant is used to soothe cough, pita, constipation, kidney diseases and urinary related problems (Narayanamoorthi et al., 2015). In Indonesia folk medicine, this plant is used

to treat fever, skin diseases and contused wound (Susilawati et al., 2015). The Siamese eat this plant as salad or as cooked vegetable to lower the cholesterol level and to prevent glaucoma (Sangsuwon et al., 2015). In the other part of world, the local people in Bangladesh, have been used the plant leaves with the aerial part to treat excited mental disorder (Khan et al. 2008; Mutee et al., 2010). According to Khan et al. (2010), Manila Medical Society in Philipine, use *Peperomia pellucida* to relieve arthritic pains however, manifest CNS depression as side effect. In Amazon and Guyana it is a popular cough suppressant, emollient diuretic and effective in the treatment of proteinuria (Oloyede et al., 2011; Sheikh et al., 2013). The Altenos Indians in Bolivia use the whole plant to stop haemorrhages, the roots to cure fevers and for dressing wounds the aerial parts are used (Egwuche et al., 2011). In Cameroon, *Peperomia pellucida* together with whole grin is used to treat fractures, and the decoctions will be taken orally or will apply as a paste to the site of fracture. While in Brazil the plant is used to skin sores, treat furuncles and conjunctivitis. Where as in north-eastern part of Brazil, this plant is used as cholesterol suppressant agent (Kartika et al., 2016). Furthermore, medically in South America, fresh juice from leaves and stem is used for eye inflammation treatment, while the infusion and decoction of parts of the plants are used for gout and arthritis (Majumder, 2011). In Caribbean and Jamaica, colds and kidney problem are treating using this herb (Narayanamoorthi et al., 2015). A decoction from this plant is used in the Philippines to treat renal problems and to decrease uric acid levels. Furthermore, this plant used to topically for skin disorders such as acne and boil. This plant also has externally used as a facial rinse for complexion problems. The pounded whole plant used as warm poultice for boils, pustules and pimples. Besides that, it is also used for headaches, rheumatic pains and impotence (Sheikh et al., 2013).

Aerial parts of *Peperomia pellucida* revealed dose-dependent analgesic and anti-inflammatory actions by interfering with prostaglandin synthesis (Mutee et al., 2010). It is also reported that a dose dependent depressant effect and antipyretic effect of *Peperomia pellucida* leaf as compared to standard aspirin (Khan et al., 2008). While the potential use of the plant reported as an antioxidative agent (Mutee et al., 2010). Ethno-botanical studies proves that since long, the plant has been used for medicine. The plant has been consumed as salads or crushed and mixed with water to form a mixture, heated and consume orally to treat disease (Alves et al., 2018).

2.1.7 Chemical Constituents

In recent years, awareness about importance of medicinal plants among human being has increased. Drugs from plants are easily accessible, cheap, effective and safe. The naturally occurring phytochemical concentration in medicinal plants plays a significant role in traditional remedies and also in modern medicines (de Moraes et al., 2014; Razmavar et al., 2014). Studies have revealed hundreds of chemical compounds from this herbs (Kartika et al., 2016). Previous phytochemical studies have revealed varieties of major classes of compounds with biological activities present in few species of *Peperomia*, including essential oils, flavonoids, phenylpropanoids, benzoic acids, benzopyran derivatives, secolignans, terpenes, arylpropanoids, phenolic compounds and a dimeric ArC_2 compound (Abere & Okpalaonyagu, 2015; Mota et al., 2011; Sangsuwon et al., 2015; Velozo et al., 2009). There are many variety of chemical constituents were found in *Peperomia pellucida*. The presence of phenols, alkaloids, sesquiterpenes, saponins, and tannins revealed during phytochemical screening (Majumder et al., 2011). Phytochemical analysis by Mensah et al. (2013) and Igwe and Mgbemena (2014), reported the presence of sesquiterpenes, fatty acids, phenol, methoxy benzene, benzodioxole, glycoside saponin, flavonoids, tannins, alkaloids and cardiac glycosides

from the leaves extracts of *Peperomia pellucida*. Some researchers have reported the polyphenolic content from this herb including dillapiole, chromene, peperomin, patuloside A, pelucidin A and quercetin patuloside is xanthone glycoside (Ahmad et al., 2017; Hamzah et al., 2012). Organic compounds are obtained from *Peperomia pellucida* including alcoholic compound, ether compound, diterpene, palmitic acid, linoleic acid, linolenic acid, phenolic unsaturated fatty acid ester, sulphur compound, ketone compound, vitamin and steroids.

The aerial part of this plant reported contain, phenols, steroids, tannin, saponins, terpenoids, alkaloids and amino acids. Tetrahydrofuran lignin, dihyronapthalene, chromene and 3',4', dihydroxy-3-5-dimethoxy flavone-7-O-β-rhamnose have been isolated (Kurniawan et al., 2016). The essential oil shows characteristic by the presence of dillapiole, β-caryophyllene and carotol. Carotol was the major hydroxlated sesquiterpene in chemical analysis of this plant (Hamzah et al., 2012; Majumder et al., 2011). In medical literature, the essential oil of the plant is primarily found. One study isolated 71 compounds from the essential oil of 10 Piperaceae species. It also documented the presences of phytosterols and flavonoids such as apigenin, isovetexin, arylpropanoids (eg, apiols), pellucidatin campesterol, acacetin and stigmasterol, substituted styrenes and dimeric ArC_2 compound or pellucidin A have been isolated (Abere & Okpalaonyagu, 2015; Majumder et al., 2011).

A study reported that new compounds were found from *Peperomia pellucida.* These include peperomins A, B and C, sesamin, and isoswertisin. The study suggested the presence of tetrahydrofuran lignin (7,8-*trans*-8,8'-*trans*-7',8'-*cis*-7,7'-bis(5-methoxy-3,4-methylenedioxypheny l0-8-cetoxymethyl-8'-hydroxymethltetrahydrofuran) exhibited estrogen agonist activity . Apart from the above chemical compounds,

Peperomia pellucida also contains ascorbic acid, protein, calcium, potassium, magnesium, phosphorus, sodium and also iron. High crude fibre and carbohydrate are also reported for the leaves. The food fibre helps in absorption of dietary minerals and helps to reduce absorption of cholesterol (Florence et al., 2017; Mensah et al., 2013).

2.1.8 Medicinal Properties

Medicinal plants are being used as therapeutic by man since ancient time. Recently, there has been great interest to study medicinal plants with extensive research around the world. This is due to the potential and diversity that medicinal plants have as a source of therapeutic product. Mainly, the purpose of the studies related to therapeutic plants is for scientific evidence of the therapeutic properties by characterize the active compounds of the plant (Sussa et al., 2013). *Peperomia pellucida* contain patuloside A, where it is important for antibacterial activity. It is potent against gram-positive and gram negative bacteria (Khan et al., 2010). Methanolic fraction from *Peperomia pellucida* destroy the *Acanthamoeba* cyst, thus revealing the presence of antiamoebic compounds (Sangsuwon et al., 2015). *Peperomia pellucida* oil contains *trans*-caryophyllene and when tested with mice, this *trans*-caryophylene had exhibited anti-inflammatory action (Ooi et al., 2012). Calcium is mineral element found in *Peperomia pellucida* which is important for bone. Fracture healing was evaluated in female Wistar rat using aqueous extract of *Peperomia pellucida*. It is reported, through stimulator effects on osteoblast differentiation and mineralization, the ethanolic extract of *Peperomia pellucida* accelerates fracture repairmen and marrow injury in rats (Florence et al., 2017; Hartati et al., 2015; Ooi et al., 2012). Alkaloids and flavanoids are reported comprises antimicrobial properties and found in *Peperomia pellucida*. The ethanolic extract of *Peperomia pellucida* reported to contain antibacterial properties which was evaluated with disc diffusion method. The plant extract is potential source of antimicrobial agent against gram positive bacteria such

as *Staphylococcus aureus, Bacillus subtilis* and *Bacillus cereus* and gram negative bacteria, such as *Escherichia coli, Proteus mirabilis, Salmonella thypi* and *Pseudomonas aeruginosa* (Akinnibosun et al*.,* 2008; Zubair et al., 2015). Flavonoids are known to contain antioxidant activity to protect the body against cancer and other degenerative disease such as arthritis and diabetes (Mensah et al., 2013). Apart from that, flavonoid also know for anti-ulcer, anti-inflammatory and anti-neoplastic properties (Majumder et al., 2011). The isolated 3', 4', dihydroxy-3-5-dimethoxy flavone-7-O-β-rhamnose in an investigation from *Peperomia pellucida*, showed a mechanism action as angiotensin converting enzyme (ACE) inhibitor. This compound is potent as a folk medicinal of antihypertensive. ACE inhibitors also contribute in the treatment of diabetes mellitus, chronic renal disorder, heart failure, myocardial infarction and stroke (Kurniawan et al., 2016; Saputri et al., 2015). The chloroform extract of *Peperomia pellucida* exhibit antifungal activity against *Trichophyton mentagrophytes.* A whole plant extract of *Peperomia pellucida* was reported for antiprotozoal activity by inhibiting the growth of chloroquine-resistant *Plasmodium falciparum* Indo strain by 95% *in vitro* at 100 mg/mL and the rodent malaria *Plasmodium vinckei petteri* by 78% *in vivo* at 1000 mg/kg (Igwe & Mgbemena, 2014; Majumder et al., 2011). Literature has reported that high amount of iron is found in *Peperomia pellucida* which will help to overcome the iron deficiency problem (Ooi et al., 2012). Carotol is an important aromachemical and can be found in *Peperomia pellucida*. One of the most popular phytotoxic fungus, *Alternaria alternate* activity can be inhibited carotol (Verma et al., 2015). Dillapiole is a potent natural insecticidal agent found in *Peperomia pellucida*. It is also possess cytotoxic activity, adulticidal activity against *Aedes aegypti* and protect against gastric ulcer activity (Paz et al., 2017; Rojas-Martínez et al., 2013; Verma et al., 2015). Plant extract of *Peperomia pellucida*, was cytotoxic against HL-60, MCF-7 and HeLa cells. It is claimed that peperomin E inhibit the growth of this cancer cells. Phytol is one of most important

diterpenes which has antimicrobial and anticancer activity (Verma et al., 2015). Cardiac glycoside is an organic compounds acts on the heart muscles and help to increase renal flow. Folk's medicine containing cardiac glycosides are used to treat congestive heart failure, cardiac arrhythmia, constipation oedema and microbial infection (Awe et al., 2013). Steroids present in *Peperomia pellucida,* are used by athletes to increase muscles and bone in women, steroids are used as hormone controller (Mensah et al., 2013). Khan et al. (2008) reported, the ethyl acetate and petroleum ether fraction of *Peperomia pellucida* leaves extract showed the presence of psychoactive substances, a CNS depressant in nature. The observations showed, ethyl acetate fraction is less active than petroleum ether fraction. At the same doses, petroleum ether fraction induced sleep on the duration of diazepam and nikethamide toxicity was better in causing latency of the death. The presence of campesterol, stigmasterol and β-sitosterol reduce the cholesterol level in body (Khan et al., 2008). The effectiveness of burn healing was tested on rabbit using *Peperomia pellucida* leaves extract gel. The different concentration of leaves extract gel showed healing effect on burn wound (Mappa et al., 2013). *Peperomia pellucida* has been using as a natural sources as antisickling remedies. The laboratory test was done by adding methanol extract into a blood sample collected from sickle cell anemia patient and the inhibition was observed (Abere & Okpalaonyagu, 2015).

2.2. Plant Tissue Culture (*In vitro* Regeneration)

The history of plant tissue culture takes its origins from the discovery of cell theory in the year 1838 by Schleiden and Schwann. According to this cell theory, cell is the simple structural unit of all living organisms. So every single cell is autonomic and thus, every plant cell has the ability to regenerate into complete individual plantlet. A breakthrough in tissue culture was achieved in 1930 by Kolte and Robbinson with successfully culturing roots and stem tips (Hussain et al., 2012).

Tissue culture means the development of plant cells through *in vitro*. In Latin *in vitro* is known as *vitreous* which means glass. For more than 55 years ago, plant cell culture techniques were established to study the biological and physiological properties of plant cells to be conducted more precisely if the cells are isolated from the plant (Purohit, 2012). Through tissue culture, we often produce the clones of plants under controlled nutritional and environmental conditions. These clones are identically similar with the selected genotype. Furthermore, through controlled conditions able to provide the necessary supply of nutrients, appropriate pH medium, suitable temperature and suitable gaseous and liquid environment for the growth and multiplications of the cells. Generally, three phases are involved in plant tissue culture. First, is the isolation of plant tissue from its natural environment; secondly, aseptic techniques is used to gain sterile materials and, thirdly, the maintenance of culture in controlled condition (Hussain et al., 2012).

According to Willmer (2015), the tissue culture technique which is being practised today can be categorized under three different parts. The first part is known as tissue culture. In tissue culture, small portion of tissue will be transferred into a suitable medium by stimulating to form colonies with their similar functions. In such culture, the tissues may lost their original constitution but the principal cells emerging into zones of outgrowth can be observed. The second part is known as cell culture. In cell culture, the cells of a tissue will be produced in a same technique as bacteria are grown. All the original constitution of tissues will be removed and only the uniform multiplication and growth of cells are essential. The different tissue's cells will become dedifferentiated and comparatively similar in appearance. The third part is known as organ culture. The development through embryological and the preservation of regular physiological functions are given the main importance in organ culture. Furthermore, normal

constitution of the tissue is important than suppressing the growth and migration of dedifferentiated cells.

The explant is the plant tissue materials used to induce plant cell culture. All plant tissues can be used as explants depending on efficiency of the plant. The wounded cells on the surface of the explant will expand, divide, dedifferentiate and form callus (Barbulova et al., 2014). The formation of new organs from cut sites is the unique feature of plant regeneration. In many different plant species, formation of roots occurs from the shoot cutting. Some plants regenerate leaves and shoots from root cutting and regeneration of shoots and roots from leaf cutting (Ikeuchi et al., 2016).

Large number of plant species including numerous medicinal or ornamental plants have been commercially propagated through plant cell and tissue cultures. This techniques offer great potential for rapid multiplication but also for conservation of valuable, elite, endangered and rare plant species. *In vitro* conservation of medicinal plant germplasm is important to support chemical analysis and genetic improvement studies due to the continuous deforestation and extensive collection, (Sahai et al., 2010; Verma et al., 2011). Apart from that, *in vitro* propagations has proved several potential advantages over conventional propagation method like high multiplication rate in short period of time, season independent production of plants, producing disease-free plant, plant genome transformation, production of plant derived metabolites of important commercial value and germplasm conservation. Maintaining true to type of the *in vitro* plant is very important in commercial industry. When using elite genomic type plant, it is important to preserve certain agronomic and horticultural traits. Nevertheless, a report shows that, tissue culture environment and condition cause modification in cellular controls which resulted genomic changes in the *in vitro* plants. Therefore, at the early

stage the *in vitro* plantlet should assessed for genetic stability to prevent deleterious effect expressed in later growth (Altpeter et al., 2016; Rai et al., 2012).

For decades, plants are studied for their vital constituents and the nutritional factors (Naik & Al–Khayri, 2016). Approximately 200,000 natural products have been identified, and some of them have been used as pharmaceutical, agrochemicals, flavours, fragrances, colours, bio pesticides and food additives. Different methods have been developed to produce natural products. Production of natural products depends on molecular complexity, demands and economic production industries. New compounds are being produced by plant and new chemical models are drawing for new drugs. This is due to unexplored plants chemistry (Naik & Al–Khayri, 2016; Ochoa-Villarreal et al., 2016). Plant tissue culture is a acknowledged technology to produce natural products especially for the production of natural products with complex structures. (Fischer et al., 2015). The *in vitro* propagation of plantlet or callus able to provide plant material proficient of producing secondary metabolites. Hence, through micropropagation large numbers of homogenous plants can be produced all year round which can be commercially lucrative and benefits the conventional horticultural propagation practices (Mota et al., 2011). Many research have focused on expending *in vitro* culture of medicinal plants for thr production of secondary metabolites (Atanasov et al., 2015; Karuppusamy, 2009). Useful bioactive compounds are identified and isolate to generate the need for continuous production. Under controlled environments, well-founded technology platform are used to produce natural products from *in vitro* cultured plant (Espinosa-Leal et al. 2018).

2.2.1 Plasticity and Totipotency

An essential concepts to understand plant tissue culture and regeneration process is through plasticity and totipotency. Due to the long life span and sessile nature of plants,

they have produce a greater strength to endure extreme conditions and predation than having animals. Through plasticity, plants able to modify their metabolism, adapting growth and development to suit the surrounding environmental condition. The important aspects of adaptation that concern for plant tissue culture and regeneration are the competences to use almost any part of the plant tissue to trigger cell division and to reconstruct the lost organs or able to go through different evolving process in response to particular condition. Typically, cell division can be initiated from any tissue of plant cells. Which can be regenerated and switch on different biosynthetic and development pathways. Generally, the cultured *in vitro* plant cells and tissues exhibit very high degree of plasticity. This allows one type of tissue or organ to be initiated from another type and subsequently regenerated. Totipotency is the maintenance of genetic potential. Thus, genetic structure of plant cell preserved to generate an entire plant. Totipotency able the cell to self-renew after series of cell divisions to produce a cell identical to adult cell. Plant regeneration and cell culture provide the most compelling evidence for totipotency (Barbulova et al., 2014; Gaillochet & Lohmann, 2015; Geneve, 2011; Ikeuchi et al., 2016).

2.2.2 Culture Environment

Regeneration of plant cell influenced by various environmental conditions with proper supply of nutrient composition, gelling agents, pH, light, temperature and suitable gaseous. In plant tissue culture, plant cells, tissues, organs or whole plant are grown *in vitro* on artificial media under controlled nutritional and environmental conditions. (Espinosa-Leal et al., 2018; Hussain et al., 2012). The important parameters in tissue culture incubation are the light, temperature and humidity. Light is important for morphogenetic processes of shoot, root induction and embryogenesis. The photoperiod, quality and intensity of light are important to achieve good culture. Normally, cool white

fluorescent light exposure for 12-16 hours are preferred. Blue light however, helps to promote shoot formation while red light helps to induce rooting in many species. The optimum temperature of 26ºC - 28ºC in culture room is usually maintained. Heat build-up can be a problem, so the temperature must be maintained and monitored. The culture room humidity level at 60-98% ± 3% and constant forced-air ventilation must be controlled (Bhojwani & Dantu, 2013; Caula, 2016; Shahzad et al., 2017).

2.2.3 Culture Media

The culture conditions too play an important role in the growth, development, production of secondary metabolites and morphogenic response of an explant. Fundamentally, a successful plant tissue culture rely on the selection of suitable medium for the plant species. The plant growth rely on genetic make-up, surrounding condition and composition of the culture medium (Bhojwani & Dantu, 2013; Yue et al., 2016; Molnár et al., 2011). The uptake of ingredient by tissue in culture influence by the pH of the medium. Usually, the pH of medium is set at pH 5.8, because at this pH all the salt will be maintained in a near buffered form (Bhojwani & Dantu, 2013). In general, salt requirement varies from one species to another. Similarly, different plant growth regulators enhance the regeneration and growth (Caula, 2016). The cell cultures can be maintained using solid or liquid medium depending on the purpose of collection and culture requirement of the plant (Ahmadian et al., 2017; Grzegorczyk-Karolak, et al., 2017; Jang et al., 2016). The culture media used for plant tissue culture are composed of three essential source of nutrition for plant to grow in nature. The first component is the necessary elements or mineral ions, which will be used as a complex mixture of salts. The second component is the organic supplement, source of vitamins and amino acids. The last component is a fixed source carbon, used in the process of photosynthesis as a source of energy which is usually supplied in the form of sucrose (Molnár et al., 2011). Several

types of culture medium are widely used with or without additional organic or inorganic supplements. One example include Murashige and Skoog medium (MS) (1962) which is widely used medium for plant regeneration from tissues and callus. These are the other medium used in plant tissue culture namely, Gamborg B5 medium (1968), Linsmaier and Skoog medium (LS) (1965), Lloyd and McCown medium (1980), Schenk and Hildebrandt medium (1972), White's medium (1963), Nitsch's medium (1969) and Kao and Michayluk medium (1975). These different media contain different concentrations of salt and minerals (Caula, 2016; Molnár et al., 2011; Thomas & Chaturvedi, 2008; Yue et al., 2016). The required ingredients for plant tissue culture are macronutrients, micronutrient, carbon source, vitamins, plant growth regulators and organic materials.

2.2.3.1 Macronutrient

Macronutrient is one of the important elements in plant tissue culture media. This macronutrients are required in milimolar quantity. Six major elements are included in macronutrient which is crucial for the development of plant cells and tissues. They are calcium (Ca), nitrogen (N), phosphorus (P), potassium (K), sulphur (S) and magnesium (Mg). Calcium helps in the movement carbohydrates and amino acids throughout the plant and promotes root development. Usually in media, calcium will be used in the form of calcium chloride or calcium nitrate. Nitrogen has profound effect on the morphology of the tissue that grow and molecular make up of chlorophyll, nucleic acid, alkaloids, plant hormone and amino acids. Nitrogen is sourced in form of ammonium nitrate (NH_4^+) and nitrate compounds (NO_3^-). In media, phosphorus works as an enzyme activator and potassium is required for normal cell division, synthesis of carbohydrates and protein, to produce chlorophyll and as nitrate reduction. Whereas, sulphur helps to promote root development and deep green foliage. Magnesium is known as the central element in

chlorophyll molecules and essential as an enzyme activator (Agarwal, 2015; Purohit, 2012).

2.2.3.2 Micronutrient

Microelements are required in extremely small quantity but essential element for plant tissue growth. The micro elements are boron (B), manganese (Mn), iron (Fe), zinc (Zn), copper (Cu), molybdenum (Mo) and cobalt (Co). In media boron helps in sugar movement in plant. Small amount of boron is added into the media in the form of boric acid (H_3BO_3). Yellow mottling of leaves is due to lack of manganese. Manganese sulphate ($MnSO_4.H_2O$) is used in the media for chloroplast membrane. Iron (Fe) in media used in the form of ferrous sulphate ($FeSO_4.7H_2O$) which involve in chlorophyll synthesis. Iron also help to convert energy in photosynthesis and respiration. A trace of zinc as zinc sulphate ($ZnSO_4.7H_2O$) involve in the formation of chlorophyll and production of auxin. Only 0.25 mg of copper in the form of cupric sulphate ($CuSO_4.5H_2O$) used in plant tissue culture media for energy conversion. Lack of copper in media can cause malformations, growth inhibition, twisted and blotched leaves. The function of molybdenum in media is to convert nitrogen to ammonia (Bhojwani & Dantu, 2013; Purohit, 2012).

2.2.3.3 Organic Nutrient

To achieve successful plant tissues in cultures, organic compounds need to be added into the media. The organic compounds contains vitamins, amino acids, proteins, and enzymes. Usually plants synthesize their own nutrient. Since plants in the culture are too small or incomplete to synthesize the organic chemical needed, organic nutrients need to be added into the tissue culture media to augment the plantlets' atmospheric. Sucrose is a carbon source for plants and can be found abundantly in plant tissues. Since plants grow in tissue culture media, they are unable to produce all the sugars that is required so high

concentration of sucrose need to be added into the media. Vitamins are synthesized endogenously by the plants and in various metabolic processes vitamins are used as catalysts. To achieve the best growth of the tissue, it is compulsory to supplement the medium with required amount of vitamins. Vitamin B complex contains essential compounds for plant metabolism and growth. Apart from vitamin B complex, thiamine, nicotinic acid, pyridoxine, folic acid, ascorbic acid, riboflavin, biotin and tocopherol also used in the media. Most of this vitamins are known as coenzymes in media for metabolic functions (Bhojwani & Dantu, 2013; Purohit, 2012).

2.2.3.4 Amino Acids

The amino acids needed for various metabolic processes are able to synthesizing by the cultured tissue. For stimulating cell growth and establishing cell lines, it is important to add amino acids into the media. The amino acids that commonly used in media as sources of reduced organic nitrogen are L-glutamine, L-asparagine, L-glycine, L-argine, L-serine, L-proline and L-cystein. These amino acids are used for inducing and maintaining somatic or nonzygotic embryogenesis (Bhojwani & Dantu, 2013; Caula, 2016).

2.2.3.5 Plant Growth Regulators

Plant growth regulators (PGRs) is necessary to regulate and trigger the initiation and development of shoots and roots on explants culture media. PGRs will also help to stimulate expansion and cell division. Even though growth regulators can be synthesized naturally by the explants, it is vital to supplement them exogenously to induce certain responses (Caula, 2016). Two main classes of PGRs used in tissue culture are auxins and cytokinins. In 1957, Skoog and Miller explain the relative effects of auxin and cytokinin ratio in tobacco tissue culture. They have revealed that, the differentiation of organs could

be regulated by changing the concentration of auxin and cytokinin in the medium (Purohit, 2012).

Auxins involve in various developmental processes in plants including stem and internodals elongation and swelling of tissue, apical dominance, abscission, adventitious root development and somatic embryogenesis. Apart from that, auxins have been used in tissue culture to induce cell division, cytodifferentiation and organogenetic differentiation. The low concentration of auxin favours the initiation of root, whereas, at high concentration of auxin induces callus (Agarwal, 2015; Caula, 2016). Commonly used auxin (Figure 2.3) in tissue culture media are indole-3-acetic acid (IAA), indole-3-butric acid (IBA), 2,4-dichlorophenoxy-acetic acid (2,4-D), naphthalene-acetic acid (NAA), and picloram. IAA and IBA is a naturally occurring auxins, whereas NAA, IBA, 2,4-D and p-CPA are synthetic auxins have been used in tissue culture (Bhojwani & Dantu, 2013). For rooting IAA, NAA and IBA are widely used (Quambusch et al., 2017; Shekhawat et al., 2015). 2,4-dichlorophenoxy-acetic acid (2,4-D) is specially used for the growth and induction of callus and somatic embryogenesis (Bhojwani & Dantu, 2013).

Cytokinins promote cell division, which trigger the shoot proliferation by releasing axillary buds from apical dominance, control seed germination, and induce adventitious shoot from callus (Bhojwani & Dantu, 2013). The commonly used cytokinins (Figure 2.4) are kinetin (KN), benzylaminopurine (BA), zeatin, thidiazuron (TDZ) and isopentenyladenine (2iP) (Caula, 2016; Smith, 2013). The higher concentration of cytokinin induce adventitious shoot formation but inhibit the root formation (Chawla, 2018). Cytokinin also promotes axillary shoot formation by opposing apical dominance regulated by auxin (Caula, 2016). BA is synthetic and most frequently used cytokinin

compared to kinetin. While, Zeatin, and 2-iP, are naturally occurring cytokinin (Chawla, 2018).

Another type of plant hormone, gibberellin is isolated from higher plants and fungi (Chawla, 2018; Purohit, 2012). There are thirty four gibberellins chemically identified (Purohit, 2012). Gibberellins are reported used in plant tissue culture to induce elongation in internodal, growth of stem and develop plantlets from *in vitro* formed adventive embryos (Bhojwani & Dantu, 2013).

Another group of plant hormone, abscisic acid (ABA) is a naturally occurring growth regulator compound. ABA usually supplemented to suppress or to stimulate callus growth, depending upon the species. ABA able to enhances shoot proliferation and suppress the later stages of embryo development. ABA functions as an endogenous regulator of abscission in plants. Abscisic acid is heat stable, but light sensitive (Bhojwani & Dantu, 2013; Chawla, 2018; Smith, 2013).

2.2.3.6 Gelling Agent

Tissue would submerge and die in liquid culture due to anaerobic conditions. To avoid this problem, suitable gelling agent is used to solidify the medium. The gelling agent for tissue culture should be inert, withstand autoclaving sterilization and in a liquid form when it is in high temperature so that the medium could be dispensed (Bhojwani & Dantu, 2013). The tissue culture media must be strong to support the culture and yet must be able to allow nutrients to diffuse to the plantlet through the medium. Agar is used in tissue culture media because it solidify into a gel. Agar obtain from several species of seaweed which contains high molecular weight of polysaccharides that can bind water. The concentration of agar influence the firmness of the media. The consistency of agar will

influence the culture growth. The higher the agar concentration, the harder the media will be and if lower concentration of agar used, the media will be soft and unable to produce successful culture (Caula, 2016; Purohit, 2012). Other alternative gelling agent also used in plant tissue culture are gelrite or gellan gum, alginate, agarose, gelatin, and isubgol. All alternative agar comprises gelling agent properties. Poor quality of agar can affect the growth of cultures (Chawla, 2018).

2.2.3.7 Antibiotics

Antibiotic is a chemical substance has the ability inhibit and destroy the growth of harmful microorganism without causing any effect to the host (Bennett & Sánchez, 2015; Singh et al., 2017). Antibiotic will be added into the medium by cold filter sterilization method. The commonly used antibiotic to eliminate bacteria on woodly plant are cefotaxime, polymyxin-B, rifampicin and tetracycline. Streptomycin, gentamysin, ribavirin are effective antibiotic used to inhibit certain microorganisms (Purohit, 2012).

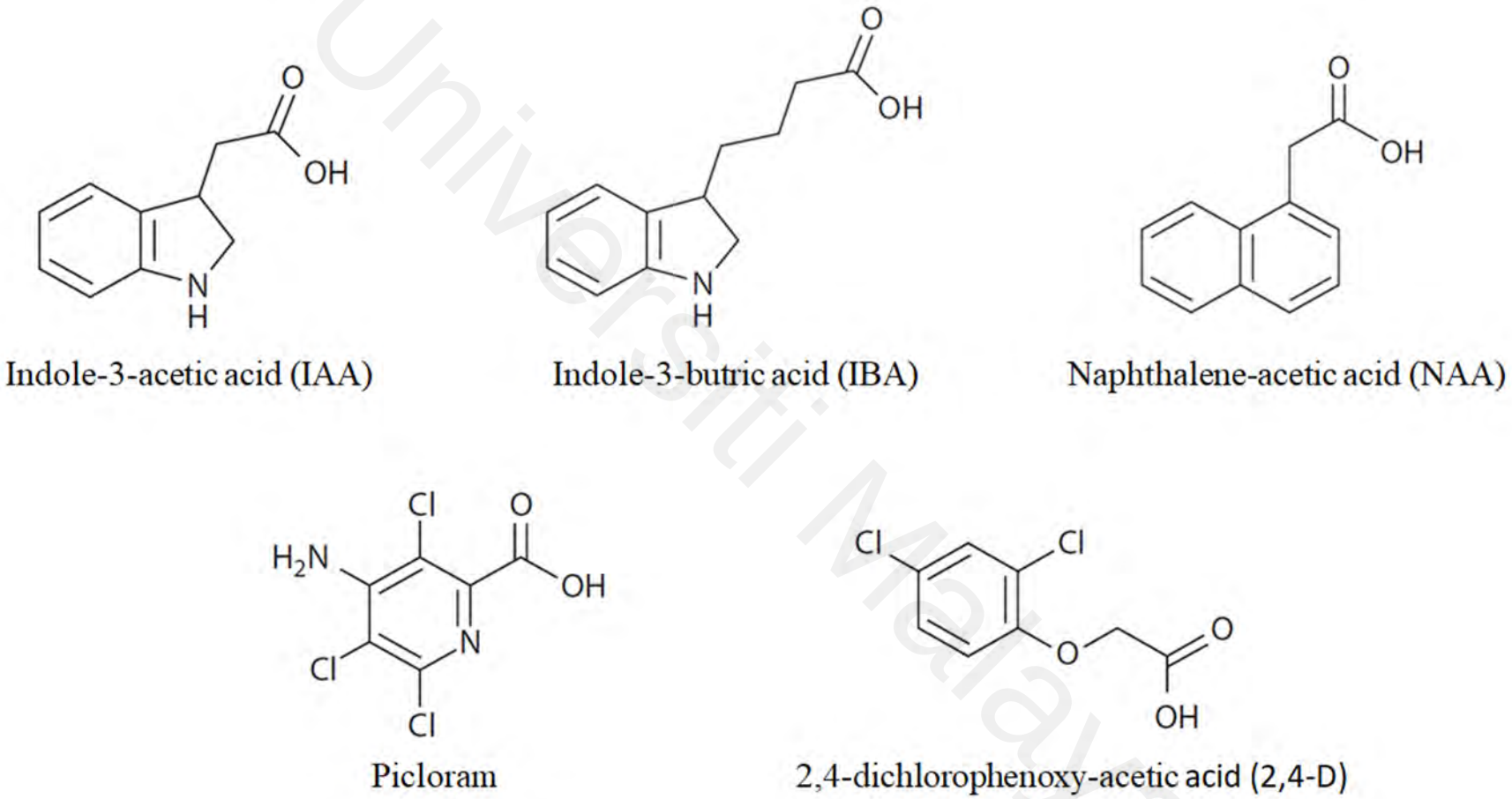

Figure 2.3: Structure of auxins used in tissue culture. **(**Caula, 2016; Smith, 2013)

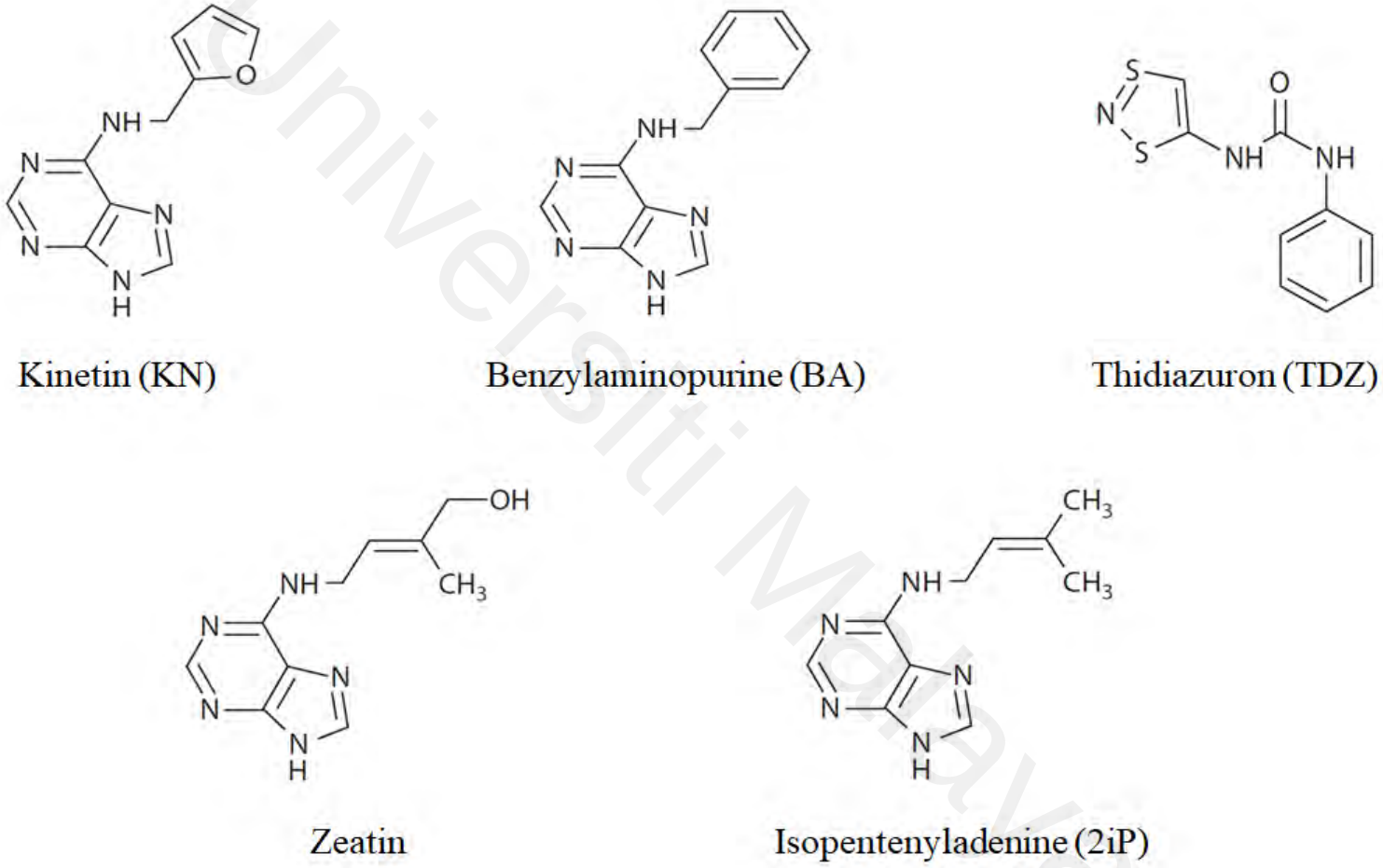

Figure 2.4: Structure of cytokinins used in tissue culture. (Caula, 2016; Smith, 2013)

2.3. Somatic Embryogenesis

Somatic embryogenesis (SE) is a specialized process of development by which the egg is fertilized through series of cell divisions and differentiation to form an embryo (Bhojwani & Dantu, 2013). In other word, embryos are regenerated from somatic cells, tissues or organs directly or *de novo* from the tissue (Chawla, 2018). Somatic embryos resembled by zygotic embryos with the competency to develop into new plants. This process happens when somatic cell differentiate into a plant without involving fertilization. To study about early stage of embryogenesis differentiation and development, somatic embryogenesis is used (Mose et al., 2017; Wu et al., 2011). Through somatic embryogenesis propagation, multiple genetically identical embryos can be formed without waiting for reproductive season (Mose et al., 2017). Reprogramming of gene expression pattern with cascades of genetic signal needed in somatic embryogenesis to turn on or of the expression of different gene group. Initiation of embryogenesis mechanism are complex but similar among different species. For somatic embryogenesis to take place, few processes need to be activated. Firstly, explant cells must be able to express totipotency. Secondly, cell must be able to respond to exogenous signals. Finally, specific signals must be able to induce by the cell and follows the embryogenic pathway (Elhiti et al., 2013). Somatic embryogenesis is essential tool to improve various system through *in vitro* propagation (Fehér, 2015; Kumar & Thomas, 2012; Ramawat et al., 2014). Media with different concentration of PGRs are able to induce embryos directly from culture explant tissues or able to initiate callus with embryo (Mose et al., 2017; Smertenko & Bozhkov, 2014). Through somatic embryogenesis, viable approach are provided for clonal propagation of elite genotypes and growth of synthetic seed technology (Prakash & Gurumurthi, 2010). Somatic cell from plants differentiate in more flexible way compared to animal cell. It is reported that state under certain circumstances differentiated plant cells can revert to an earlier development state

and will regain pluripotency or totipotency (Fehér, 2015). Steward et al. (1958) and Schraudolf and Reinert (1959) reported the first origin and development of *in vitro* adventitious embryos in a cell suspension. The presented embryo like structure aroused from a root callus of carrot. Since the successful discovery, many researcher reported somatic embryogenesis induction in many other plants (Purohit, 2012 & Shahzad et al., 2017).

2.4. Secondary Metabolites

Phytochemists have been investigating secondary metabolites from plants. Secondary metabolites refer to the bioactive compounds filtered by natural selection in plants, microorganisms or animals. Since the beginning humans have been using secondary metabolite product for different uses (Espinosa-Leal et al., 2018; Wink, 2015). Only since last 100 years natural product been replaced with synthetic drugs (Karuppusamy, 2009). Secondary metabolites from plants are a fascinating class of phytochemicals exhibiting immense chemical diversity (Gandhi et al., 2015). Secondary metabolites from higher plant produce substances that have been a source for pharmaceuticals, agrochemical, insecticides, flavouring, fragrance and food colorants (Karuppusamy, 2009; Yue et al., 2016).

It is interesting to know that our ancestors from ancient times have used medicinal plants for therapeutic purposes. Today, the research with regards to medicinal plants have taken the research field by storm due to their potential as well as the diversity of these plants. The effects observed from these plants originated essentially from the active compounds that are prevalent in the said plants and characterizing these compounds will help us understand and subsequently elucidate their respective scientific properties (Shanmugam et al., 2012; Sharma et al., 2012; Street, 2012; Sussa et al., 2013).

There are numerous therapeutically active chemical compounds in such medicinal plants and these include alkaloids tannins, flavonoids, sugars phenols, coumarins, terpenoids, saponins. The advantage of these compounds is that they can be precisely studied and dosage on par to the current pharmaceutical standards, thereby presenting as the next wave of therapeutic products in pharmaceutical industry (Oloyede et al., 2011).

Biotechnology approaches are being used by researchers to understand and exploit the secondary metabolites from plants due the never ending source of novel chemical structures with pharmacological activities (Gandhi et al., 2015). Many researchers done investigating the biological and bioactive properties of the plant (Dias et al., 2016).

Secondary metabolites is characterized based on the chemical structure with multiple chiral centres and labile bonds. For continuous production of secondary metabolite, a method need to be generated to identify and isolate useful bioactive compound (Pickens et al., 2011). Researchers have developed various methods to produce secondary metabolites depending on molecular complexity, market demand and economic production (Ochoa-Villarreal et al., 2016).

Through biotechnological approach, plant tissue culture promising for the production of valuable plant secondary metabolites which promotes a higher rate of metabolism than that found in field grown plants (Jang et al., 2016). The plant grown in tissue culture produces same valuable secondary metabolites as the parent plant (Karuppusamy, 2009). Micropropagation provides advantages in horticultural propagation practices by easing large numbers of homogenous plant all year round (Espinosa-Leal et al., 2018).

2.5. Antimicrobial Properties

The word antimicrobial was derived from the Greek words “anti” means “against”, “mikros” mean “little” and “bios” mean “life”. This refer to all type of agents that act against microbial organisms. The term antimicrobials is used overall for all the microorganism, such as bacterial, viruses, fungi and protozoa. (https://amrls.cvm.msu.edu/pharmacology/historical-perspectives/tools/module-pdf-files/pharmacology). Antimicrobial molecules acts as an agent to kill microorganisms. This agent is a chemical compounds or physical agent used to kill the microorganisms (https://www.omicsonline.org/antimicrobial-agents.php). For decades, antimicrobial served as modern medicine to treat a various diseases and help to support in medical procedures (Padiyara et al., 2018). Consciousness about the importance of medicinal plants have increased over the years. Over thousands of years, plants have been used for therapeutic purposes. Drugs obtained from plants are easily available, reasonable price, safe, effective and has less side effects (Razmavar et al., 2014). Medicinal plants with their bioactive compounds harbour potential endophytic microbes (Egamberdieva et al., 2017). As previously mentioned, society use medicinal plants to treat various diseases caused by microorganisms such as bacteria, fungal and viruses. The presence of natural compounds in plants as antimicrobial agents was shown in numerous studies (Gupta et al., 2016; Passari et al., 2015; Wink, 2015).

CHAPTER 3: METHODOLOGY

3.1 *In vitro* Regeneration of *Peperomia pellucida* L. Kunth.

3.1.1 Plant Collection and Identification

Peperomia pellucida plants were collected and identified from a population growing at Institute of Biological Science's garden of University of Malaya as well as from various areas around University of Malaya (UM). Then, the plants were maintained in the Institute of Biological Sciences garden of the Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Herbarium prepared specimen was prepared and kept at the University of Malaya Herbarium (KLU) with the voucher specimen No. KLU 47794. Fresh intact plants were collected before culturing.

3.1.2 Preparation of Explants and Sterilization

In general, above ground vegetative shoots of *Peperomia pellucida* were collected from the ISB garden, UM. Surface sterilization was performed on the plants to eliminate epiphytes or microbial contamination and to maintain the growth of the plant. Firstly, the *Peperomia pellucida* plants were washed under running tap water for 30 minutes. From this step onwards, the sterilization process was done in a laminar flow. The plants were subsequently transferred into a sterile jam jar. The plants were soaked and washed with three drops of teepol with sterile distilled water for two minutes. After that, the plants were rinsed thoroughly with sterile distilled water for five times. Further, the plants' surfaces were sterilized with 50% sterile sodium hypochlorite for two minutes and followed by rinsing with sterile distilled water for five times. Then, plants were washed in a sterile 50% dettol for 10 minute and followed by rinsing the plant with sterile distilled water for five times. Finally, the *Peperomia pellucida* plants were treated with an antifungal, 0.01% carbendazime diluted in 70% alcohol for 30 seconds and rinsed with

sterile distilled water for five times. Then, the sterile plants were cultured on culture medium.

3.1.3 Preparation of Media

The MS medium (Murashige & Skoog, 1962) (Duchefa) was used in which 4.2 g of MS medium were dissolved in 1 liter of distilled water with 30 g/L sucrose (Systerm) and 3.3 g gelrite and subsequently mixed well. Carbendazime (0.01%) an antifungal was also added into the mixture. Plant growth regulators were added as required. The pH of medium was then adjusted to 5.8 by using sodium hydroxide (NaOH). The medium was heated up till boiling with hot plate heater. Later, the medium was autoclaved at 121ºC for 20 minutes. After autoclaved, the medium was poured into sterile tubes (5.5 cm x 4 cm) in laminar flow chamber. Preparation of the media was carried out before culturing work and the medium must be cooled prior to use.

3.1.4 Effect of Seed Germination under *In vivo* and *In vitro* Conditions

The sterilized seeds were cultured on MS medium (Murashige and Skoog 1962) and sterilized soil. Then the cultures were maintained at 25 ± 2ºC, 16 h/8 h (light/ dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity.

3.1.5 Effect of Plant Growth Regulators on Shoot Induction from Internodal Explants of *Peperomia pellucida* L. Kunth

Plant growth regulators 10 mg: BA, KN, and ADE were dissolved gradually in a few drops of sodium hydroxide (NaOH) or ethanol, and added with 100 mL distilled water. The plant growth regulators were then pipetted into the MS media with various concentrations (0-2.0 mg/L).

For shoots, the sterilized internodals were intersected into small pieces (10 mm) and inoculated onto the MS medium (Murashige & Skoog 1962) as control and also inoculated on MS medium supplemented with the different plant growth regulators such as cytokinins [BA (benzylaminopurine and KN (kinetin)], and ADE (adenine). All the media were added with 0.33% gelrite and 3% sucrose. Carbendazim (0.01%), an antifungal were added into the medium. Then, the pH of the medium was adjusted to 5.8 by using sodium hydroxide (NaOH). The media were subsequently autoclaved at 121ºC for 20 minutes after adjusting the pH. All the cultures were maintained at 25 ± 2ºC, 16 h/8 h (light/ dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity.

The media and combination of plant growth regulators used were as listed below:

1. MS basal medium (without hormone)
2. MS media + 0.5 mg/L BA
3. MS media + 1.0 mg/L BA
4. MS media + 1.5 mg/L BA
5. MS media + 2.0 mg/L BA
6. MS media + 0.5 mg/L KN
7. MS media + 1.0 mg/L KN
8. MS media + 1.5 mg/L KN
9. MS media + 2.0 mg/L KN
10. MS media + 0.5 mg/L ADE
11. MS media + 1.0 mg/L ADE
12. MS media + 1.5 mg/L ADE
13. MS media + 2.0 mg/L ADE

3.1.6 Effect of Plant Growth Regulators Combinations on Shoot Regeneration.

Several combinations of plant growth regulators were tested for plant regeneration of *Peperomia pellucida.* MS medium supplemented with different combinations of plant growth regulators were prepared. Then, the cultures were maintained at 25 ± 2ºC, 16 h/8 h (light/dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity. The following are the list of media with different combinations of hormones that were used in this study.

1. MS media + 1.0 mg/l BA + 0.5 mg/L KN
2. MS media + 1.0 mg/l BA + 1.0 mg/L KN
3. MS media + 1.0 mg/l BA + 1.5 mg/L KN
4. MS media + 1.0 mg/l BA + 2.0 mg/L KN
5. MS media + 1.0 mg/l BA + 2.5 mg/L KN
6. MS media + 1.0 mg/l BA + 3.0 mg/L KN
7. MS media + 1.5 mg/l BA + 0.5 mg/L KN
8. MS media + 1.5 mg/l BA + 1.0 mg/L KN
9. MS media + 1.5 mg/l BA + 1.5 mg/L KN
10. MS media + 1.5 mg/l BA + 2.0 mg/L KN

3.1.7 Effect of Charcoal on Shoot Regeneration at Different Temperatures

The MS medium (Murashige & Skoog, 1962) (Duchefa) was used in which 4.2 g of powdered MS medium was dissolved in 1 liter of distilled water with 30 g/L sucrose (Systerm) and 3.3 g gelrite and subsequently mixed well. 0.01% of carbendazime were also added into the mixture. Plant growth regulators were added as required. Different concentrations of charcoal (0%-25%) were added into the media. Then, the pH of medium was adjusted to 5.8 by using sodium hydroxide (NaOH). The media was heated up till boiling with hot plate heater. The media were autoclaved at 121ºC for 20 minutes. After autoclaved, the media were poured into sterilised tubes in laminar flow. Preparation of

the media must be done prior to before culturing work. The media must be cool prior to use.

The sterilized internodal segments were intersected into small pieces (10 mm) and inoculated into the media with different concentration of charcoal. Then, all the cultures were maintained at 13 ± 2°C and 25 ± 2°C, 16 h/8 h (light/dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity.

The following are the list of different concentrations of charcoal used:-

1. MS media + 0% charcoal
2. MS media + 1% charcoal
3. MS media + 5% charcoal
4. MS media + 10% charcoal
5. MS media + 15% charcoal
6. MS media + 20% charcoal
7. MS media + 25% charcoal

3.1.8 Root Induction

For root induction, auxin plant growth regulators 10 mg: IBA, NAA and IAA were dissolved gradually in a few drops of sodium hydroxide (NaOH) or ethanol, and added with 100 mL distilled water. Mainly, auxins were used in root induction and their effects varied with type and concentration used in different plant species. Then, the plant growth regulators were pipetted into the MS media with various concentrations (0-2.0 mg/L). All the media were added with 0.33% gelrite and 3% sucrose. 0.01% of carbendazim an antifungal were added into the mixture Then, the pH of medium was adjusted to 5.8 by

using sodium hydroxide (NaOH). The media were autoclaved at 121ºC for 20 minutes after adjusting the pH.

The healthy adventitious shoots, from aseptic seedlings were transferred into a different medium with rooting hormone IBA, NAA and IAA for root formation. The cultures were then maintained in a culture room at 25ºC ± 2ºC. All the cultures were maintained at 25 ± 2ºC, 16 h/8 h (light/ dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity. After two weeks, the adventitious shoots started to form adventitious root.

The media and combinations of plant growth regulators used were as listed below:

1. MS media + 0.5 mg/L IAA
2. MS media + 1.0 mg/L IAA
3. MS media + 1.5 mg/L IAA
4. MS media + 2.0 mg/L IAA
5. MS media + 0.5 mg/L NAA
6. MS media + 1.0 mg/L NAA
7. MS media + 1.5 mg/L NAA
8. MS media + 2.0 mg/L NAA
9. MS media + 0.5 mg/L IBA
10. MS media + 1.0 mg/L IBA
11. MS media + 1.5 mg/L IBA
12. MS media + 2.0 mg/L IBA

3.1.9 Acclimatization

The rooted plantlets were removed from the culture tubes in the laminar flow. Then, the roots were washed with running tap water to remove the attached medium. Plantlets with fully developed leaves and roots were transferred into a pot containing soil, sand and vermiculite (1:1:1). The plantlets were grown in a culture room for two weeks for hardening with the photoperiod consisting of 16 hour light and 8 hours dark at 25 ± 2°C. The hardened plantlets were transferred to the natural environment in the green house.

3.1.10 Data collection and analysis

A total of 30 replicates were used for each treatment. Visual observations of the culture were performed every week. Data related to frequency of organogenesis of regeneration frequency, shoot induction, number of shoots and root induction were collected. All collected data were analysed and compared using Duncan's multiple range test (DMRT). Mean followed by the different letters in each column are significantly different at $P < 0.05$. Standard errors (SE) of the difference between treatments were also calculated.

3.2 Callus Induction and Somatic Embryogenesis of *Peperomia pellucida* L. Kunth. from Explants

3.2.1 Plant materials and culture condition

The *Peperomia pellucida* plant was collected from different parts of University Malaya, and maintained in the Institute of Biological Sciences, garden of UM, Kuala Lumpur, Malaysia. The *Peperomia pellucida* plants will be washed under the running tap water for 30 minutes. The plants were then soaked and washed with three drops of teepol with water mixture for two minutes. The plants were rinsed thoroughly with sterile distilled water for five times. Further, the plants were sterilized with 50% prepared sterile sodium hypochlorite for two minutes, followed by rinsing with sterile distilled water for

five times. Later, plantlets were washed in a sterile 50% dettol for 10 seconds and followed by rinsing the plant with sterile distilled water for five times. Finally, the *Peperomia pellucida* plants must be treated with an antifungal, 0.01% carbendazime diluted in 70% alcohol for 30 seconds and rinsed with sterile distilled water for five times.

The sterilized internodals were then inoculated onto the MS medium (Murashige and Skoog 1962) (Duchefa) with 0.33% gelrite and 3% sucrose to grow and form plants. The pH of medium was adjusted to 5.8 by using sodium hydroxide (NaOH). The media were autoclaved at 121ºC for 20 minutes after adjusting the pH. A total of 30 culture replicates were prepared. The culture were maintained at 25 ± 2ºC, 16 h/8 h (light/ dark) photoperiod with 25 μmol $m^{-2}s^{-1}$ of light intensity for four weeks.

3.2.2 Effect of Plant Growth Regulators on Callus Induction

Plant growth regulators 10 mg: BA, KN, 2,4-D, NAA, IAA, IBA, GA_3 and ADE were dissolved gradually in a few drops of sodium hydroxide (NaOH) or ethanol, and added with 100 mL distilled water. The plant growth regulators were then pipetted into the MS media with various concentrations (0-3.0 mg/L).

For callus induction, the aseptic seedlings leaves and stems were intersected into small pieces (5 mm^2 or 10 mm^2) and inoculated on the MS medium (Murashige & Skoog, 1962) as control and were also inoculated on MS medium supplemented with the different plant growth regulators such as cytokinins [BA (6-benzylaminopurine and KN (kinetin)], auxins [2,4-D (2,4-dicholorophenoxyacetic acid), NAA (1-naphthaleneacetic acid), and IAA (Indole-3-acetic acid)], gibberellins and adenine. All the media were added with 0.33% gelrite and 3% sucrose. 0.01% carbendazime, an antifungal were added into the medium. Then, the pH of medium was adjusted to 5.8 by using sodium hydroxide

(NaOH). The media were autoclaved at 121ºC for 20 minutes after adjusting the pH. Callus cultures were maintained at 25 ± 2ºC, 16 h/8 h (light/ dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity.

In this experiment, various concentrations and combinations of plant growth regulators were used. The media and combination of plant growth regulators used were as follow:

1. MS basal medium (without hormone)
2. MS media + 0.1 mg/L - 0.6 mg/L BA
3. MS media + 0.1 mg/L - 0.6 mg/L KN
4. MS media + 0.1 mg/L - 0.6 mg/L 2,4-D
5. MS media + 0.1 mg/L - 0.6 mg/L IAA
6. MS media + 0.1 mg/L - 0.6 mg/L NAA
7. MS media + 0.1 mg/L - 0.6 mg/L GA_3
8. MS media + 0.1 mg/L - 0.6 mg/L ADE
9. MS media + 0.1 mg/L 2,4-D + 0.1 mg/L BA
10. MS media + 0.2 mg/L 2,4-D + 0.1 mg/L BA
11. MS media + 0.1 mg/L 2,4-D + 0.1 mg/L KN
12. MS media + 0.2 mg/L 2,4-D + 0.1 mg/L KN
13. MS media + 0.1 mg/L 2,4-D + 0.1 mg/L IAA
14. MS media + 0.2 mg/L 2,4-D + 0.1 mg/L IAA
15. MS media + 0.1 mg/L 2,4-D + 0.1 mg/L NAA
16. MS media + 0.2 mg/L 2,4-D + 0.1 mg/L NAA
17. MS media + 0.1 mg/L 2,4-D + 0.1 mg/L GA_3
18. MS media + 0.2 mg/L 2,4-D + 0.1 mg/L GA_3
19. MS media + 0.1 mg/L 2,4-D + 0.1 mg/L ADE
20. MS media + 0.2 mg/L 2,4-D + 0.1 mg/L ADE

3.2.3 Identification of Embryogenic Callus Using Double Staining Method

3.2.3.1 Preparation of 2% Acetocarmine

The preparation was done as decribed previously (Gupta & Durzan, 1987). The preparation of 2% acetocermine was done in fume cupboard. Glacial acetic acid (45 mL) was dissolved in 55 mL of distilled water in a conical flask. Slowly, 2 g of carmine was added into the mixture and stirred using magnetic stirrer. The solution was gently heated for 5 minutes. After the solution cool down, it was filtered using Whatman filter paper and stored in a screw capped container at room temperature.

3.2.3.2 Preparation of 0.5% Evan's Blue

The preparation was done as decribed previously (Gupta & Durzan, 1987). Evan's Blue powder (0.5 g) was added into 100 mL disttilled water in a conical flask. Then, the flask was covered with stopper and gently swirled the flask by hand to mix the solution. The solution was stored in a screw capped container at room temperature.

3.2.3.3 Double Staining Technique

Through double staining method, embryogenic callus could be easily distinguished and differentiated from non embryogenic callus (Gupta & Durzan, 1987). Callus (1 mm^2) was placed on a clean glass slide. Two drops of 2% acetocermine were dropped on the callus. The callus was gently divided into smaller pieces using needles. For a few seconds the slide was heated over a low flame. Subsequently, the callus cells were carefully rinsed 3 times with distilled water and all the excess liquids was eliminated from the cells. Two drops of Evan's Blue were added onto the acetocarmine stained callus cells. After 30 seconds,the stained callus cells were washed 3 times with the distilled water. The excess water was removed. Lastly, 2 drops of Glycerol were added to the stained callus cells to

prevent the cells fro drying. The cells were covered with cover slips. The prepared samples were observed under the microscope.

3.2.3.4 Examination of Somatic Embryo Stages

Six-week-old somatic embryo of *Peperomia pellucida* were observed and compared by examining the sturucture, type and colour under image analyzer for identification of somatic embryo stages such as globular, heart, torpedo and cotyledonary shapes.

3.2.4 Data analysis

All data were collected and were statistically analysed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p < 0.05$.

3.3 Phytochemical Analysis

3.3.1 Plant Materials

For the preparation of extraction, the intact plants without the roots were collected from the ISB garden UM, and the *in vitro* regenerated plantlets from MS medium were used as comparison. The plants were washed clean from soils and media. The clean intact plants were freeze-dried for five days. After which, the dried samples were ground to a uniform powder and kept in -20ºC freezer.

3.3.2 Soxhlet Extraction

The preparation of plant extract was as described previously with slight modification (Yadav & Agarwal, 2011). In brief, dry powdered plant material (10 g) was weighed out and filled into a thimble. The thimble was then inserted into soxhlet apparatus and extracted successively with 300 mL ethanol for 48 hours and followed by methanol.

Separately, all the extracts were then concentrated by using a rotary evaporator. The concentrated extracts were kept in air tight containers and preserved at -20°C until further use.

3.3.3 Determination of Plant Extracts Chemical Composition

The phytochemical properties of treated extractions were identified by using Agilent gas chromatography – mass spectrometry (GC-MS). Slight modification were done from previous described method for the GC-MS analysis (Wei et al., 2011). GC-MS was carried out on 7890A GC/ MS Agilent 5975, Shimadzu QP2010 ULTRA GCMS equipped with RTX-5 column. Sample injected: 2 µl. The oven column temperature pre-set at 60°C, with an initial hold time of 2 minutes, to 280°C at the rate of 5°C/minute with a final hold time of 9 minutes. Injector temperature set to 240°C. The total GC running time was 47 minutes. Library was used NIST Version – Year 2011. Interpretation on GC-MS was conducted using the database of National Institute Standard and Technology (NIST). The name, retention time, molecular weight and molecular structure of the compounds of the test substances were ascertained.

3.4 Determination of Antimicrobial Properties of Extracted Compounds

3.4.1 Plant Materials

For antimicrobial study, the *in vivo* and *in vitro Peperomia pellucida* plants were used. The plants without the roots were collected from the ISB garden, UM and the *in vitro* plants were obtained from the regenerated plantlets from MS medium. The cultures were incubated and maintained at 25 ± 2°C with 16 hours light and 8 hours dark photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity. Then, the plants were washed clean from soils and media. The clean plants were freeze-dried for five days, after which, the dried samples were ground to uniform powder and stored in air tight container at -20°C freezer.

3.4.2 Preparation of Extraction

Ten gram of the freeze dried powdered plant material was weighed out and filled into a thimble. The thimble was then placed in soxhlet apparatus and extracted successively with 300 mL ethanol for 48 hours and followed by methanol. All the extracts were then concentrated separately by using a rotary evaporator under reduced pressure at 20ºC relatively low temperature. The concentrated extracts were kept in air tight container and preserved at -20ºC until further use.

3.4.3 Preparation of The Media

3.4.3.1 Nutrient Agar

Twenty three gram of the Nutrient Agar powder (Difco) were dissolved in 1000 mL of distilled water in a conical flask. Then, it was mixed appropriately and dissolved by heating over hot plate stirrer. The neck of the conical flask was then plugged with cotton wool and wrapped with aluminium foil. The media were autoclaved at 121ºC for 20 minutes after adjusting the pH. Finally, the sterilized medium was poured on sterile petri dish and allowed to cool and solidify.

3.4.3.2 Nutrient Broth

Eight gram of the Nutrient broth powder (Difco) were dissolved in 1000 mL of distilled water in a conical flask. Then, it was mixed properly and dissolved by heating over hot plate stirrer. Then, Nutrient broth mixture were equally poured into 5 conical flask. The neck of the each conical flask was then plugged with cotton wool and wrapped with aluminium foil. The media were autoclaved at 121ºC for 20 minutes. Finally, the sterilized medium was allowed to cool down.

3.4.4 Bacterial Culture

All the isolated bacteria were provided by Microbiology Laboratory, Institute of Biological Sciences, Faculty of Science, UM. Purely isolated bacteria used in this study are as follow: *Salmonella typhii, Pseudomonas aeruginosa, Escherichia coli* and *Staphylococcus aureus.* All the bacteria were cultured in Nutrient broth and incubated overnight at 37^{o}C. After 24 hours, serial dilution were done to all the bacteria before culturing bacteria for disc diffusion method. One mililitre of the bacterial broth culture were then added into 9 mL saline solution to dilute the bacteria. A total of nine serial dilution were done to the bacteria with dilution factor of ten.

3.4.5 Disc Preparation

The disc diffusion method by Bauer et al. 1966, was used with slight modification. A stock solution of *in vivo* and *in vitro* extracts was prepared by dissolving 0.1 g extract with 100 mL of sterile distilled water to produce a final concentration of 100 mg/mL. The stock solution were then diluted to concentration of extracts 10 mg/mL. 10 mg/mL of Chloramphenicol were also prepared as a positive control for bacterial activity. Sterile blank discs 6 mm in diameter (Oxoid) were impregnated with 20 µL of each diluted extracts and Chloramphenicol. The impregnated discs were allowed to fully dry in laminar flow before the application on bacterial lawn.

3.4.6 Disc Diffusion Method (Antibiotic Sensitivity Testing)

The serially diluted bacteria were aseptically swabbed on the surface of sterile nutrient agar plates using sterile cotton swabs. By using sterile forceps, aseptically the prepared antibiotic discs were placed over the bacteria seeded agar plates. The discs were separated sufficiently from each other to avoid overlapping of inhibition zones. The plates were

then incubated at 37ºC for 24 hours. Finally, antibacterial activity was expressed as the mean zone inhibition diameter (mm) produced by the leaf extract.

3.4.7 Data Analysis

All data were collected and were statistically analysed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p < 0.05$.

3.5 Determination of Anticancer Properties of Extracted Compounds

3.5.1 Sample Preparation

For the purpose of testing the anti-cancer properties, both the methanol-extracted and ethanol-extracted compounds from *in vitro* and *in vivo* conditions were prepared at seven different concentrations, serially diluted with dilution factor of two. As such, the final concentrations of each condition is as follow: 30 µg/mL, 15 µg/mL, 7.5 µg/mL, 3.75 µg/mL, 1.875 µg/mL, 0.9375 µg/mL, and 0.469 µg/mL.

3.5.2 Cell Line Culture

The cell culture works were performed as described previously with slight modification (Shaghayegh et al., 2016) The anti-cancer properties of extracted compounds were tested on two different types of commercially available cancer cell lines namely adenocarcinomic human alveolar basal epithelial cells (A549, ATCC, USA) and human breast adenocarcinoma cell line (MCF-7, ATCC, USA). They were seeded at 1000 cells/100 uL/well in 96 well plate format (Corning, USA). The anti-cancer effects of the aforementioned compounds were evaluated by means of viability assay using 10% Alamar Blue reagent (Thermofisher scientific, MA, USA). Cell viability was analysed via absorbance at 450 nm with reference wavelength set at 590 nm by Tecan micro plate

reader (Infinite 200 PRO Tecan, Life Sciences, Switzerland). From the viability profile, the respective half maximal inhibitory concentration (IC_{50}) was calculated from the general equation y=mx+c to indicate the toxicity level of each compound. All assays were performed in triplicates to ensure consistency.

CHAPTER 4: RESULTS

4.1. *In vitro* Regeneration of *Peperomia pellucida* L. Kunth.

4.1.1. Effect of Seed Germination Under *In vivo* and *In vitro* Conditions

At the beginning of the experiment, seeds from *Peperomia pellucida* L. Kunth were germinated on MS basal medium and also on soil. Both germinating seeds were maintained in culture room at temperature of 25 ± 2°C. Based on Table 4.1, the seeds under i*n vivo* and *in vitro* conditions gave positive responses with 100% of seeds germinated successfully after 4 weeks.

Based on the results in Table 4.1, also under both conditions the plants grew well. In terms of height, the plants in *in vitro* (2.06 cm) condition showed better results as compared to the plants in *in vivo* (1.34 cm) condition. The plant morphology in both *in vitro* and *in vivo* condition were slightly different in week 4. The 4-week-old plant leaves in both cultures had different shapes. The leaf under *in vitro* condition was in globular heart shape, whereas the leaves in *in vivo* conditions were in globular shape (Figure 4.1). However, in week 5, the *in vivo* plant has globular heart shape leaves. The plants under both conditions have similar type of succulent stem and fibrous root. Thus, *Peperomia pellucida* was capable to germinate and grow well in *in vitro* conditions as well as *in vivo* condition.

Table 4.1: *In vivo* and *in vitro* seeds germination after 4 weeks.

Medium	Germination rate (%)	Mean stem height (cm)	Plant morphology
In vitro (MS basal)	100 ± 0.0[a]	2.06 ± 0.09[a]	Green leaves and in globular heart shape Succulent and light green shoots White roots
In vivo (Soil)	100 ± 0.0[a]	1.34 ± 0.04[b]	Green and globular shape leaves Succulent and light green shoots White roots

Mean values with different letters in the same column are significantly different at $p < 0.05$, according to Duncan's multiple range test (DMRT).

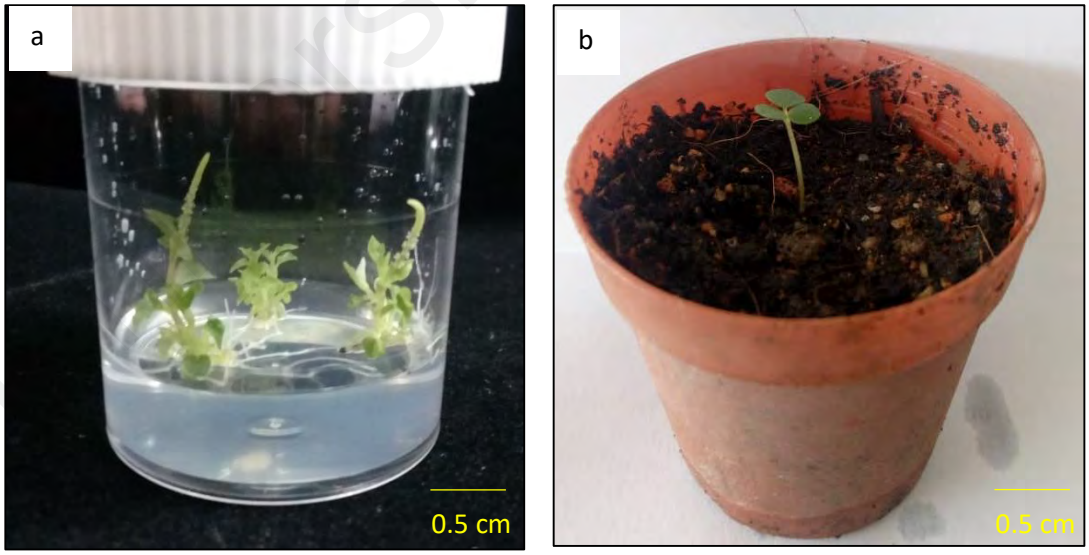

Figure 4.1: Germination of seed in a) *in vitro* and b) *in vivo* conditions.

4.1.2. Shoot Induction from Internodal Explants of *Peperomia pellucida* L. Kunth.

In this study, explants managed to rejuvenate shoots directly and grow on cultured media. The explants produced multiple shoots from internodal segments. Multiplication of shoots were studied on MS medium as a control and MS medium supplemented with different PGRs.

The main focus of this study was to identify which PGR and concentrations were producing optimum number of shoots and highest length of shoots. The number of new emerging shoots and the percentage of culture response varied according to the type and the concentration of cytokinins used. Table 4.2 and Figure 4.2 showed the response of shoots induction against the concentration of different plant growth regulators on internodal explants of *Peperomia pellucida* at 6th week. Internodal explants cultured on free plant growth regulator MS medium regenerated into complete plantlets. The number of shoots produced by internodal explants relying on the concentration of PGRs used in MS medium. Out of various PGRs used, MS medium supplemented with 0.5 mg/L Kinetin showed higher number of shoot induction (6.2 shoots). This was followed by MS (4.20 shoots) and MS supplemented with 2.0 mg/L Adenine (4.0 shoots). Meanwhile, the longest shoot length was observed in MS medium (1.48 cm), followed by MS medium supplemented with 0.5 mg/L Kinetin (0.70 cm). From Table 4.2, it can be suggested that MS medium supplemented with 0.5 mg/L Kinetin is the suitable hormone to obtain higher number of shoots coupled with tallest shoots.

Table 4.3 summarizes the response of shoot induction using MS medium with different concentrations and combinations of plant growth regulators. Maximum number of multiple shoots was induced in MS medium supplemented with combinations of 1.5 mg/L BA and 1.5 mg/L KN with 10.3 shoots (Figure 4.2(f)), whereas, the maximum shoot

elongation was showed by MS medium supplemented with 1.0 mg/L BA and 3.0 mg/L KN which gave 0.77 cm height. This is not significant when compared with MS medium supplemented with 1.5 mg/L BA and 1.5 mg/L KN which resulted in 0.73 cm height. MS medium supplemented with equal amount of BA and KN 1.0 mg/L also did not show high number of shoot. From the results, can be suggested that 1.5 mg/L BA and 1.5 mg/L KN able to induce maximum number of multiple shoots and produced maximum height of shoots.

Following this, Table 4.4 showed the response of shoots with combinations of cytokinin and auxin. The shoots were cultured on BA and KN with equal concentration of IAA and NAA to analyze the height and number of shoot production. It was observed that combination of 1.00 mg/L BA with 1.00 mg/L IAA produced higher number of shoots (38.7 ± 0.86 shoots/explant), followed by 1.00 mg/L BA with 1.0 mg/l NAA (29.9 ± 0.57 shoots/explant). Combination of 1.00mg/L KN with 1.00 mg/L IAA produced highest shoot elongation (0.81 ± 0.11 cm). This indicated that the number of shoots initiation in the culture affected the height of plant shoots.

Table 4.5 and Table 4.6 show the comparison of shoot induction under different concentrations of charcoal and different temperatures (13ºC and 25ºC). *In vitro* shoot induction resulted its superior ability to grow with higher number of shoots under 25ºC without charcoal (2.60 ± 0.25), which was not significantly different comparable with number of shoots under 13ºC (2.53 ± 0.25) without charcoal. While, the tallest shoot was observed in 15% charcoal under 13ºC (4.72 ± 2.42).

Subsequently, elongated shoots were separated and cultured in rooting media to achieve root formation in MS media with different concentrations of indole-3-butyric acid

(IBA), 1-naphthaleneacetic acid (NAA) and indole-3-acetic acid (IAA). Table 4.7 shows the percentage of root response using MS media with IBA, NAA and IAA, at 6th week. The best medium for root induction was observed (96.67%) in MS medium supplemented with IBA (2 mg/L) (Figure 4.3(a)) at 6th week. Rooted plantlets were successfully acclimatized to pot containing soil, sand and vermiculite (1:1:1) and further established in tissue culture room (25^{o}C ± 2^{o}C). The harden plantlets, then transferred to natural environment in the green house. The percentage of plantlets surviving was 85% under natural environment.

Generally, all the explants successfully produced shoots on all the hormones tested. Formation of roots was also observed from different hormones. Six-week-old plantlets were cultured on different hormones and induction of roots were observed. The cultured plantlets for root induction also grew well and transferred to green house.

Table 4.2: The effects of different concentration of PGRs in shoot induction from internodal explants of *Peperomia pellucida* L. Kunth.

MS + Plant growth regulators (mg/L)		No. of shoot (mean ± SE)	Shoot length (cm) (mean ± SE)
MS		4.20 ± 0.51^{b}	1.48 ± 0.10^{a}
BA	0.5	2.00 ± 0.00^{f}	0.40 ± 0.02^{fg}
	1.0	2.90 ± 0.33^{de}	0.41 ± 0.03^{fg}
	1.5	2.67 ± 0.09^{ef}	0.33 ± 0.01^{g}
	2.0	2.10 ± 0.18^{f}	0.56 ± 0.07^{bcde}
KN	0.5	6.20 ± 0.30^{a}	0.70 ± 0.07^{b}
	1.0	3.60 ± 0.19^{cd}	0.66 ± 0.06^{bc}
	1.5	3.40 ± 0.19^{cde}	0.64 ± 0.04^{bcd}
	2.0	3.20 ± 0.22^{de}	0.50 ± 0.05^{def}
ADE	0.5	2.83 ± 0.19^{e}	0.40 ± 0.03^{fg}
	1.0	3.23 ± 0.17^{de}	0.59 ± 0.03^{bcde}
	1.5	3.60 ± 0.21^{cd}	0.47 ± 0.02^{efg}
	2.0	4.00 ± 0.21^{bc}	0.55 ± 0.03^{cdef}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

Table 4.3: The effects of different combination of PGRs in shoot induction from internodal explants of *Peperomia pellucida* L. Kunth.

MS + Plant growth regulators (mg/L)		No. of shoot tips (mean ± SE)	Shoot length (cm) (mean ± SE)
BAP	**KN**		
1.0	0.5	3.33 ± 0.33^{d}	0.43 ± 0.03^{bc}
1.0	1.0	3.00 ± 0.58^{cd}	0.42 ± 0.04^{d}
1.0	1.5	2.30 ± 1.20^{f}	0.23 ± 0.12^{e}
1.0	2.0	3.67 ± 0.33^{bc}	0.47 ± 0.03^{b}
1.0	2.5	4.67 ± 0.67^{a}	0.43 ± 0.07^{bc}
1.0	3.0	4.00 ± 0.58^{b}	0.77 ± 0.09^{a}
1.5	0.5	4.00 ± 0.50^{cd}	0.30 ± 0.10^{bc}
1.5	1.0	7.00 ± 2.08^{b}	0.60 ± 0.21^{b}
1.5	1.5	10.30 ± 0.67^{a}	0.73 ± 0.19^{a}
1.5	2.0	4.30 ± 2.40^{c}	0.17 ± 0.03^{d}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

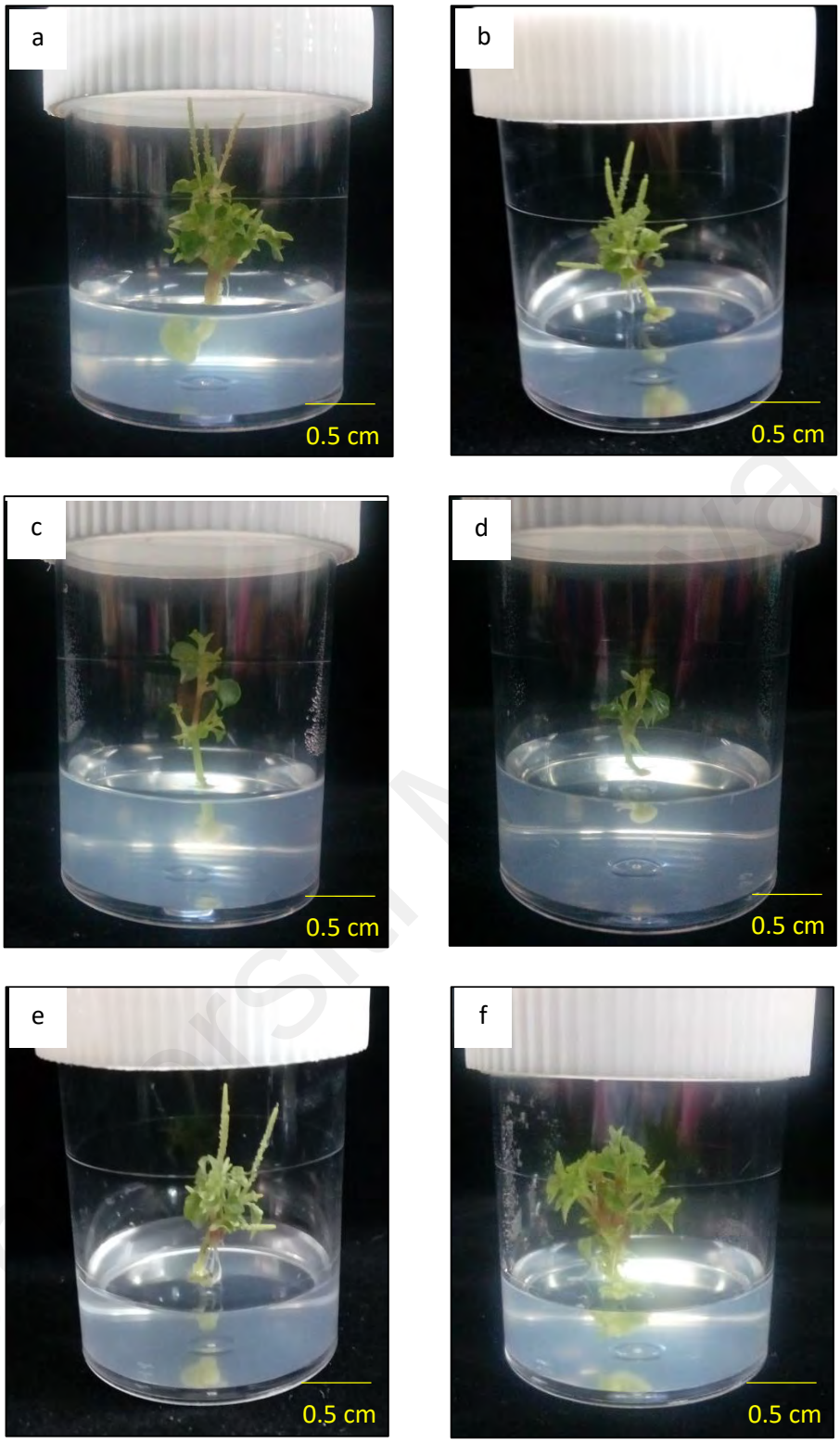

Figure 4.2: Shoots response against the concentration of different plant growth regulators on node explants of *Peperomia pellucida* a) MS basal. b) 0.5 mg/L KN. c) 1.0 mg/L BA. d) 1.0 mg/L ADE. e) 1.0 mg/L BA + 3.0 mg/L KN f) 1.5 mg/L BA + 1.5 mg/L KN.

Table 4.4: The effects of different combination of PGRs (auxin and cytokinin) on shoot induction at 6th week.

MS + Plant growth regulators (mg/L)		No. of shoot tips (mean ± SE)	Shoot length (cm) (mean ± SE)
Cytokinin	**Auxin**		
BA 1.00 mg/L	NAA 1.0 mg/L	29.9 ± 0.57^{b}	0.68 ± 0.08^{a}
BA 1.00mg/L	IAA 1.0 mg/L	38.7 ± 0.86^{a}	0.59 ± 0.11^{a}
KN 1.0 mg/L	NAA 1.0 mg/L	24.4 ± 0.97^{c}	0.75 ± 0.10^{a}
KN 1.0 mg/L	IAA 1.0 mg/L	26.7 ± 0.92^{bc}	0.81 ± 0.11^{a}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

Table 4.5: The effect of charcoal on shoot elongation under different temperatures.

MS + Percentage of charcoal	Shoot length (cm) (mean ± SE)	
	13°C	**25°C**
0%	0.81 ± 0.08^{b}	1.48 ± 0.51^{bc}
1%	0.53 ± 0.08^{b}	1.04 ± 0.15^{c}
5%	1.01 ± 0.22^{b}	1.67 ± 0.19^{bc}
10%	1.75 ± 0.43^{b}	1.39 ± 0.22^{bc}
15%	4.72 ± 2.42^{a}	3.38 ± 0.13^{a}
20%	1.21 ± 0.25^{b}	1.61 ± 0.14^{bc}
25%	1.16 ± 0.3^{b}	2.45 ± 0.66^{ab}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

Table 4.6: The effect of charcoal on shoot multiplication under different temperatures.

MS + Percentage of charcoal	No. of shoots (mean ± SE)	
	13°C	25°C
0%	2.53 ± 0.25^{a}	2.60 ± 0.25^{a}
1%	1.27 ± 0.21^{ab}	1.80 ± 0.34^{b}
5%	1.53 ± 0.45^{b}	1.67 ± 0.33^{b}
10%	1.13 ± 0.29^{b}	1.67 ± 0.25^{b}
15%	1.13 ± 0.43^{b}	1.20 ± 0.17^{bc}
20%	1.20 ± 0.42^{b}	1.53 ± 0.22^{bc}
25%	2.20 ± 0.5^{ab}	0.87 ± 0.09^{c}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

Table 4.7: Percentage of root formation on MS media supplemented with IAA, NAA and IBA.

MS + Plant growth regulators	Percentage of root formation (%)	No. of roots (mean ± SE)
IAA 0.5	0	0.0 ± 0.0^{c}
IAA 1.0	0	0.0 ± 0.0^{c}
IAA 1.5	6.67	0.36 ± 0.26^{c}
IAA 2.0	10	0.4 ± 0.23^{c}
NAA 0.5	60	2.8 ± 0.48^{b}
NAA 1.0	53.33	2.53 ± 0.51^{b}
NAA 1.5	46.67	2.83 ± 0.58^{b}
NAA 2.0	43.33	2.57 ± 0.56^{b}
IBA 0.5	80	4.97 ± 0.53^{a}
IBA 1.0	93.3	5.70 ± 0.45^{a}
IBA 1.5	90	5.03 ± 0.42^{a}
IBA 2.0	96.67	5.33 ± 0.41^{a}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

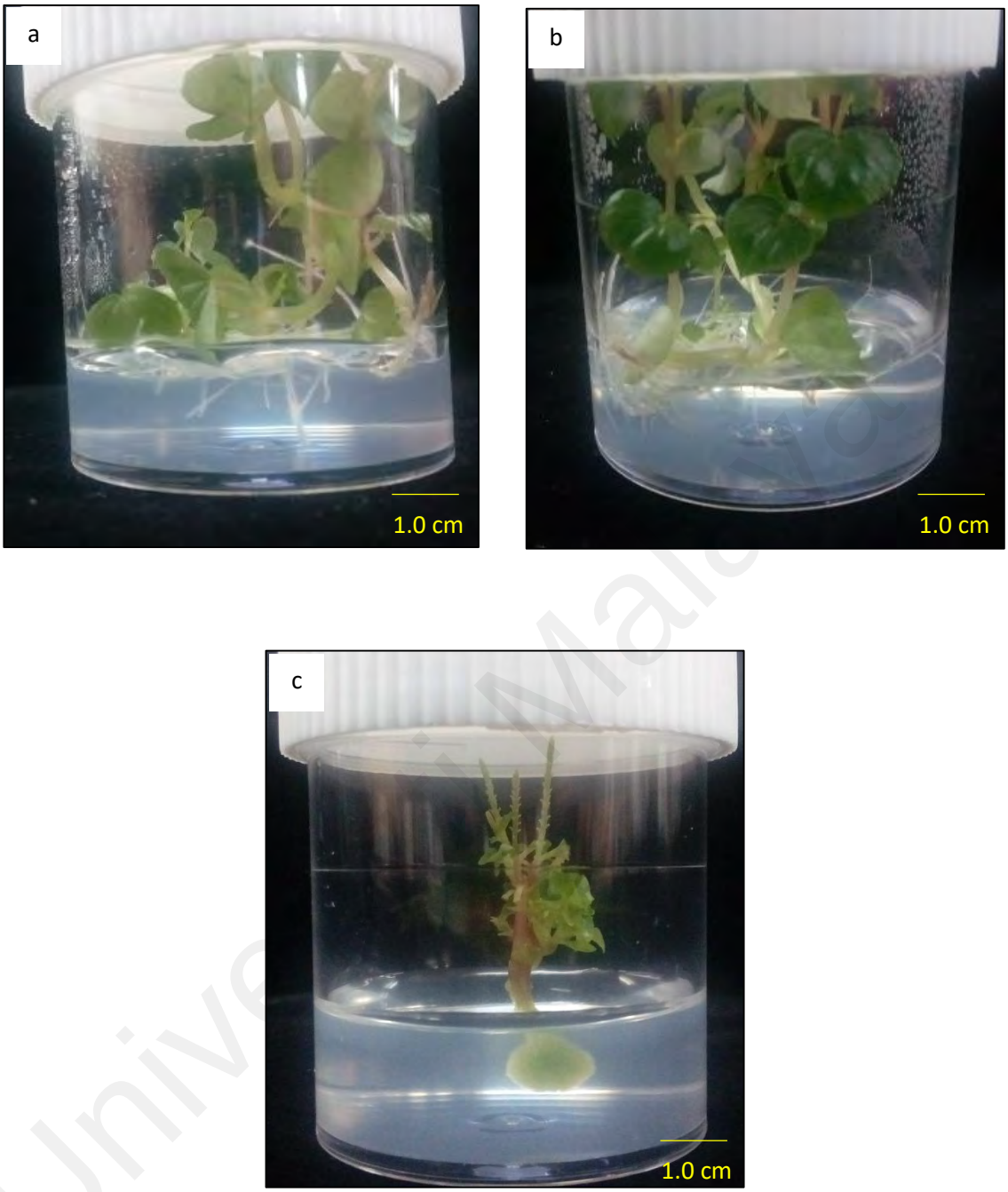

Figure 4.3: Root responses against the different concentrations of plant growth regulators on nodal explants of *Peperomia pellucida* a) 2.0 mg/L IBA. b) 0.5 mg/L NAA. c) 1.0 mg/L IAA.

4.2. Callus Induction and Somatic Embryogenesis

4.2.1. Determination of Embryogenic and Non-embryogenic Callus

After 1 week the edge of the explants became swollen to form callus structures. After 8 weeks, all the callus were tested using double staining method for identification of embryogenic and non-embryogenic callus. Observation under microscope showed that all the callus nuclei were stained blue (Figure 4.4(a)). This reflected that there was no embryogenic callus formed in week 8 and all the callus were in juvenile stage. After 12 weeks, when the callus tested using double stain in method, and observed under microscope, the callus nuclei were stained intense bright red with acetocarmine (Figure 4.4(b)). This indicates that the callus were in embryogenic stage. After a 16 weeks, the embryogenic callus changed to cotyledonary stage and formed shoot. Somatic embryogenesis pathway offered an ideal process for *in vitro* production of plants.

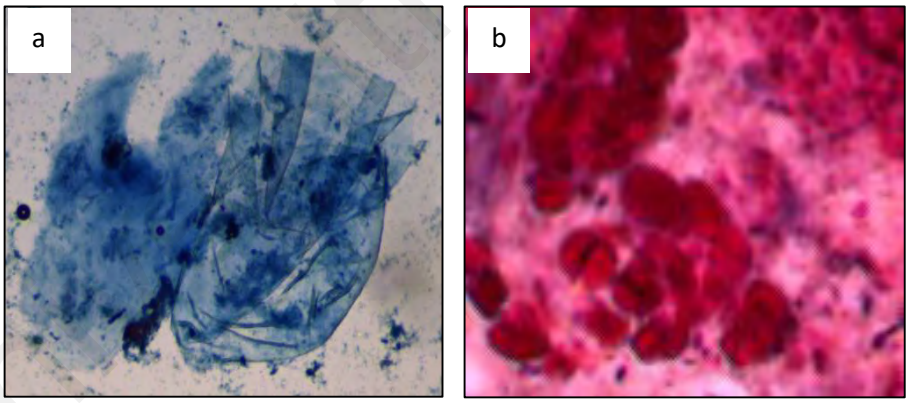

Figure 4.4: Non-embryogenic and embryogenic callus identification from leaf explants of *Peperomia pellucida* using double staining method observed under microscope under 40x. a) Non-embryogenic young callus cell (8 weeks old) with blue cell and nucleus stained by Evan's blue. b) Embryogenic callus cell with red nucleus stained by acetocarmine.

4.2.2. Callus Induction

Leaf and stem explants from *Peperomia pellucida* were cultured on MS medium supplemented with different concentrations of single PGRs such as 2, 4-D, BA, KN, GA_3, and ADE and combinations of 2,4-D with BA, KN, IAA. After 2 weeks, only leaf explant from 2, 4-D started to produce callus and explants from BA, KN, GA_3 and ADE media and combinations of PGRs 2,4-D with BA, KN, IAA did not produce any callus till week 12. Table 4.8 shows the response of callus formation from leaf on MS medium supplemented with different plant growth regulators after 12 weeks. It was noted that 80-90% of callus formation were observed in MS medium supplemented with 2, 4-D. The callus formed in 2, 4-D were white friable and light green in colour. On the other hand, the explants in BAP, KN, GA_3, ADE, IAA, NAA, IBA and combinations of 2,4-D with BA, KN, IAA, NAA, IBA and GA_3 were observed to have undergone necrosis after 5 weeks. Figure 4.5 shows the callus induction after 8 weeks and Figure 4.6 shows cream color callus induction on MS supplemented with 0.3 mg/L 2,4-D.

The maturation of somatic embryo stages (globular, heart shape, torpedo) were observed in the callus (Figure 4.7). *Peperomia pellucida* took 12 to 16 weeks to complete the maturation of somatic embryos. The callus structure was also harder.

Table 4.8: Effect of plant growth regulators on callus induction from leaf explants.

MS + Plant growth regulators (mg/L)		Observation	Callus induction (%)
MS	0.0	No formation of callus	0.0 ± 0.0^{d}
2,4-D	0.1	Cream and light green callus	90 ± 1.21^{a}
	0.2	Cream and light green callus	90 ± 1.25^{a}
	0.3	Cream and light green callus	80 ± 2.43^{c}
	0.4	Cream and light green callus	85 ± 2.05^{b}
	0.5	Cream and light green callus	90 ± 1.29^{a}
	0.6	Cream and light green callus	85 ± 1.98^{b}
BAP	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}
	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}
KN	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}
	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}

Table 4.8, continued.

ADE	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}
	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}
GA_3	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}
	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}
IAA	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}
	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}
NAA	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}

Table 4.8, continued.

NAA	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}
IBA	0.1	Necrosis	0.0 ± 0.0^{d}
	0.2	Necrosis	0.0 ± 0.0^{d}
	0.3	Necrosis	0.0 ± 0.0^{d}
	0.4	Necrosis	0.0 ± 0.0^{d}
	0.5	Necrosis	0.0 ± 0.0^{d}
	0.6	Necrosis	0.0 ± 0.0^{d}
2,4-D	**BA**		
0.1	0.1	Necrosis	0.0 ± 0.0^{d}
0.2	0.2	Necrosis	0.0 ± 0.0^{d}
2,4-D	**KN**		
0.1	0.1	Necrosis	0.0 ± 0.0^{d}
0.2	0.1	Necrosis	0.0 ± 0.0^{d}
2,4-D	IAA		
0.1	0.1	Necrosis	0.0 ± 0.0^{d}
0.2	0.1	Necrosis	0.0 ± 0.0^{d}
2,4-D	**NAA**		
0.1	0.1	Necrosis	0.0 ± 0.0^{d}

Table 4.8, continued.

2,4-D	**NAA**		
0.2	0.2	Necrosis	0.0 ± 0.0^{d}
2,4-D	**GA_3**		
0.1	0.1	Necrosis	0.0 ± 0.0^{d}
0.2	0.1	Necrosis	0.0 ± 0.0^{d}
2,4-D	**ADE**		
0.1	0.1	Necrosis	0.0 ± 0.0^{d}
0.2	0.1	Necrosis	0.0 ± 0.0^{d}

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

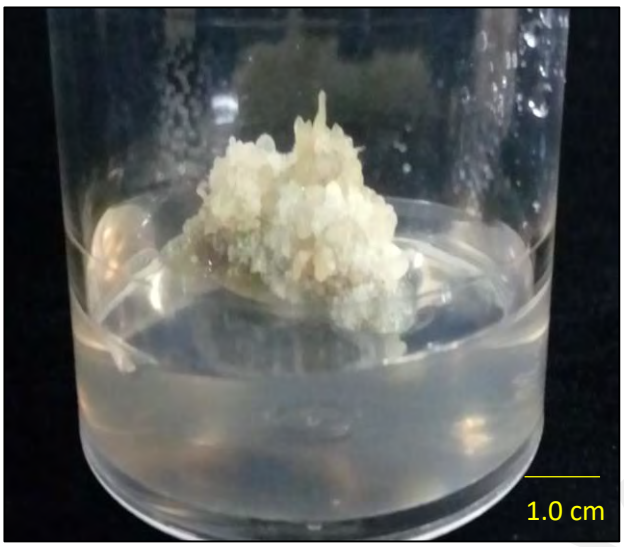

Figure 4.5: Induction of callus from leaf explants on MS medium supplemented with 2,4-D, after 8 weeks.

Figure 4.6: Induction of callus from leaf explants on MS medium supplemented with 0.3 mg/L 2,4-D.

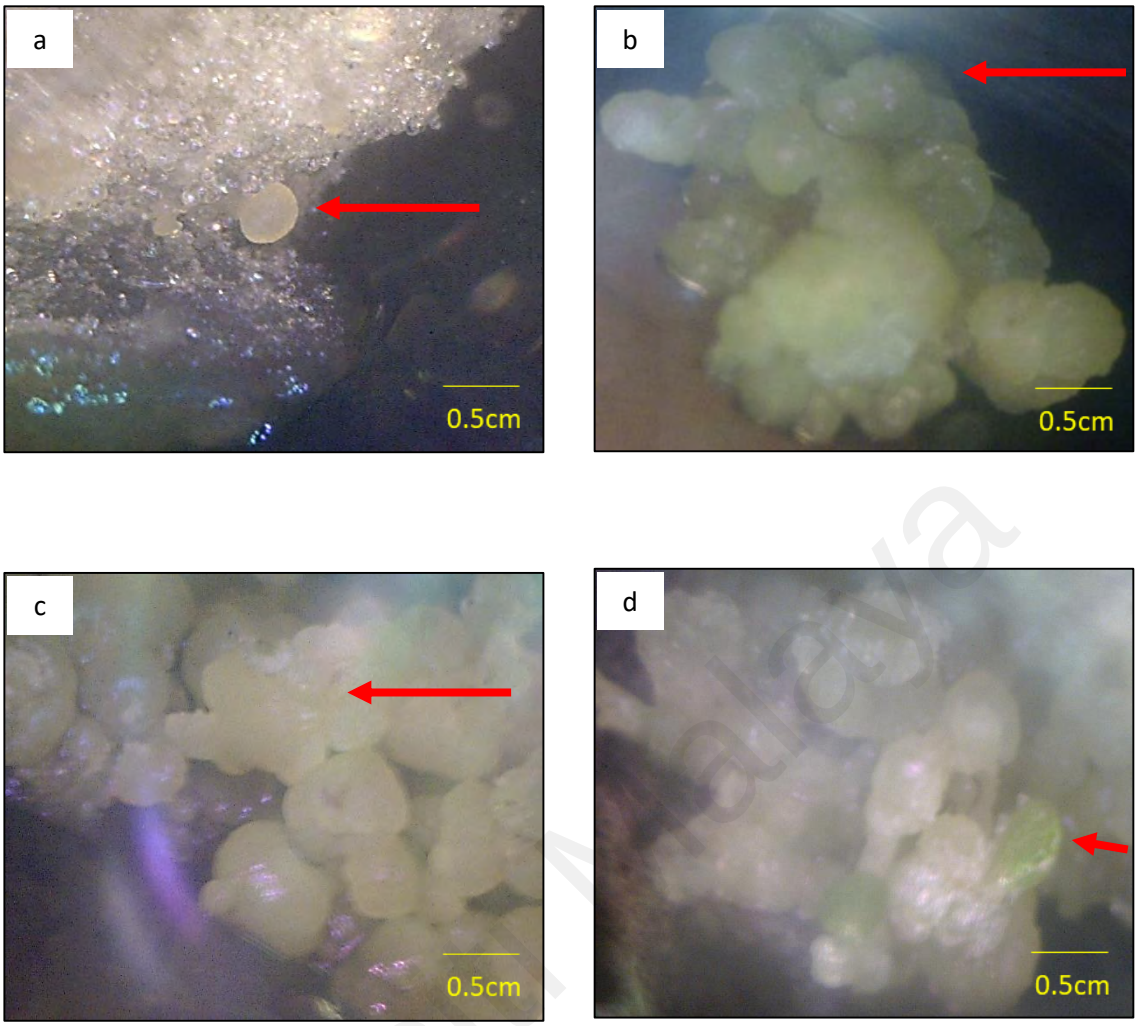

Figure 4.7: Somatic embryos of *Peperomia pellucida* cultured on MS medium supplemented with 0.2 mg/L 2,4-D. a) Globular stage. b) Heart-shape. c) Torpedo shape. d) Cotyledonary stage.

4.3. Gas Chromatography – Mass Spectrometry (GC-MS)

The GC-MS study was carried out using *in vitro* and *in vivo* ethanol and methanol extracts. The GC-MS analysis of *Peperomia pellucida* identified the presence of chemical compounds that could contribute to the medicinal value of the plant. The phytochemical compounds was identified and confirmed based on the peak area, retention time, retention index and molecular formula of the compounds. The active principle with their retention time (RT), area and molecular weight (MW) are presented in Tables below.

The ethanol extract of *in vitro* grown *Peperomia pellucida* showed 24 peaks from the chromatogram of the extract (Figure 4.8). These peaks indicated the presence of 24 compounds in the extract (Table 4.9). The retention time, molecular formula, area and molecular weights of these compounds are shown in Table 4.9.

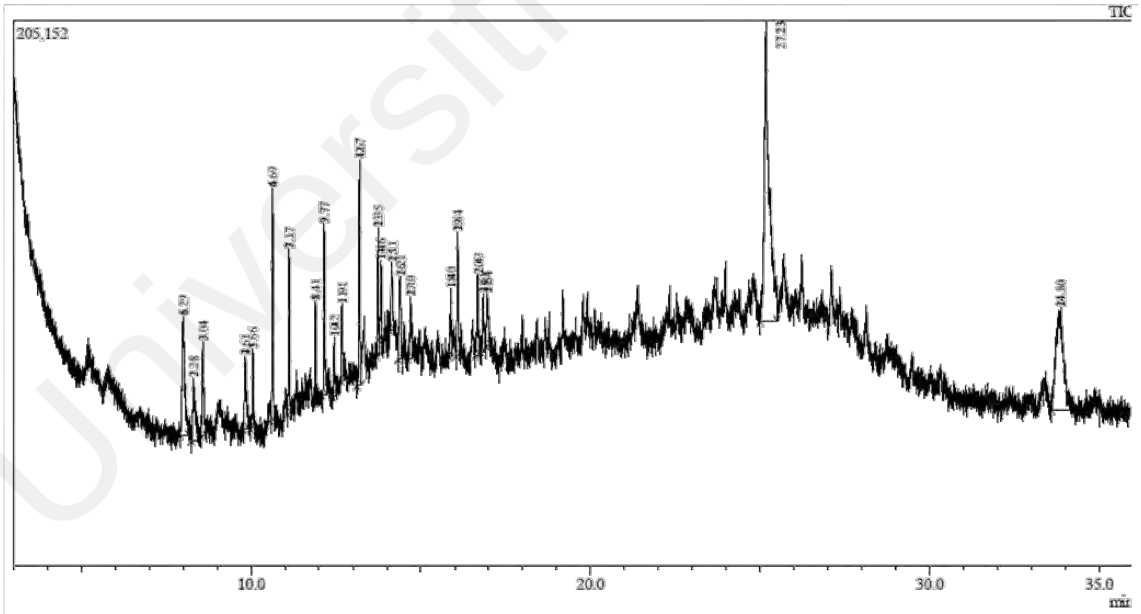

Figure 4.8: GC-MS Chromatogram of ethanol extract of *in vitro* grown *Peperomia pellucida*.

Table 4.9: GC-MS of ethanol extract analysis revealed the presence of phytochemical components in *in vitro* grown *Peperomia pellucida*.

No	RT	Compound	Molecular formula	Area	MW
1	8.004	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	14930	296
2	8.299	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	10933	206
3	8.573	Dodecane, 1-iodo-	$C_{12}H_{25}I$	15065	296
4	9.819	Apiol	$C_{12}H_{14}O_4$	13615	222
5	10.056	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	$C_{14}H_{42}O_5Si_6$	21897	458
6	10.631	Heneicosane	$C_{21}H_{44}$	38616	296
7	11.125	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	24149	296
8	11.903	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	23116	578
9	12.147	Phytol, acetate	$C_{22}H_{42}O_2$	11401	338
10	12.452	6-Octen-1-ol, 3,7-dimethyl-, propanoate	$C_{13}H_{24}O_2$	9386	212
11	12.685	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	4074	296
12	13.195	Heneicosane	$C_{21}H_{44}$	31253	296
13	13.740	Eicosane	$C_{20}H_{42}$	16281	282
14	13.829	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	21128	576
15	14.146	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	15246	312
16	14.391	3,5-Decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-	$C_{18}H_{30}$	957	246
17	14.713	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-	$C_{30}H_{50}O$	15834	426
18	15.893	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	$C_{12}H_{38}O_5Si_6$	16124	430
19	16.088	Octadecane, 1-iodo-	$C_{18}H_{37}I$	20998	380
20	16.681	Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	8105	242
21	16.681	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	15201	276
22	16.963	2-Bromotetradecane	$C_{14}H_{29}Br$	4178	276

Table 4.9, continued.

23	25.175	Pentanedioic acid, 3-phenyl-, dibenzyl ester	$C_{25}H_{24}O_4$	77051	388
24	33.837	1,3,5-Cycloheptatriene, 2,4-di-t-butyl-7,7-dimethyl	$C_{17}H_{28}$	5528	232

Note: Retention time (RT) and molecular weight (MW).

The ethanol extract of *in vivo* grown *Peperomia pellucida* showed 23 peaks from the chromatogram of the extract (Figure 4.9). These peaks indicated the presence of 23 compounds in the extract (Table 4.10). The retention time, molecular formula, area and molecular weights of these compounds are shown in Table 4.10.

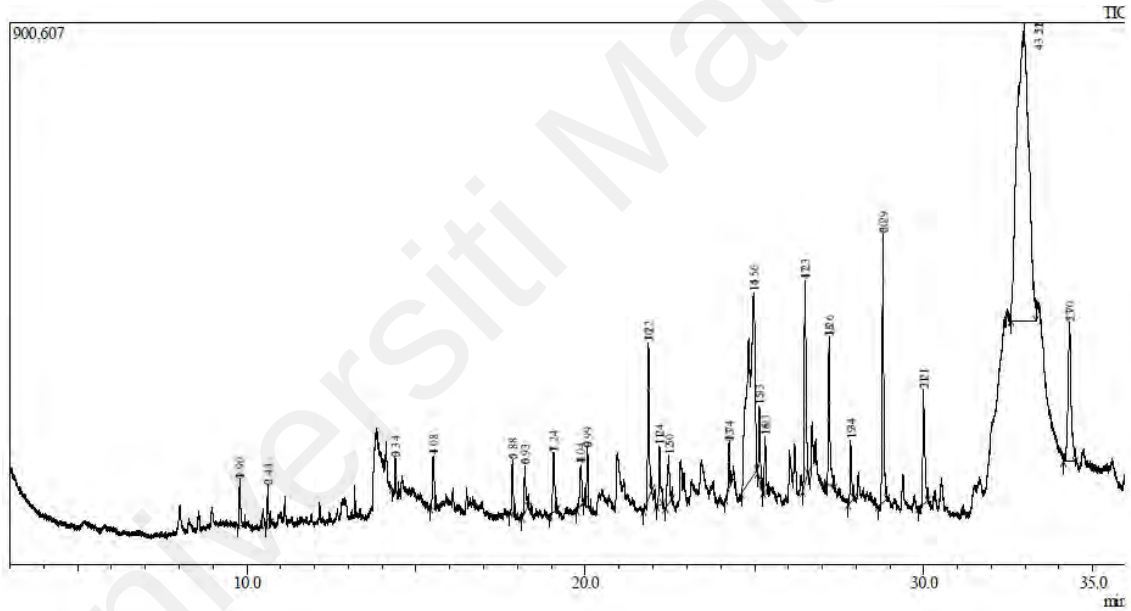

Figure 4.9: GC-MS Chromatogram of ethanol extract of *in vivo* grown *Peperomia pellucida*.

Table 4.10: GC-MS of ethanol extract analysis revealed the presence of phytochemical components in *in vivo* grown *Peperomia pellucida*.

No	RT	Compound	Molecular formula	Area	MW
1	9.780	Apiol	$C_{12}H_{14}O_4$	26511	222
2	10.620	Heneicosane	$C_{21}H_{44}$	24850	296
3	14.395	4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	$C_{21}H_{32}O_3$	14673	332
4	15.507	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	78017	578
5	17.849	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	79189	576
6	18.209	2-Propenoic acid, pentadecyl ester	$C_{18}H_{34}O_2$	31124	282
7	19.053	Azetidine, 3-methyl-1-(phenylmethyl)-	$C_{11}H_{15}N$	222955	161
8	19.855	1-Benzylazetidine	$C_{10}H_{13}N$	93849	147
9	20.058	Heptasiloxane, hexadecamethyl-	$C_{16}H_{48}O_6Si_7$	83943	532
10	21.865	Cyclopentanone, 2-(4-benzyloxy-3-methoxybenzylidene)-	$C_{20}H_{20}O_3$	399631	308
11	22.189	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	$C_{14}H_{42}O_5Si_6$	84704	458
12	22464	Benzene, (1-ethylpropyl)-	$C_{11}H_{16}$	113270	148
13	24.241	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	81360	532
14	24.973	Oxazolo[4,3-a]isoquinolin-3-one, 1-benzyl-8,9	$C_{22}H_{25}NO_4$	646578	367
15	25.149	Naphtho[2,3-b]norbornadiene	$C_{15}H_{12}$	97222	192
16	25.316	trans,trans-Dibenzylideneacetone	$C_{17}H_{14}O$	20241	234
17	26.498	3-Pyrrolidinone, 1-(phenylmethyl)-	$C_{11}H_{13}NO$	372102	175
18	27.200	2-Benzylaminonicotinonitrile	$C_{13}H_{11}N_3$	85393	209
19	27.839	2-Propenoic acid, 3-phenyl-, phenylmethyl ester	$C_{16}H_{14}O_2$	27490	238
20	28.788	1-Imidazolidinecarboxylic acid, 2-(1,1-dimethylethyl)-4-oxo-3-(phenylmethyl)-, phenylmethyl ester, (s)-	$C_{22}H_{26}N_2O_3$	77422	366

Table 4.10, continued.

21	30.000	1,3-Dibenzyl-2-(3-nitro-phenyl)-hexahydro-pyrimidine	$C_{24}H_{25}N_3O_2$	339136	387
22	32.987	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	$C_{57}H_{104}O_6$	754734	884
23	34.303	beta.-Phenylpropiophenone	$C_{15}H_{14}O$	325594	210

Note: Retention time (RT) and molecular weight (MW).

The methanol extract of *in vitro* grown *Peperomia pellucida* showed 13 peaks from the chromatogram of the extract (Figure 4.10). These peaks indicated the presence of 13 compounds in the extract (Table 4.11). The retention time, molecular formula, area and molecular weights of these compounds are shown in Table 4.11.

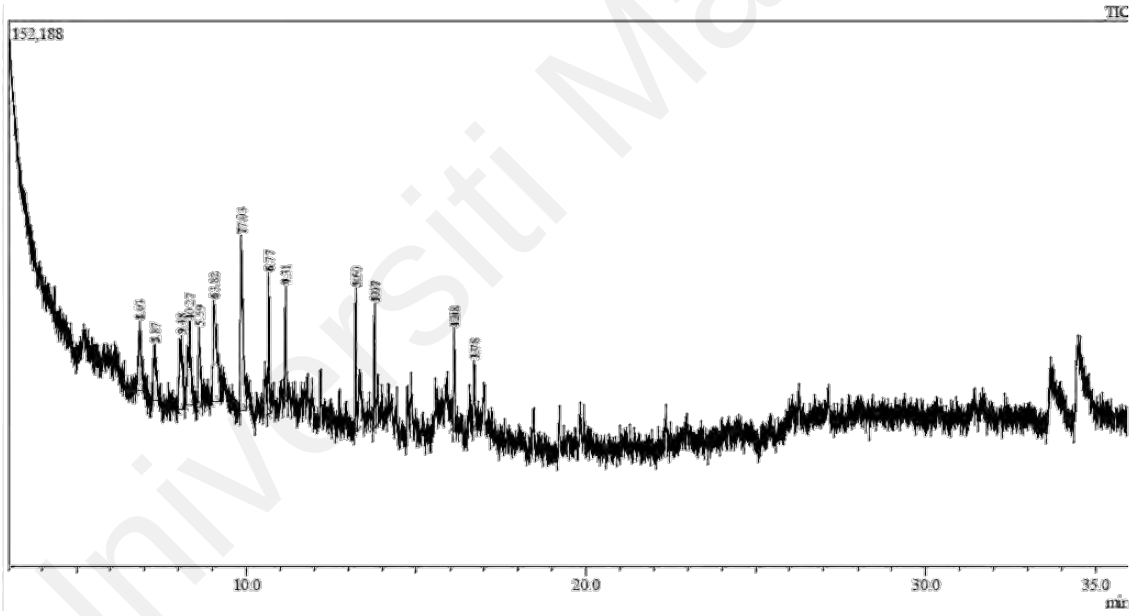

Figure 4.10: GC-MS Chromatogram of methanol extract of *in vitro* grown *Peperomia pellucida*.

Table 4.11: GC-MS of methanol extract analysis revealed the presence of phytochemical components in *in vitro* grown *Peperomia pellucida*.

No	RT	Compound	Molecular formula	Area	MW
1	6.939	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	$C_{15}H_{24}$	920	204
2	7.295	Caryophyllene	$C_{15}H_{24}$	9467	204
3	8.031	Octadecane, 1-chloro-	$C_{18}H_{37}Cl$	15936	288
4	8.336	(7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	$C_{15}H_{26}O$	15000	222
5	8.612	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	5219	296
6	9.053	1,2-Dimethoxy-4-(2-methoxyethenyl)benzene	$C_{11}H_{14}O_3$	20096	194
7	9.829	Apiol	$C_{12}H_{14}O_4$	1994	222
8	10.663	Octadecane	$C_{18}H_{38}$	9479	254
9	11.156	Dodecane, 1-iodo-	$C_{21}H_{44}$	15003	296
10	13.227	Hexadecane, 1-iodo-	$C_{12}H_{25}I$	14503	352
11	13.772	Eicosane	$C_{20}H_{42}$	11266	282
12	16.116	Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	3024	352
13	16.710	Pentadecane, 8-hexyl-	$C_{21}H_{44}$	9511	296

Note: Retention time (RT) and molecular weight (MW).

The methanol extract of *in vivo* grown *Peperomia pellucida* showed 15 peaks from the chromatogram of the extract (Figure 4.11). These peaks indicated the presence of 15 compounds in the extract (Table 4.12). The retention time, molecular formula, area and molecular weights of these compounds are shown in Table 4.12.

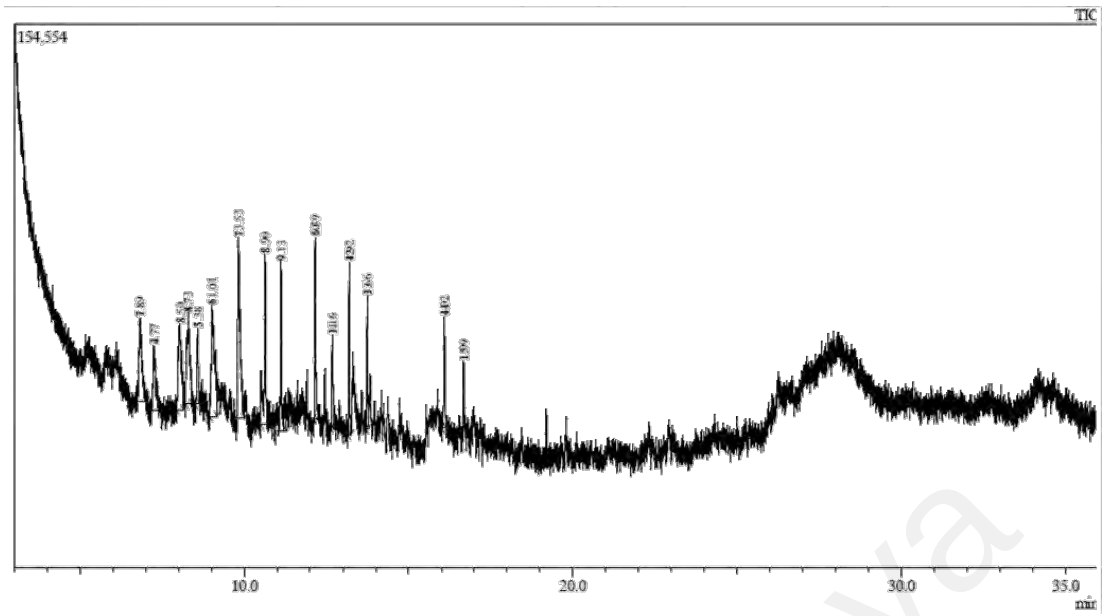

Figure 4.11: GC-MS Chromatogram of methanol extract of *in vivo* grown *Peperomia pellucida*.

Table 4.12: GC-MS of methanol extract analysis revealed the presence of phytochemical components in *in vivo* grown *Peperomia pellucida*.

No	RT	Compound	Molecular formula	Area	MW
1	6.813	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	$C_{15}H_{24}$	11775	204
2	7.251	Caryophyllene	$C_{15}H_{24}$	5400	204
3	8.006	Decane, 2,3,5,8-tetramethyl-	$C_{14}H_{30}$	26432	198
4	8.321	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	18982	206
5	8.595	Pentadecane, 2,6,10,14-tetramethyl-	$C_{19}H_{40}$	2448	268
6	9.017	1,2-Dimethoxy-4-(2-methoxyethenyl)benzene	$C_{11}H_{14}O_3$	21853	194
7	9.810	Apiol	$C_{12}H_{14}O_4$	6059	222
8	10.631	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	17031	296
9	11.127	Heneicosane	$C_{21}H_{44}$	22904	296
10	12.143	Phytol, acetate	$C_{22}H_{42}O_2$	4197	338
11	12.696	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	6933	296

Table 4.12, continued.

11	12.696	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	6933	296
12	13.199	Hexadecane, 1-iodo-	$C_{16}H_{33}I$	17462	325
13	13.746	Heneicosane	$C_{21}H_{44}$	16601	296
14	16.087	2-Bromotetradecane	$C_{14}H_{29}Br$	13482	276
15	16.685	5-Butyl-5-ethylpentadecane	$C_{21}H_{44}$	7061	296

Note: Retention time (RT) and molecular weight (MW).

4.4. Antimicrobial Activities of *Peperomia pellucida* L. Kunth. Extracts.

Table 4.13 shows the antibacterial activities of ethanolic and methanolic extract from *in vivo* and *in vitro* grown of *Peperomia pellucida.* The antibacterial analysis of crude plant extract found to be effective against the following bacteria: *Salmonella typhii, Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus aureus*.

The results showed that different extracts had unique inhibitory effects towards the various bacterial species tested. Among the various extracts used, the methanol extract from *in vitro* grown plant shows highest inhibition zones (12.5 mm) against *Staphylococcus aureus.* The ethanol extract from *in vitro* grown plant shows (12 mm) inhibition zones against *Salmonella typhii* and *Pseudomonas aeruginosa.* The ethanol extract from *in vivo* plant shows 11.5 mm inhibition zones against *Escherichia coli*.

From the inhibition zone obtained for controls, it was observed that the chloramphenicol had the highest inhibitory zones as compared to methanol and ethanol plant extracts.

Table 4.13: Antibacterial activities of ethanolic and methanolic extracts of *in vivo* and *in vitro Peperomia pellucida.*

Bacteria	Inhibition zone diameter (mm)				
	Methanol		Ethanol		Chloramphenicol
	In vivo	***In vitro***	***In vivo***	***In vitro***	
Salmonella typhii	11.75 ± 1.25[a]	11.25 ± 0.75[a]	9.25 ± 0.63[ab]	12.00 ± 0.41[a]	15.75 ± 0.85[a]
Staphylococcus aureus	11.00 ± 0.91[a]	12.5 ± 1.19[a]	6.75 ± 1.31[b]	10.75 ± 0.48[a]	16.75 ± 0.95[a]
Escherichia coli	9.50 ± 0.65[a]	10.00 ± 1.08[ab]	11.5 ± 0.65[a]	8.00 ± 0.41[b]	17 ± 0.82[a]
Pseudomonas aeruginosa	10.00 ± 0.91[a]	7.50 ± 0.65[b]	10.75 ± 0.85[a]	12.00 ± 1.47[a]	17 ± 0.91[a]

Mean values with different letters in the same column are significantly different at $p < 0.05$, to Duncan's multiple range test (DMRT).

4.5. Anticancer Properties of *Peperomia pellucida* L. Kunth. Extracts

Apart from the aforementioned characteristics of *Peperomia pellucida* the anti-cancer properties of the said extracts was tested on two commercially available cancer cell lines namely; adenocarcinomic human alveolar basal epithelial cells (A549) and human breast adenocarcinoma cell line (MCF-7). In addition to exposing these cells to a variety of extract concentrations and evaluate their viability, the half maximal inhibitory concentration (IC_{50}) was also calculated from the general equation $y = mx+c$ to indicate the toxicity level of each compound. As shown in Figure 4.12, a general decrement of viability was observed with increasing concentration of *in vitro* ethanol extract. At 30 µg/mL, the viability of A549 cells was reduced from 100 % to 45.3%. The IC_{50} of this extract was found to be 13.02 µg/mL based on the equation $y = -7.335\ln(x) + 68.828$.

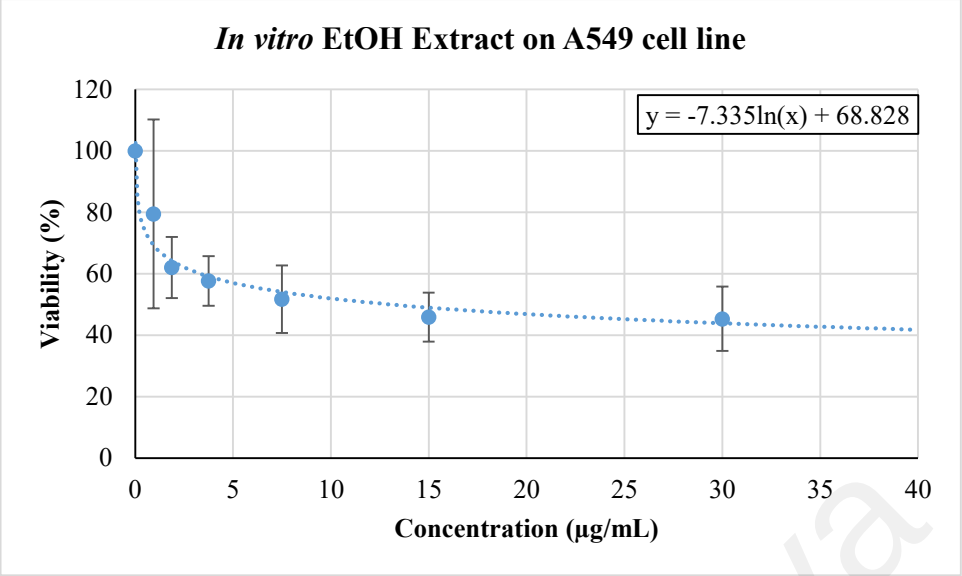

Figure 4.12: Viability assay of A549 cells in response to *in vitro* ethanol extract.

Similarly, the viability of these cells was evaluated in the presence of *in vivo* ethanol extract. As observed in Figure 4.13, it was shown that a general decrement of viability was again noted and at 30µg/mL, the viability of A549 cells was reduced from 100 % to 35.4%. The IC_{50} of this extract was found to be 15.97 µg/mL based on the equation y = -8.097ln(x) + 72.435.

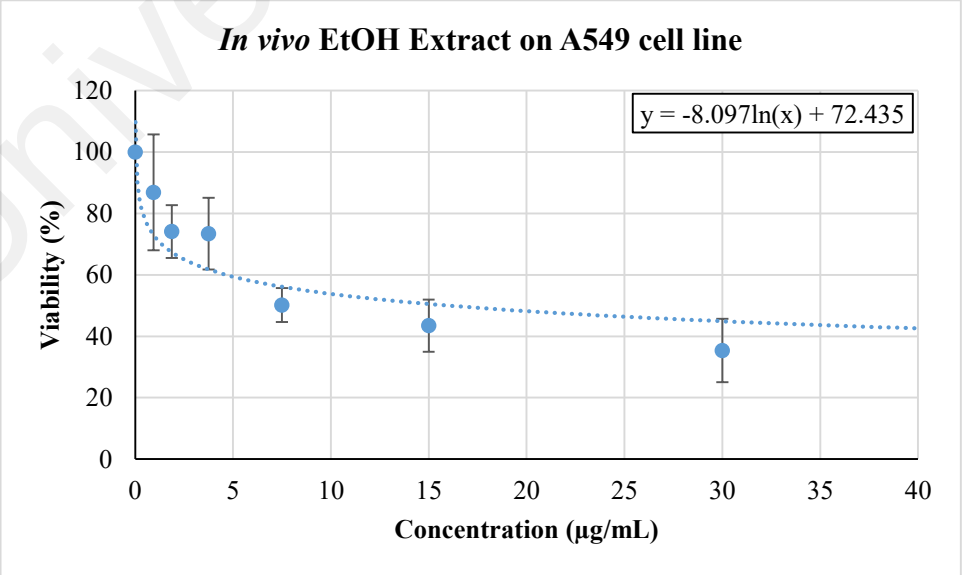

Figure 4.13: Viability assay of A549 cells in response to *in vivo* ethanol extract.

Subsequently, the viability of A549 cells were also checked upon exposure of both *in vitro* and *in vivo* methanol extract. With regards to *in vitro* extract as depicted in Figure 4.14, it was observed that a similar trend of viability which decreased as the concentration was increased. In Figure 4.15, similar observation was noted but with steeper trend line as compared to Figure 4.14. The viability of A549 cells at 30 µg/mL for both *in vitro* and *in vivo* methanol extracts were 35.8% and 33.3%, respectively whereby indicating the potency of this extract especially upon methanol extraction. The IC_{50} for the extracts were found to be 8.11 µg/mL and 6.76 µg/mL, respectively.

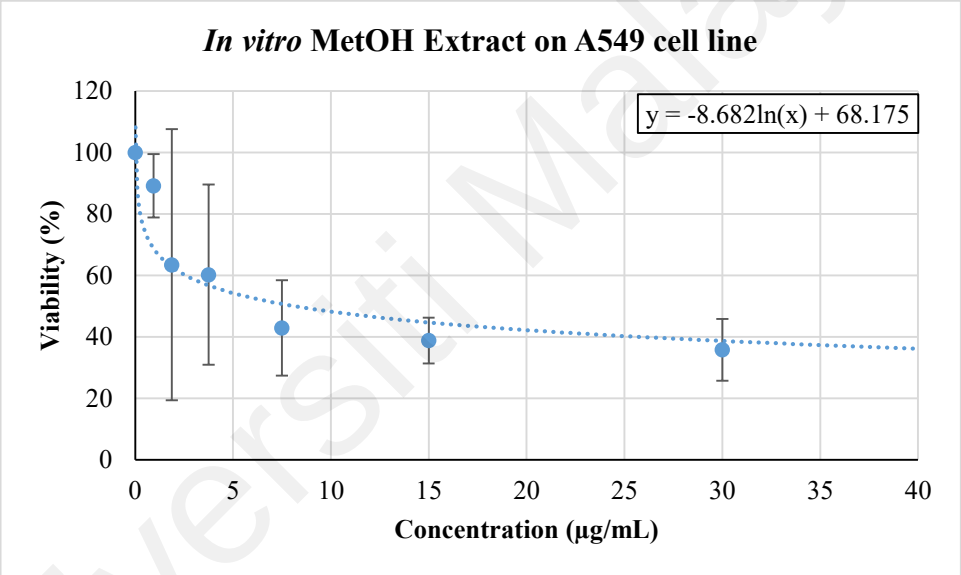

Figure 4.14: Viability assay of A549 cells in response to *in vitro* methanol extract.

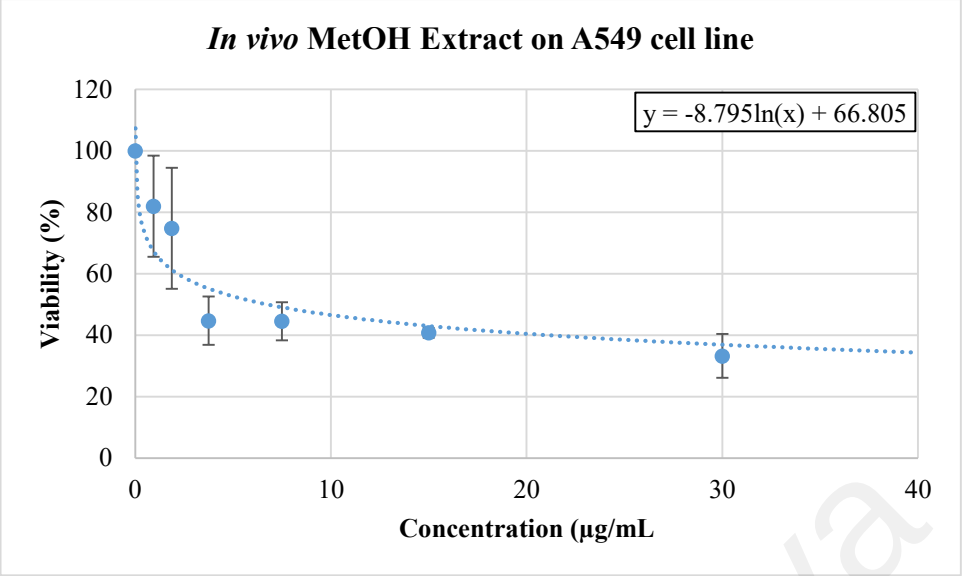

Figure 4.15: Viability assay of A549 cells in response to *in vivo* methanol extract.

In addition to assessing the viability of A549, a similar assay was also conducted on another robust cancer cell line, MCF-7. Upon exposure of *in vitro* ethanol extract, the viability trend showed a typical decrement with increasing concentration, as showed in Figure 4.16. Intriguingly, the viability percentage was more affected, unlike how it was observed in A549 cells. For instance, with exposure of 30 µg/mL of extract, the viability percentage was observed to be at 38.6%, as opposed to 45.3% in its A549 counterpart. The IC_{50} for this extract was observed to be 22.88 µg/mL based on equation y = -7.627ln(x) + 73.875.

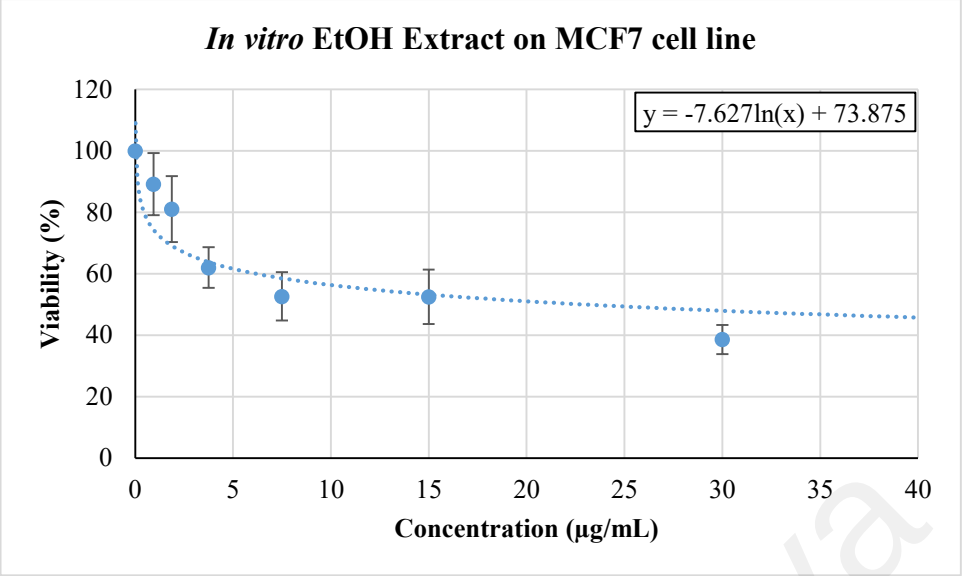

Figure 4.16: Viability assay of MCF7 cells in response to *in vitro* ethanol extract.

The viability for MCF7 cells upon exposure of *in vivo* ethanol extract began to reflect more resistance (i.e more viable cells). As shown in Figure 4.17, the percentage of viable cells at extracts concentration of 30 µg/mL was found to be 57.3%, as compared to 35.4% in its A549 counterpart. The IC_{50}, however was found to be 143.02 based on y = -5.623ln(x) + 77.907.

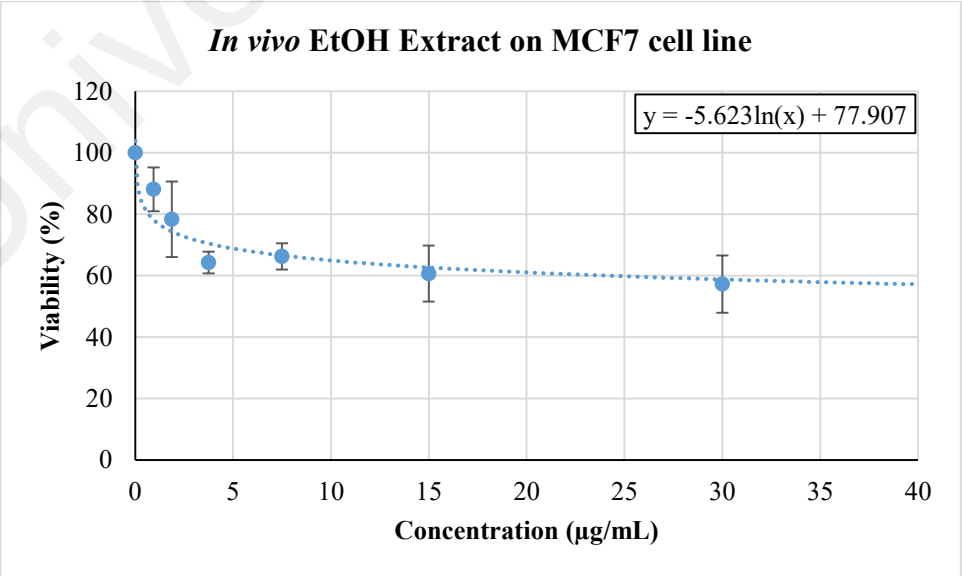

Figure 4.17: Viability assay of MCF7 cells in response to *in vivo* ethanol extract

The exposure with *in vitro* methanol extract displayed the most resistance in terms of viability, as showed in Figure 4.18. This was evident whereby in the presence of 30 µg/mL of extract, the viability percentage was observed to be at 70.5%, as opposed to 35.8% in its A549 counterpart. The survival rate in this case was doubled as compared to A549 cells. Moreover, the IC_{50} for this particular extract was found to be 10738.3 µg/mL based on y = -3.928ln(x) + 86.458, thereby verifying the survival profile of MCF7 upon exposure of extract obtained via methanol *in vitro*.

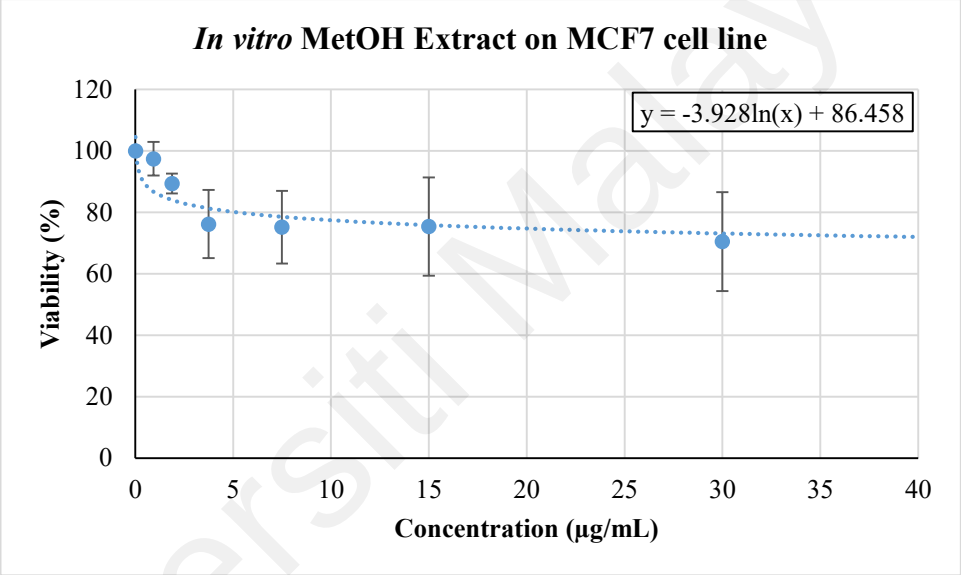

Figure 4.18: Viability assay of MCF7 cells in response to *in vitro* methanol extract

Finally, the viability of MCF7 upon exposure of *in vivo* methanol extracts also displayed augmented survival pattern as compared to its A549 counterpart, as showed in Figure 4.19. For instance, the exposure of 30 µg/mL of extract revealed a viability percentage of 49.94%, as compared to 33.3% in A549 cells. Additionally, the IC_{50} of the said extract was found to be 52.56 µg/mL based on y = -6.208ln(x) + 74.596.

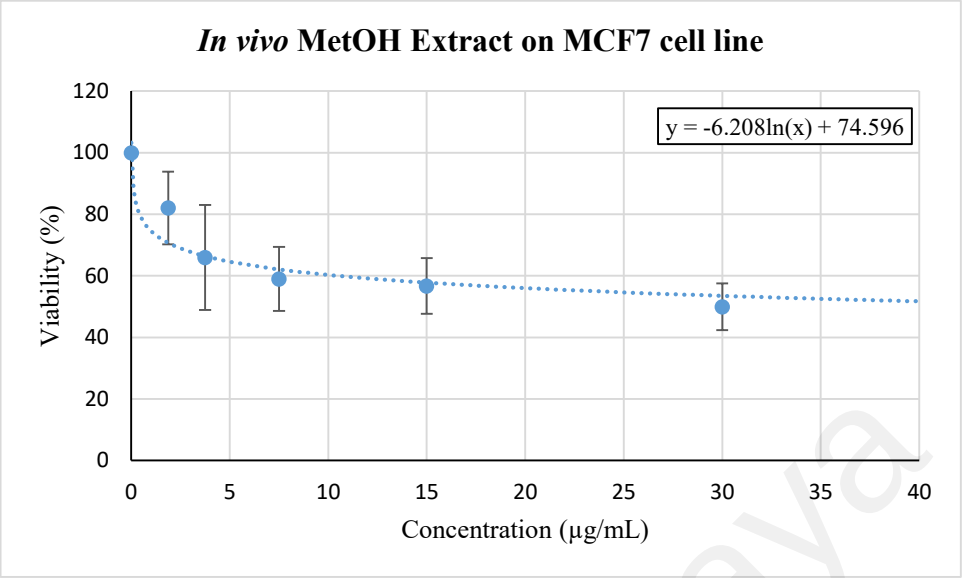

Figure 4.19: Viability assay of MCF7 cells in response to *in vivo* methanol extract

CHAPTER 5: DISCUSSION

Tissue culture studies have been reported in many medicinal plant species. However, *Peperomia pellucida* used in the present research can be considered as under exploited. This herb can be found in many parts of the world. The entire humankind is reliant on plants as sources of proteins, carbohydrates, vitamins and also as food. As a result of global resurgence in traditional medicine, the market for herbal drug is on high demand. Herbals needed in mass quantity to meet the global demand and during certain seasons, the demand for herbal was very challenging to fulfil. Through *in vitro* method, the impact of the weather can be eliminated and can be produced all year round. Apart from that, new cultivar can be also developed commercially in short time and abundant in mass. Through *in vitro* micropropagation, new and improved genetically engineered plants can be produced. There are many medicinal plants with valuable chemicals used in drug making but miropropagation procedures are missing for this plants. The current study was embarked to focus on the *Peperomia pellucida* through tissue culture for mass propagation and callus induction. The successfully regenerated *in vitro* plantlets were analysed for antibacterial, anticancer and secondary metabolites to compare with the intact plants.

Efficiency of *in vitro* micropropagation and transformation protocols produce successful generation of plants. Suitable protocol development using biotechnological methods is needed to improve genetic of plants. In this study, tissue culture techniques were used for plant regeneration by manipulation and exploiting different plant growth regulators. To establish a good regeneration system for the species, various factors were taken into account. In this study MS (Murashige & Skoog, 1962) medium was used in all

the experiments. Additionally, 0.01% of carbendazim was incorporated into all the culture media to prevent fungal contamination.

The objective of this study was to establish suitable tissue culture technique for mass production of *Peperomia pellucida.* All the factors that are involved in tissue culture are taken into account. The suitable culture medium and plant growth regulators for shoot initiation are investigated in this study. Suitable and effective sterilization techniques of the explants is the crucial step in plant tissue culture as exposure to the strong chemicals will affect the growth and survival rate of the explants (Ray & Ali, 2017; Singh, 2018).

Seed germination through *in vitro* technique to produce aseptic seedlings or axenic culture is a powerful and effective method to preserve native and rare species (Huh et al., 2016). The seeds of the *in vitro* plant were sterilized with suitable method and cultured in MS medium and soil. After 4 weeks of culture, the seed gave a positive response with 100% seed germination. The seedlings in MS medium and also grown on soil grew well in the culture room with 25^{o}C ± 2^{o}C, 16 h/8 h (light/dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity. The seeds in the *in vitro* condition germinated as fast as the *in vivo* seeds in 4 to 6 days. The i*n vitro* plants grew in the MS medium formed the highest shoot length (2.06) compare to the *in vivo* plant (1.34). This has proven that *Peperomia pellucida* was able to germinate and grow well on MS medium (Loc et al., 2010). According to Guney et al. (2016), seed can germinate on MS medium without any PGRs. According to their findings, seeds can germinate in MS medium but in the presence of PGRs, seeds will germinate and produce higher number of shoots. Agarwal, (2015) has reported that 100% seed germination in MS medium for *Momordica charantia* L. According to a study, MS medium is a suitable medium for the seed to germinate and grow well. Seeds also able to germinate in different concentrations of MS medium. The

germinated seeds will then develop into new shoots and then into adult plants (Huh et al., 2016). Seed will be able to germinate when they reach optimum condition (Bhattarai et al., 2016). Using fungicide in culture media will help to reduce fungal infection, and there is possibility to delay seed germination (Justamante et al., 2017).

The plant morphology in both *in vitro* and *in vivo* conditions were the same except for the leaves in week 4. The 4-week-old plant leaf in both cultures had different shape. The leaf in *in vitro* condition is in globular heart shape, whereas the leaves in *in vivo* conditions were in globular shape. Nevertheless, after 5 weeks, the *in vivo* plants have a globular heart-shaped leaves. This shows that, the *in vivo* plant leaves is not fully developed in week 4. The plants in both conditions have similar type of succulent stem and fibrous roots. Thus, *Peperomia pellucida* was capable of germinating and grow well in both *in vitro* and *in vivo* conditions. The most widely used medium for the vegetative propagation of various plant species through *in vitro* technique is MS medium (Hussain et al., 2012).

Micropropagation at most time guarantee good supply of medicinal plant through effective alternative method of asexual propagation within minimum space and limited time by producing numerous plants from single individual throughout years (Park et al., 2017; Sidhu, 2011). The right choice of explant materials is related to the success of tissue culture (Celik & Atak, 2009). Apart from that, media and plant growth regulators, age and genotype are very important factors for successful tissue culture (Farsi et al., 2012). Interaction between the plants genotype and the cultural environments caused of genotype dependent effects (Celik & Atak, 2009).

In this study, *Peperomia pellucida* were subjected to tissue culture tecnique and to develop a good protocol for micropropagation to assist in proliferation and conservation.

The *in vitro* propagation studies, the culture of internodal explants on defined culture media under standard growth conditions. All the collected explants were sterilized and cultured on medium with different and combination of various PGRs. Then, the cultures were incubated in culture room under controlled conditions. The culture were incubated at 25 ± 2ºC, 16 h/8 h (light/ dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ of light intensity.

In the present study, a well-organized micropropagation protocol was established for shoot regeneration and multiplications of *Peperomia pellucida* Table 4.2 from earlier chapter four shows the multiplication of shoots and shoot elongation on MS medium supplemented with different concentrations of single PGRs. In most of the plant cell culture medium, cytokinins plays an important role and known as critical components. Full strength MS will give best result in tissue culture (Bekircan et al., 2018). In this study, full strength MS medium was used and produced the best result as expected. Results shows that internodal explants cultured on MS medium supplemented with 0.5 mg/L KN gave higher shoot formation with 6.20 shoots per explant. This followed by MS medium supplemented with 2. 0mg/L ADE with 4 shoots. MS medium as a control medium produced 4.2 shoots. In this study, less number of shoots were produced by MS supplemented with 0.5 mg/L BA with 2 shoots. The best shoot elongation was with KN at low concentration, 0.5 mg/L. However, a sharp decrease was apparent with 0.5 mg/L BA and 0.5 mg/L ADE. The use of kinetin alone was sufficient to induce shoots from *Solanum melongena*. Highest percentage of shoots were induced in 2.0 mg/L KN (65%) (Foo et al., 2018). Bekircan et al. (2018) reported the best mean shoot elongation was achieved with lower concentration of 0.1 and 0.5 mg/L KN and higher number of shoots from 0.5 mg/L KN in *Thymus leucotrichus*.

In the previous report, the highest number of shoot and elongation was produced in 2.0 mg/L BA with 12.6 shoots and 4.2 cm length and less number of shoot in 0.5 mg/L KN with 1.5 shoots and 2.1 cm length shoot were reported on *in vitro* culture of *Peperomia pellucida* (Shekhawat & Manokari, 2015). This result was in contrast with the present study. This might be because of different sterilization technique used in both studies. In the study by Shekhawat and Manokari (2015), explants were soaked into 0.1% bavistin solution (fungicide) for 8-10 minutes as part of sterilization technique, whereas in this study, during sterilization process, 0.01% carbendazim (antifungal) were added into 70% alcohol and explants were soaked for 30 seconds. Apart from that, 0.01% carbendazim was added into the medium. Using fungicide into culture medium will help to reduce fungal infection and possibility to delay growth (Justamante et al., 2017). According to Singh, (2018) strong sterilization agent will affect the plant growth in the culture. A study has reported that various tested antibiotics in plant tissue culture affects the plant growth (Pollock et al., 1983). Debergh et al. (1993) has reported that in this, study they cultured *Cordyline terminalis* on control medium and medium with (40, 80 and 160 µg/g) of different concentrations of carbendazim. Upon comparing the shoots height on medium with carbendazim and control, the shoots on the three different carbendazim medium showed no significant difference but they were significantly shorter compared to the shoots in the control medium. This study shows that, the antifungal affects the growth of plant in tissue culture.

The most commonly used PGRs in explants regeneration at different combination and ratios are cytokinin and auxin, and this PGRs would form significant difference in regeneration processes. Cytokinin takes part in various plant physiological processes and mostly used to induce shoot formation and cell growth (Foo et al., 2018).

In this study, standard culturing media was used as basic tissue culturing medium for plant regeneration of *Peperomia pellucida.* The nodal explants were cultured on MS medium supplemented with different concentration of cytokinins (BA and KN). The response in multiplication and development of multiple shoots from mother plant showed good results for mass propagation. The maximum number of shoots and shoot elongation was observed with equal concentration of cytokinin, 1.5 mg/L BA and 1.5 mg/L KN. Previous regeneration studies in *Peperomia pellucida* have reported the use of KN in combination with BA showed better multiplication of shoots regeneration (Shekhawat & Manokari, 2015). The present result is similar in terms of the use of BA and KN in shoot multiplication and elongation. The results also are supported by the research on *Solanum melongena* by producing higher number of shoots in combination hormone of BA and KN (Foo et al., 2018). Another research on *Passiflora foetida* also gave better shoots multiplication with combination of BA and KN (Shekhawat et al., 2015). A better effect of combination BA and KN on *Exacum bicolor* has also been reported (Ashwini et al., 2015). Combination of BA and KN resulted in increase in shoot production with 98.3 shoots and 13.3cm on 2.0 mg/L BA and 2.0 mg/L KN in *Rhinacanthus nasutus* (Gouthaman et al., 2016). Cyotkinin helps to activate the axillary bud at the nodal region of the explants (Shekhawat et al., 2015).

In the recent study, different concentrations of cytokinin and auxin were used to initiate multiplication of shoots. The highest number of shoot multiplication observed in 1. 0mg/L BA and 1.0 mg/L IAA with 38.7 shoots with lowest shoot height 0.59 cm. Whereas, the lowest number of shoot were produced by 1.0 mg/L KN and 1.0 mg/L NAA with highest shoot height. Based on the results, it can be suggested that the number of shoot produced was significantly related with the height of shoot. In other words, the more number of shoots produced, more inhibition of growth and shoot shunted were observed. The

number of shoot produced in combination of cytokinin and auxin was significantly higher than combination of cytokinin alone. The results were also supported by a study on jojoba which revealed that when BA was combined with IAA, highest shoot number was observed. However, NAA enhanced the heights of shoots. This study also confirmed, the number of shoot produced was related with the height of shoots (Bekheet et al., 2018). Jeong and Sivanesan, (2015) have obtained maximum number of shoot when cultured *Scrophularia takesimensis* on 2.1 mg/L BA and 1.0 mg/L IAA. Another study has reported that usage of BA and IAA resulted in good response in shoot initiation (Kumar et al., 2016). Multiple shoots initiation were reported on *Ocimum basilicum* with 0.5 mg/L BA and 0.5 mg/L KN medium (Daniel et al., 2010). *Mentha viridis* L. achieved highest number of shoots and length on 2.0mg/L BA with 0.5 mg/L IAA (Rahman et al., 2013). *Ajuga bracteosa* showed 100% shoot regeneration on 5.0mg/L BA and 2mg/L IAA (Kaul et al., 2013). A study also reported that the plant *Salvia nemorosa* produced best shoot proliferation on 8.9 μM BA and 2.9 μM IAA. Different results were obtained due to the effect of different species, culture medium composition and environmental condition. Manan et al. (2016) had reported excess PGRs are toxic and might cause genetic alteration and morphological and physiological transformation, which will result in reduction of proliferation rate.

Activated charcoal essentially contains small particles of carbon. In plant tissue culture, charcoal is used to improve cell growth and development by absorbing inhibitory elements from culture medium (Thomas, 2008). The advantages of activated charcoal in the culture medium is adsorbing accumulated toxic substances and secondary metabolites (Olah, 2017). Apart from that, activated charcoal will modify the pH of the medium and provide soil-like condition by darkening the medium environment (Ahmadian et al., 2013). In recent study, different concentrations of charcoal were added into MS medium

and the cultured were maintained at two different temperatures (13ºC and 25ºC) to check the ability of the shoot multiplication and shoot elongation. The highest shoot induction were observed in free charcoal medium at 25ºC with 2.6 shoots which is not significantly different, comparable with free charcoal medium at 13ºC with 2.53 shoots. The overall highest shoot length was observed in 15% charcoal medium under 13ºC (4.72 shoots). At 25ºC, 15% charcoal medium showed the highest shoot length. Based on this observations, it can be suggested that for shoot elongation, 15% of charcoal can be added in the medium for optimal outcome. For shoot multiplication there is no significance by adding charcoal. A study also reported that to promote shoot elongation in *Exacum Sp*., 0.06 g/L of activated charcoal has to be added into the MS medium with BA and NAA (Unda et al., 2007). The use of activated charcoal in MS medium supplemented with BA, KN, NAA for *Blyttia spiralis* produces healthy shoots with significant multiplication rate (Patel et al., 2016). In *Glossonema edule* study, activated charcoal added into the culture medium and successful seed germination and shoot formation was obtained (Al-Hadidi et al., 2017).

In this study, an efficient micropropagation protocol was developed with high shoot regeneration and multiple shoots. Micropropagated adventitious shoots were then transferred to root initiation medium. Root induction in *in vitro* propagation from regenerated shoots and acclimatization are crucial steps. Transferring *in vitro* regenerated plantlets to greenhouse is a crucial process and need specific environment condition. This is because plantlets have been developed on culture medium under controlled environment. In this study, the six weeks old elongated shoots were separated to achieve adventitious root induction in MS medium with different concentration of auxins (IBA, IAA, and NAA). 1.0 mg/L IBA produce better rooting induction compare to IAA and NAA. Similar observation were reported by Shekhawat and Manokari (2015) whereby

the *in vitro* regenerated shoots of *Peperomia pellucida* cultured on different concentration of IBA and IAA. MS medium with IBA was reported to show effective roots induction. This result supported the recent study and showed that IBA is the suitable PGRs for root induction in *Peperomia pellucida*.

According to Singh, (2018), the two most commonly used auxins in rooting are NAA and IBA. Another study has reported after 20-25 days, regenerated shoots were cultured on media containing 1.0 mg/L and 1.5 mg/L IBA to induce roots, the latter condition produced better rooting (Vani et al., 2016). IBA was used in early induction phase of root in *Prunus avium,* (Quambusch et al., 2017). When various concentration of IBA and IAA were tested on root regeneration of *Passiflora foetida,* maximum *in vitro* rooting was achieved (Shekhawat et al., 2015). Individual microshoots of *Tylophora indica* were transferred to medium containing half strength MS with different concentrations of IBA or NAA. The best results for rooting were obtained on half strength MS with IBA (Sahai et al., 2010). Agarwal, (2015) reported, in *Momordica charantia* and *Limonium altaica*, IBA was identified to be effective in root induction. While another study reported that root induction on *in vitro* lemon showed maximum rooting on IBA as compared to IAA and NAA. Overall, IBA showed higher root induction in these studies.

The term 'acclimatization' refers to transferring of *in vitro* explants to the green house. The successful plant propagation depends on the survival rate in the greenhouse. The rooted plantlets were detached from the culture and the roots were carefully washed to remove medium. Then the whole plantlets were transferred into a pot containing soil, vermiculate and sand in the ratio (1:1:1) and maintained in tissue culture room. The fully hardened plantlets then transferred to natural environment and 85% survival rate was

recorded. Morphology of the *in vitro* grown plant was observed and there was no significant morphological variation when compared against wild grown plant.

Tissue culture success depends mainly on the correct choice of explant materials (Park et al., 2017). Cytokinin and auxin are widely used and known PGRs to date. The different combination ratios of different PGRs will cause significant difference in regeneration process (Foo et al., 2018). Dakah et al. (2013) has reported that the concentration of KN influence the initiation of multiplication of shoots. An increase in KN concentration can lead to a decrease in the number of shoots. Apart from that, cytokinin helps to promote the growth of axillary buds. However, extreme amount of PGRs will lead to morphological and physiological abnormalities (Sidhu, 2011).

Callus is an undifferentiated tissue which has same function as dedifferentiation. From somatic undifferentiated cells, callus undergoes dedifferentiation but gets heterogeneity of cells. Callus cells are naturally heterogeneous, thus it is difficult to produce continuous and stable callus cell for metabolite production (Warghat et al., 2018).

In the present study, the best response for callus formation was obtained from leaf cultured on MS supplemented with different concentrations of 2,4-D which showed callus initiation from the cut edge of the leaves after 2 weeks. The calluses on 2,4-D formed cream friable callus and light green colour callus. At 8^{th} week all the callus were transferred into new medium with same concentration of PGRs. After 12^{th} weeks the maturation of somatic embryos stages were observed. After 16^{th} weeks, the callus started to form multiple shoots. There was no sign of callus was observed from explants on different concentrations of BA, KN, GA_3, ADE, IAA, NAA, IBA and combinations of 2,4-D with BA, KN, IAA, NAA, IBA and GA_3 medium. After 5^{th} weeks all the leaves

started to undergo necrosis. For this study callus did not form in the presence of all cytokinin and auxin. In this study, it showed that 2,4-D play an vital role in callus induction.

The leaf explant of *Wedelia trilobata* were found to have highest callus formation in 2.0 mg/L 2,4-D followed by 1.5 mg/L 2,4-D. The study has proven that 2,4-D as the best regulator for callus induction (Thakur et al., 2011) and was supported by another study by Irvani et al. (2010) which reported callus induction on media containing 2,4-D.

Another study has reported that MS as the most suitable medium to induce callus due to the high concentration of macronutrient present in the MS medium. In *Barringtonia racemosa*, leaves produced high percentage of callus. Thus, it is said to be good sources of explants and PGRs for callus induction. MS supplemented with 2,4-D induced high percentage of callus from *Barringtonia racemossa.* Therefore, 2,4-D is important in callus induction (Dalila et al., 2013).

2,4-dicholorophenoxyacetic acid (2,4-D) is a synthetic auxin and known as an important plant growth regulator. Mainly 2,4-D used in embryogenic cell, callus induction and tissue culture system. In *Santalum album*, 2,4-D showed callus initiation from cut end of nodal explants. After 2 weeks of culture, the entire surfaces of nodal explants were covered with callus. It was also reported that, at the lower concentration of 1.5 mg/L 2,4-D high, percentage of callus was produced (Singh et al., 2016).

For callus induction and percentage of callus formation, variation of days do play vital role. The callus formation is influenced by the gene that affects the structure and plant growth. This describes the contribution of inheritance in callus growth. The ability of

specific tissue to form callus is determined by many factors. These include chemical, light, temperature and humidity (Ali et al., 2010).

In this study, callus was failed to be produced in cytokinin medium. In many studies, BA, and KN are used to induce callus. Irvani et al. (2010) reported MS medium supplemented with cytokinin produced callus in *Dorem ammoniacum* and later described that high concentration of KN favours callus formation. Irvani also reported that BA is important for callus regeneration.

Kumar & Thomas, (2012) reported, 2,4-D was chosen for callus induction in *Clitoria ternatea* Linn. *Clitoria ternatea* Linn, was cultured in various concentration of 2,4-D for callus induction and it was reported that the maintained callus on MS medium supplemented with 2.0 mg/L 2,4-D produced somatic embryos upon transfer to BA and KN.

Initially, after 2 weeks, leaf explants produced callus only in MS medium supplemented with 2,4-D. The embryogenic and non-embryogenic callus were determined by double staining technique (Gupta and Durzan, 1987). In double staining method, the calli nucleus were stained intense bright red with acetocarmine, thereby known as embryogenic callus (Mahmad et al., 2016). Meanwhile, the non-embryogenic calli nucleus would be stained in blue. The 12-week-old callus in different concentrations of 2,4-D were tested for any formation of embryogenic and non-embryogenic. The entire callus showed positive for embryogenic callus. Multiple shoots were initiated from the embryogenic callus. According to Phulwaria et al. (2013) optimum embryogenic callus was induced in media with 1.0 mg/L 2,4-D with 0.5 mg/L BA. The effective auxin 2,4-D for callus induction usually less metabolized by cells. Results of present study shows,

callus undergo maturity somatic embryos after callus were transferred onto 2,4-D media. This is supported by a similar study, done on *Ceropegia bulbosa*. The study showed that, when callus were transferred from callus proliferation medium to shoot multiplication medium, shoots started to regenerate.

The basic for traditional medicine system is the medicinal and aromatic plants. Drugs from plants are preferred more than the synthetic medicine because of their availability, efficacy, affordable and less side effects. Secondary metabolites from plants are unique source for pharmaceuticals, food additives, flavour and other industrial materials (Vijendra et al., 2017). Plant raw materials with consistent quality of valuable natural products become difficult to obtain due to the increase in need to consume such natural products. Thus, attempts were made to produce secondary metabolites from plants tissue culture in laboratories for commercial applications as alternative to produce plants in field or greenhouse (Dakah et al., 2014). Plant tissue culture is known as promising technologies for producing valuable plant metabolites (Jang et al., 2016).

For continuous production of secondary metabolites, method for identification and isolation need to be immediately generated. A complex structure is used to characterize the secondary metabolites (Espinosa-Leal et al., 2018). Gas chromatography and mass spectrometry produce precise information in quantitative analysis. Gas chromatography with flame ionization detector and GC-MS are used in quantitative determination (Sermakkani & Thangapandian, 2012).

There are factors need to be taken into account for secondary metabolites production. The important factor in production of secondary metabolites is stress. Stress determines the therapeutic activity and chemical content of medicinal plants. The plant stress is

actively stimulating or eliciting to induce the preferred chemical reaction of the plant. The stress factor helps to induce and improves the biosynthesis of definite compound in the plant to adapt the stress conditions. The stress can be produced from light, salinity, pH, osmotic stress, drought stress, thermal stress, contamination and hormones (Naik & Al–Khayri, 2016).

In the recent study, GC-MS analysis identified the existence of phytochemical compounds in *in vivo* and *in vitro* methanol and ethanol extract of *Peperomia pellucida*. Extracts from ethanol revealed more chemical compounds compare to extracts from methanol. These identified compounds possess biological properties. Ethanol extract of *in vitro* grown *Peperomia pellucida* revealed the presence of heptadecane, phenol, 3,5-bis(1,1-dimethylethyl)-, dodecane, 1-iodo-, apiol, 1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane, Heneicosane, Heptadecane, 2,6,10,15-tetramethyl-, octasiloxane, phytol, acetate, 6-octen-1-ol, 3,7-dimethyl-, propanoate, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, heneicosane, eicosane, 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane, octadecanoic acid, ethyl ester, 3,5-decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-,1,6,10,14,18,22-tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-, octadecane, 1-iodo-, tridecanol, 2-ethyl-2-methyl-,7,9-di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione, 2-bromotetradecane and pentanedioic acid, 3-phenyl-, dibenzyl ester. Ethanol extract of *in vivo* grown *Peperomia pellucida* revealed the presence of apiol, heneicosane, 4-(3,5-di-tert-butyl-4-hydroxyphenyl)butyl acrylate, octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-, 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane, 2-propenoic acid, pentadecyl ester, azetidine, 3-methyl-1-(phenylmethyl)-, 1-benzylazetidine, heptasiloxane, hexadecamethyl-, cyclopentanone, 2-(4-benzyloxy-3-methoxybenzylidene)-,

1,1,1,3,5,7,7,7-octamethyl-3,5-bis(trimethylsiloxy) tetrasiloxane, benzene, (1-ethylpropyl)-, 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane, oxazolo[4,3-a]isoquinolin-3-one, 1-benzyl-8,9, naphtho[2,3-b]norbornadiene, trans,trans-dibenzylideneacetone, 3-pyrrolidinone, 1-(phenylmethyl)-, 2-benzylaminonicotinonitrile, 2-propenoic acid, 3-phenyl-, phenylmethyl ester, 1-imidazolidinecarboxylic acid, 2-(1,1-dimethylethyl)-4-oxo-3-(phenylmethyl)-, phenylmethyl ester, (s)-, 1,3-dibenzyl-2-(3-nitro-phenyl)-hexahydro-pyrimidine, 9-octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)- and beta.-phenylpropiophenone. The methanol extract of *in vitro Peperomia pellucida* possess cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, caryophyllene, octadecane, 1-chloro-,(7a-isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol, heptadecane, 2,6,10,15-tetramethyl-, 1,2-dimethoxy-4-(2-methoxyethenyl)benzene, apiol, dodecane, 1-iodo-and hexadecane, 1-iodo-. Methanol extract of *in vivo* grown *Peperomia pellucida* revealed the presence of cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, caryophyllene, decane, 2,3,5,8-tetramethyl-, phenol, 3,5-bis(1,1-dimethylethyl)-, pentadecane, 2,6,10,14-tetramethyl-, 1,2-dimethoxy-4-(2-methoxyethenyl)benzene, apiol, heptadecane, 2,6,10,15-tetramethyl-, heneicosane and phytol, acetate. The presence of certain compounds in *in vitro* and *in vivo* extracts are nearly similar. The ethanol extract from *in vivo* and *in* vitro plant shows show more number of chemical compound compare to methanol extract. In this study, *in vitro* and *in vivo* methanol and ethanol extracts contain apiol. In general, apiol has an antimicrobial property and has been reported to have antifungal activity against *Trichphytin mentagrophytes* (Majumder, et al. 2011). Apart from that, apiol also used in abortion and to treat menstrual disorder (Daradkeh & Essa, 2016). The presence of phenol is detected in methanol extracts of *in vivo* grown plant whereby it has been previously reported the presence of phenol in *Peperomia pellucida* (Mensah et al., 2013). In recent study, cyclohexane, 1-ethenyl-1-methyl-2,4-

bis(1-methylethenyl)- and caryophyllene are detected in methanol extract of *in vitro* and *in vivo* grown *Peperomia pellucida*. Narayanamoorthi et al. (2015) reported the presence of cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)- and caryophyllene in *in vivo* ethanol extract of *Peperomia pellucida* whole plant. This two compounds are used as anticancer, analgesic, antibacterial, anti-inflammatory, sedative and also as fungicide. In this study, 9-octadecenoic acid (z) detected in ethanol extract of *in vivo* grown *Peperomia pellucida*. Omotoso et al. (2014) reported, 9-octadecenoic acid (z) contains anticancer, lubricant, anti-inflammatory, haemolytic 5-α reductase inhibitor, diuretic, immunostimulant, antiandrogenic, antifungal, antibacterial and lipoxygenase inhibitor activities. Decane is normally used in chemical test as a function of concentration and temperature (Hollamby et al., 2018). Phytol is detected in methanol extract of *in vivo* grown *Peperomia pellucida*. Kartika et al. (2016) has reported the presence of phytol in *Peperomia pellucida*. Phytol contains antimicrobial, anticancer, diuretic and anti-inflammatory activity.

In previous study, Wei, (2011) reported the presence of phytol, 2- naphthalenol, decahydro-, hexadecanoic acid, 9,12-octadecadienoic acid and methyl ester from methanol extraction of *Peperomia pellucida.* Verma et al. (2014) has reported that a total of 39 compounds were detected from essential oil of *Peperomia pellucida.* Among the 39 compounds, apiol was also present in the essential oil. (Majumder et al., 2011). Another study also reported that dillapiole were isolated from *Peperomia pellucida.* Then, the extract was tested on Wistar rat for gastroprotection. Dillapiole was then recognised as the most active gastroprotective agent of *Peperomia pellucida* (Rojas-Martínez et al., 2013). Many studies have reported the presences of various chemical compounds in *Peperomia pellucida. Peperomia pellucida* has many chemical compounds with medicinal property. Many diseases have been treated using *Peperomia pellucida.*

Peperomia pellucida is known as medicinal plant and widely used for skin, eye, throat, and gout as well as for antibacterial activity. In the present study, the methanol extract from *in vivo* and *in vitro* plant and ethanol extracts from *in vivo* and *in vitro* plant found to be effective against all the tested organisms. Overall, the methanol extract from *in vitro* plant shows highest inhibition zones (12.5 mm) against *Staphylococcus aureus.* The ethanol extracts from *in vivo* plant shows contrast result with the methanol extract from *in vitro* plant with less effective (6.75 mm) against *Staphylococcus aureus.* However, the ethanol extract from *in vitro* plant shows highest inhibition zones (12 mm) against *Salmonella typhii* and *Pseudomonas aeruginosa* compare with methanol extracts from *in vivo* and *in vitro* plant and the ethanol extracts from *in vivo* plant. The ethanol extract from *in vivo* plant shows 11.5 mm inhibition zones against *Escherichia coli*. There are no studies reported the antibacterial activity of *in vitro Peperomia pellucida* plant extract. The result obtained in this study on the antimicrobial efficacy of dried crude extracts of *in vitro Peperomia pellucida* showed the highest inhibitory activity using ethanol and methanol as the medium of extraction. Akinnibosun et al. (2008) have reported that ethanol has stronger extraction capacities which were able to extract greater active components for antibacterial activity. This suggest that, the active components are highly polar compounds and able to dissolve in alcohol. Comparing results with earlier study by Zubair et al. (2015) and Ibibia (2012), the zone of inhibition obtained for *in vivo* methanol and ethanol extracts shows least active on *Staphylococcus aureus, Escherichia coli, Salmonella typhii and Pseudomonas aeruginosa.* Also in this study the inhibition zone is lesser as compared to Zubair et al. (2015) and Ibibia (2012), because in current study, the dry plants were extracted using methanol and ethanol and after extraction the ethanol and methanol were removed using rotary evaporator. Then, the crude extract was diluted using sterile distilled water for the antibacterial test. In Zubair et al. (2015) and Ibibia (2012), they have used ethanol to dilute the extracts. Ethanol is reported as a powerful

sterilizing agent and highly phytotoxic which can kill bacteria (Abbasi et al., 2016; Samadi et al., 2015). Thus, in earlier study, the bacteria growth inhibition has effect from the ethanol and methanol together with the extracted compound. In the current study, the bacteria inhibition was rather by the plant components. The presence of various active compounds in the plants, is the cause of antibacterial activity. The presence of polyphenols and flavonoids from the plants extracts could be the antimicrobial components (Zubair et al., (2015).

Staphylococcus aureus is known as human pathogen and cause mild skin and soft tissue infections and also life threating diseases such as endocarditis, pneumonia or bacteria (Kobayashi et al., 2015; McGuinness et al., 2017). It is reported, *Peperomia pellucida* has been used traditionally to treat skin disorder (Hamzah et al., 2012; Oloyede et al., 2011; Susilawati et al., 2015). Thus, this study proven that *in vitro* methanol extract of *Peperomia pellucida* will be effective to treat skin related diseases caused by *Staphylococcus aureus*. *Salmonella Typhi* are associated with systemic infection and a pathogen known to cause typhoid fever, a life threatening disease in human (Galán, 2016; Wong et al., 2015). *Peperomia pellucida* can be used to treat disease caused by *Salmonella Typhi*. Escherichia coli causes diarrhoea and urinary tract infection among children and adults (Bryce et al., 2016; Patzi-Vargas et al., 2015). Zubair et al. (2015) and Mensah et al. (2013) reported, the *Peperomia pellucida* plant was used by folks to treat diarrhea. Majumder et al. (2011) also reported, the whole plant have been used traditionally to treat diarrhea and gastrointestinal infections. In this study, it is proven *Peperomia pellucida* extract can be used to treat diarrhea. *Pseudomonas aeruginosa* opportunistic pathogen that causes lung infections such as pneumonia. It is also causes urinary tract, skin and soft tissue infection and blood stream infection (Egamberdieva et al., 2017; Gonçalves-de-Albuquerque et al., 2016). From the antibacterial activity of

recent study, it shows that of *Peperomia pellucida* can be used to treat disease caused by *Pseudomonas aeruginosa*.

Due to the increasing demand for medicinal plant, many pharmaceutical companies intend to use medicinal plant for medicine production. The phytochemical investigations identified the occurrence of alkaloids, flavonoids, tannins, saponins and carotol in *Peperomia pellucida* (Ibibia, 2012). All parts of *Peperomia pellucida* have potent antibacterial activity. Polyphenol and flavonoid in plant extracts could be antibacterial components (Zubair et al., 2015). It is also reported that crude methanol extract of *Peperomia pellucida* has broad spectrum of antimicrobial activity (Abere & Okpalaonyagu, 2015). *In vivo* and *in vitro* grown plants produce various compounds which show different bioactivity prospective (Khorasani et al., 2010). Antimicrobial activities differ in *in vivo* and *in vitro* extracts, probably due to the inherent characteristic of the fully grown plants and the maturity of its chemically active constituents.

Apart from the aforementioned properties of *Peperomia pellucida,* it is also very intriguing to note the anti-cancer feature as observed in the current study. Extracts from both *in vitro* and *in vivo* and via two solvents namely methanol and ethanol have reflected differential outcome in terms of proliferation. Cancer cells are well known for their extensive proliferation as well as their ability to form life-threatening tumour (Vincent-Chong et al., 2018). Despite the medical advancement in oncology, specifically in identifying possible ways to control the proliferation of cancer cells is improving rapidly; the need of alternative medicine to supplement current treatment is inevitable (Keene et al., 2019).

In this study, two different types of cancer cells namely adenocarcinomic human alveolar basal epithelial cells (A549) and human breast adenocarcinoma cell line (MCF-7) were tested against the methanol and ethanol extracts from both *in vitro* and *in vivo* grown plants. These cell types were chosen based on the frequency of such cancers to occur among man and woman (Siegel et al., 2019). In general, the increment in extract concentration resulted in poor survival of cancer cells over time. However, the total effect varies among the type of cancer as well as the origin of extract being used. For instance, in A549 cells, extracts from methanol have showed lower survival rate upon exposure at 30 µg/mL for 24 hours. This could reflect that the active compounds that were concentrated via methanol extraction have high cytotoxicity on A549 cells as compared to those from ethanol extraction. This was also supported with IC_{50} values found alongside. Contrastingly in MCF7 cells, compounds via ethanol extraction presented higher cytotoxicity coupled with lower IC_{50} values, as compared to those of methanol extraction. Similar studies which utilize *Peperomia pellucida* as anti-cancer agent are rather limited in the literature but based on the cell type of study, they are trends which can be observed using other plant extracts. As an example, a study by Macedo et al. (2019) have presented that compounds (extracted via methanol) of plants of the genus *Piper* have great potential for further development as anticancer drugs for basal cell carcinoma due to that fact that the cytotoxicity effect observed was even greater than that of carboplatin. It was further described that the major constituent of the best performing compounds were identified as dibenzylbutyrolactone and dibenzylbutyrolactol lignans, which are chemical classes recognized for their cytotoxicity activities.

From the perspective of MCF-7 cells, the cytotoxicity effect seems to be less apparent as compared to A549 cells, especially with those extracted via methanol. Extracts obtained via ethanol however, showed higher cytotoxicity behaviour than that of

methanol. Of particular interest, a study by Wei et al. (2011) has described the potential of methanol extract of *Peperomia pellucida* against MCF-7 cells and it was reported that the IC_{50} obtained was 10.4 µg/mL. This was in contrast with the current findings and it is believed that this could be due to duration exposed with the extract while conducting MTT assay. The data from current study was obtained after 24 hours while the authors from cited study have incubated the cells for 48 hours before assessing their viability. A much older study has reported that the ethanol extract of *Peperomia pellucida* has stronger cytotoxicity effect against Hela, MCF-7 and HL-60 cancer cell lines, which was in line with the current study (Xu et al., 2006).

Additionally, a study by Nurrani et al. (2016) had showed that the ethanol-extract fraction of *Crotalaria sp*. was found to inhibit MCF-7 breast cancer cells with lowest IC_{50} value of 29.67 µg/mL. Meanwhile in the current study, the compound obtained from ethanol extract had IC_{50} value of 22.88 µg/mL; thereby presenting itself as a more potent cytotoxic agent. It is interesting to note that by eye-balling all the IC_{50} values obtained from this study, it is apparent that extracts of *Peperomia pellucida* is more potent against basal cell carcinoma than that of breast cancer. This indicates that the concentration of the extract has to be optimised should the target cancer to address is of breast origin.

Though in this study, the possible mechanism behind the cytotoxicity effect of these compounds was not addressed, it is speculated that key cellular pathways such as TGF-β/Smad and PI3K/Akt/mTOR signalling pathways could be one of which the active compounds exert their effects (Luo et al., 2019). Further studies are indeed warranted to address this area should these compounds are to be translated for clinical and therapeutic usages. Anticancer activities differ in *in vivo* and *in vitro* extracts, probably due to the

inherent characteristic of the fully grown plants and the maturity of its chemically active constituents.

CHAPTER 6: CONCLUSION AND FUTURE RECOMMENDATION

The present study has revealed the potential of *Peperomia pellucida* L. Kunth. to be introduced as medicinal plant in Malaysia and to be cultivated at a larger scale using tissue culture technique. This species can be considered as under exploited plant. Initially, the seed germination was observed in MS medium and soil at 25 ± 2ºC. The seeds germinated and grew well in both conditions. The seedlings in MS medium showed a fast growth into a complete plant with maximum elongation in 4th week.

Plant regeneration of *Peperomia pellucida* involved the shoot multiplication and shoot elongation from internodal explants and rooting. In this study, internodal explants managed to rejuvenate shoots directly and grow well on culture medium. MS supplemented with 0.5 mg/L KN shows the best response by inducing 6.2 shoots. MS medium as a control produce 4.2 shoots. Meanwhile, the highest shoot elongation was obtained in control MS medium (1.48 cm) and highest shoot elongation with PGRs was obtained in MS medium supplemented with 0.5 mg/L KN (0.7 cm). MS supplemented with 0.5 mg/L KN is the most suitable medium for *Peperomia pellucida* shoot multiplication and elongation. Apart from above, for shoot multiplication and shoot elongation, internodal explants were tested on different concentrations and combinations of cytokinin. The best result was achieved with 1.5 mg/L BA and 1.5 mg/L KN with 10.3 shoots and 0.73 cm height after 6 weeks of culture. Further studies were carried out to test the shoot multiplication and shoot elongation rate with combination of cytokinin and auxin. The highest shoot multiplication was observed on MS supplemented with 1.0mg/L BA and 1.0mg/L IAA with 38.7 shoots. Meanwhile, the highest shoot elongation was observed on MS supplemented with 1.0 mg/L KN and 1.0 mg/L IAA (0.81 cm). Furthermore for root formation, 6-week-old adventitious shoots were subcultured on

rooting medium. MS supplemented with 2.0 mg/L IBA showed the highest number of roots (5.33) production. Internodal explants of *Peperomia pellucida* was also tested in different concentrations of charcoal and under different temperatures for shoot multiplication and elongation. MS supplemented with 15% charcoal at 13ºC gave the highest shoot elongation with 4.72 cm in height, whereas, free charcoal MS medium showed the highest shoot multiplication with 2.60 shoots at 25ºC.

Acclimatization of *in vitro* rooted plantlets of *Peperomia pellucida* was carried out successfully and the plantlets had survived the hardening process in the culture room. Then, the hardened plants were transferred to natural environment in green house. Out of this, only 85% of the plants survived and grew well.

Leaf explants of *Peperomia pellucida* showed better response for callus formation. Optimum callus induction of *Peperomia pellucida* was obtained on MS supplemented with 2,4-D. White and light green callus was induced. However, no callus induction in BA, KN, ADE, GA_3, IAA, NAA, IBA and combinations of 2,4-D with BA, KN, IAA was observed. The callus in MS supplemented with 2,4-D showed positive for embryogenic callus. The embryogenic callus formed through four stages namely globular, heart, torpedo and cotyledonary. Multiple shoots were initiated from the embryogenic callus.

Plants naturally produce their own phytochemical substances. A total of twenty four compounds were observed from the GC-MS analysis of *in vivo* and *in vitro* grown *Peperomia pellucida*. Both ethanol and methanol extract of *Peperomia pellucida* contains apiol which is proven to contain medicinal property. Many chemical compound were detected in both ethanol and methanol extract of *Peperomia pellucida.* Most of this

chemical compounds have medicinal properties. Further investigation is warranted to reveal the rationale behind the wide applicability of the plants in herbal medicine.

Through tissue culture system, *in vitro* regenerated plants were tested for antibacterial activity and compared with the *in vivo* grown plants. The results of antibacterial activity showed the highest inhibition zone of *Peperomia pellucida* from methanol extract of *in vitro* plant with 12.5 mm against *Staphylococcus aureus*. This followed by the ethanol extract from *in vitro* plant shows (12 mm) inhibition zones against *Salmonella typhii* and *Pseudomonas aeruginosa.* The result obtained in this study on the antimicrobial efficacy of dried crude extracts of *in vitro Peperomia pellucida* showed the highest inhibitory activity using ethanol and methanol as the medium of extraction.

Cancer is the most devastating disease and leading cause of death in many countries. Many herbaceous plants are used traditionally to treat cancer. In this study, *in vivo* and *in vitro* ethanol and methanol extracts were tested on A549 and MCF-7 cancer cell lines. *In* vitro methanol extract showed better anticancer activity on A549 cells, even though not significant as compared to *in vivo* methanol and ethanol extracts. In MCF-7, *in vitro* ethanol extract showed best anticancer activity. Based on this study, it can be suggested that, *Peperomia pellucida* contains anticancer property and the plant extracts can be used in breast cancer and alveolar cancer treatments.

For future work, animal studies need to be done to test anticancer property of *Peperomia pellucida.* Cellular behaviour of these species is lacking and hence, cytological studies of *Peperomia pellucida* is needed to understand the cellular behaviour of this plant. The studies on Mitotic index, ploidy level, cell cycle, chromosome number,

nuclear and cell area can be further explored to determine variations in plant genotype and phenotype grown *in vivo* and *in vitro*.

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LIST OF PUBLICATIONS AND PAPER PRESENTED

1. **L. Teoh** and R. M Taha. (2017). Identification of Pigments from *Peperomia Pellucida* L. and Its Potential in Coating Technology. Paper presented at 6th International Conference on Functional Materials and Devices (ICFMD 2017). 15-18th August 2017, Melaka, Malaysia. Award: Best 2 minute Oral Presenter.

2. **Teoh Lydia** and Rosna Mat Taha. *In Vitro* Plant Regeneration and Production of Secondary Metabolites from *Peperomia Pellucida* (L.). Paper presented at International Conference on Agriculture and Environment: Food, Water, Soil, Air (ICAE 2016). 25-27th May 2016, Kuala Lumpur, Malaysia.

3. **Teoh Lydia** and Rosna Mat Taha. High frequency plant regeneration and secondary metabolites detection in *Peperomia pellucida* (L.) – A medicinal plant. Paper presented at 20th Biological Sciences Graduate Congress (20th BSGC), 9-11th December 2015, Bangkok, Thailand.