PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF EXPLANT AND CALLUS EXTRACTS OF *Lippia nodiflora* (L.) Michx.

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PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF EXPLANT AND CALLUS EXTRACTS OF *Lippia nodiflora* (L.) Michx.

ABSTRACT

The present study reports a protocol for efficient *in vitro* callus induction of *Lippia nodiflora* (L.) Michx. which belongs to Verbenaceae family, was found to possess natural product source that contains anticancer properties including remarkable bioactivity depends on its bioactive compounds and found to have flavonoids, sugar, steroids, essential oil, resins, tannins and other medicinally valuable constituents. Callus induction and shoot regeneration were achieved through culturing leaf and stem explants after disinfected with 70% ethanol for 3 minutes, 0.10% mercury chloride for 3 minutes and 20% sodium hypochlorite for 2 minutes on Murashige and Skoog (MS) medium (pH5.8) supplemented with single or combinations of auxins and cytokinins, which are naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and benzyladenine (BA), kinetin (KN). The highest shoot regeneration response was observed from MS medium without plant growth regulators (90%) while shoot regeneration from MS media supplemented with combination of IAA and BA which gave the highest multiple shoots with three shoots per explant. Maximum (92.50%) callus response was obtained on single plant growth regulators (PGRs) which was 2.50 mg/L NAA (118.60 mg/L) with light green friable callus, 90.00% response from 1.00 mg/L BA (42.70 mg/L) with green compact callus and 89.20% response from combination of 3.00 mg/L NAA: 2.00 mg/L KN (165.00 mg/L) with green compact and friable callus and 2.50 mg/L IBA: 1.00 mg/L BA (139.60 mg/L) with green compact callus. PGRs were evaluated for their influence on callus induction with biomass parameters in regular interval for *in vitro* callus growth curves which were plotted. Maximum biomass of the callus achieved on the third week. The callus was extracted with methanol using soxhlet apparatus and screening for stigmasterol and β-sitosterol was run using TLC, GC-MS, HPLC and HPTLC. Traces of steroids was found

on TLC, while HPTLC exhibited clear bands of steroids on the R_f value of 0.46 minute. HPLC confirms the presence of stigmasterol at RT 8.69 and 8.71 minutes of standard stigmasterol and extracts, respectively. GC-MS analysis of the methanolic extract of *L. nodiflora* showed 15 peaks in total and the highest component in leaf extracts, octadecanoic acid with 93% and docosanoic acid with 80% in stem extracts. The extracts were tested on *Escherichia coli, Bacillus subtilis, Aspergillus niger* and *Candida albicans* for antimicrobial assay and DPPH radical scavenging activity was used for antioxidant evaluation. The methanolic extracts of *in vivo* leaf and stem explants with *in vitro* callus showed prominent antifungal activity against *A. niger* and *C. albicans*. The 50% (IC_{50}) inhibition concentration of leaf, stem and callus extracts on the free radical scavenging DPPH were determined as 8231, 3959 and 16534 µg/ml respectively, showing there are high antioxidant activity on stem of *L. nodiflora* with 92.2% of scavenging activity. The inhibition of colon cancer cells production on HCT116 was successful with strong level of cytotoxity against cancer cells where dried leaf extracts gave the highest inhibition (4.19 µg/mL). Hence, *Lippia nodiflora* can be suggested to have a high potential to assist in the medical and pharmaceutical industry, especially on anticancer to aid successful cure for cancer.

Keywords: *Lippia nodiflora*, ultrastructure, callus induction, metabolic analysis, biological activities

AKTIVITI-AKTIVITI FITOKIMIA DAN BIOLOGI DARI EKSTRAK EKSPLAN DAN KALUS *Lippia nodiflora* (L.) Michx.

ABSTRAK

Kajian ini melaporkan protokol yang berkesan untuk induksi kalus *in vitro* pada *Lippia nodiflora* (L.) Michx. yang tergolong dalam keluarga Verbenaceae, didapati mempunyai sumber produk semulajadi yang mengandungi ciri-ciri anti-kanser termasuk mempunyai pelbagai bioaktiviti bergantung kepada sebatian bioaktif dan didapati mempunyai flavonoid, gula, steroid, minyak pati, resin, tanin dan pelbagai unsur- unsur berharga dalam industri perubatan. Induksi kalus dan pertumbuhan semula pucuk telah dicapai melalui pengkulturan eksplan daun dan tangkai, di mana ia dibasmi dengan 70% etanol selama 3 minit, kemudian 0.10% merkuri klorida selama 3 minit dan 20% natrium hipoklorit selama 2 minit pada media Murashige dan Skoog (MS) (pH5.8) yang ditambah dengan satu atau kombinasi auksin dan sitokinin, menggunakan asid naftalena asetik (NAA), asid 2,4-diklorofenoksiasetik (2,4-D), asid indol-3-asetik (IAA), asid indol-3-butirik (IBA) dan benziladenina (BA), kinetin (KN). Respon mikropropagasi tertinggi dicapai oleh MS media tanpa hormon (90%) manakala mikropropagasi dari media MS ditambah dengan gabungan IAA dan BA memberi jumlah pucuk tertinggi dengan tiga pucuk setiap eksplant. Maksimum 92.50% respon telah dapat memperolehi kalus daripada satu jenis hormon iaitu 2.50 mg/L NAA (118.60 mg/L) yang mempunyai kalus bewarna hijau yang rapuh, dan seterusnya 90.00% respon daripada 1.00 mg/L BA (42.70 mg/L) dengan kalus bewarna hijau yang padat serta 89.20% respon daripada gabungan hormon 3.00 mg/L NAA: 2.00 mg/L KN (165.00 mg/L) dengan kalus bewarna hijau yang padat dan rapuh, 2.50 mg/L IBA: 1.00 mg/L BA (139.60 mg/L) dengan kalus bewarna hijau yang padat. Pengaruh hormon dinilai persamaan untuk induksi kalus dengan parameter biomas dalam jarak waktu tetap di mana graf pertumbuhan *in vitro* kalus telah diplot. Biomas maksimum kalus telah dicapai pada minggu ketiga. Kemudian, kalus diekstrak dengan metanol menggunakan soxhlet dan kompaun stigmasterol dan β-

sitosterol sebagai rujukan telah dijalankan menggunakan TLC, GC-MS, HPLC dan HPTLC. Kesan- kesan steroid ditemui di TLC manakala analisis HPTLC mempamerkan tompok yang jelas untuk steroid pada nilai R_f 0.46 minit. HPLC mengesahkan kehadiran stigmasterol di dalam ekstrak di mana kompaun stigmasterol serta pecahan daripada ekstrak tersebut dengan masa retensi RT 8.69 dan RT 8.71 minit. Analisis GC- MS daripada ekstrak metanol *L. nodiflora* mempunyai 15 puncak keseluruhan dan komponen yang paling tinggi dijumpai di dalam ekstrak daun, iaitu asid oktadekanoik dengan 93% manakala asid dokosanoik dengan 80% di dalam ekstrak stem. Ekstrak *L. nodiflora* telah diuji pada *Escherichia coli*, *Bacillus subtilis*, *Aspergillus niger* dan *Candida albicans* untuk ujian aktiviti antimikrob dan aktiviti antioksida menggunakan ujian DPPH. Ekstrak metanol daripada eksplan *in vivo* daun, batang dan *in vitro* kalus menunjukkan aktiviti anti-kulat terhadap *A. niger* dan *C. albicans*. Kepekatan perencatan 50% (IC_{50}) bagi ekstrak daun, batang dan kalus untuk memerangkap DPPH radikal bebas adalah 8231, 3959 dan 16.534 μg/mL masing-masing, menunjukkan terdapat aktiviti antioksida yang tinggi pada batang *L. nodiflora* dengan 92.2% aktiviti antioksida. Manakala, perencatan pengeluaran sel-sel kanser usus besar pada HCT116 telah berjaya dengan tahap tinggi terhadap sel-sel kanser di mana ekstrak daun kering memberikan perencatan tertinggi (4.19 μg/mL). Kesimpulannya, *Lippia nodiflora* boleh dicadangkan untuk mempunyai potensi yang tinggi untuk membantu dalam industri perubatan dan farmaseutikal, terutama pada kajian antikanser untuk membantu mengelak dan mengubati kanser.

Kata kunci: *Lippia nodiflora*, ultrastruktur, induksi kalus, analisis metabolik, aktiviti-aktiviti biologi

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%	:	Percentage
µg/mL	:	Microgram per mililitre
µL	:	Microlitre
µm	:	Micrometer
0°C	:	Centigrade
0°C/min	:	Centigrade per minute
2,4-D	:	2,4- dicholoro phenoxy acetic acid
B5	:	Gamborg B5 media
BA	:	Benzyl Adenine
cm	:	centimeter
CO_2	:	Carbon dioxide
DMRT	:	Duncan's Multiple Range Test
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
eV	:	Electron volt
FBS	:	Fetal Bovine Serum
FESEM	:	Field Emission Scanning Electron Microscopy
g/L	:	Gram per litre
g/mL	:	Gram per millilitre
GC-MS	:	Gas Chromatography- Mass Spectrometry
h	:	Hour
H_2SO_4	:	Sulphuric acid
$HgCl_2$	:	Mercury (II) Chloride
HPLC	:	High Performance Liquid Chromatography
HPTLC	:	High Performance Thin Layer Chromatography

IAA	:	Indole - 3 - acetic acid
IBA	:	Indole - 3- butric acid
IC_{50}	:	Median Inhibition Concentration
KN	:	6 – furfuryl amino purine / Kinetin
(L.)	:	Linn
L	:	Litre
M	:	Molarity
mg/kg	:	Milligram per kilogram
mg/L	:	Milligram per litre
min	:	Minute(s)
mL	:	Milliliter
mL/min	:	Milliliter per minute
mm	:	Milimeter
MS	:	Murashige and Skoog media
MSO	:	MS media without plant growth regulators (control)
MTT	:	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide
NAA	:	Naphthalene acetic acid
NIST	:	National Institute of Standards and Technology
nm	:	Nanometer
No.	:	Number
PBS	:	Phosphate Buffer Saline
PGRs	:	Plant growth regulators
POI	:	Percentage of inhibition
ppm	:	Parts per million
psi	:	Pounds per square inch
R_f	:	Retardation factor

RI	:	Resonance Ionization
RP	:	Reverse Phase
rpm	:	Revolutions per minute
RT	:	Retention time
SE	:	Standard error
SH	:	Schenk and Hildebrandt media
TLC	:	Thin Layer Chromatography
UV	:	Ultraviolet
(Var.)	:	Variety
v/v	:	Volume/volume
WHO	:	World Health Organization
WPM	:	Woody plant media
w/v	:	Weight/volume
X	:	Times of magnification
xg	:	Relative centrifugal force (gravity)

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CHAPTER 1: INTRODUCTION

1.1 Background

Figure 1.1: Habit of *Lippia nodiflora*.

Lippia nodiflora (L.) Michx. (Verbenaceae) is a small perennial herb, a creeping plant with branched stems, numerous leaves and it bears small white flowers with scanty roots (Ahmed et al., 2011a; Amir et al., 2011). *Lippia nodiflora* can be found particularly in South Africa, Central America, sub-continent and most of the tropical and subtropical regions, particularly in maritime areas close to rivers (Chopra & Nayar, 1956; Manjunath, 1962; Jayaweera, 1980; Terblanche & Kornelius, 1996; Mako & Noor, 2006; Siddiqui et al., 2007). In Malaysia, *Lippia nodiflora* was found in Kota Kinabalu, Sabah and Seremban, Negeri Sembilan (Teoh et al., 2013; Cheng et al., 2015). *Phyla canescense* shows similar characteristics to *Lippia nodiflora,* however it can be distinguished by its canescent stems and leaves as well as blunt short teeth on its leaf tips (Munir, 1993). Marx et al. (2010) reported that the boundaries of genus *Lantana*, under Verbenaceae family has been historically weak where the genus consists of small trees, five-lobed corollas or

four in *Aloysia* genus, including the dry fruits dividing into two one-seeded mericarps. Taxonomic revision must be interpreted in light of the current taxonomic classification as there may be overlapped in the morphology and habitat of these two species (Leigh & Walton, 2004). There are very few taxonomic reports on *Lippia nodiflora* in Malaysia. Additional taxonomic data based on its foliar micromorphology can improve the identification of this two species.

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant, then were introduced on artificial media. Plant tissue culture can be categorized into two; organized growth and unorganized growth. Organized growth contributes towards the creation or maintenance of defined structure. This usually occurs when introducing plant organs into artificial media, mostly practice using the apical meristems of plants which are shoots and roots, leaf initials, young flower buds or small fruits, to continue growing and preserving their structure. An indirect propagation could also occur during the culture of previously unorganized tissue, which the growth of cells is coherently organized when organs were induced. This process of *de novo* organ formation can be called as organogenesis or morphogenesis (the development of form).

Unorganized growth is the growth of differentiated organs, modifies and specialized to enable them to undertake their essential roles. Unorganized growth can seldom be found in the nature, but can be observed fairly frequently when parts of plants were cultured *in vitro*. Cell aggregates which are then formed, in unorganized structure which only contain a limited number from many kinds of specialized and differentiated cells that are found in an intact plant. Callus can be increased in their volume by subculture and maintained on semi-solid or liquid media for long periods. Induction of its environment can initiate organogenesis, where callus were differentiated into distinct organs.

Organ cultures includes aseptic inoculation from parts of plant such as leaf primordia, immature flowers and fruits including *in vitro* cultures. For the purpose of plant propagation, the most important types of organ cultures are meristem cultures (grown from very small excised shoot apices), shoot tips, node cultures (of separate lateral buds), isolated root cultures and embryo cultures (fertilized or unfertilized zygotic seed). The culture of unorganized tissues that are most generally recognized are callus cultures (growth and maintenance of largely unorganized cell masses), suspension cultures (small cell clumps were dispersed in an agitated liquid medium), protoplast cultures (culture of plant cells that has been isolated without cell wall) and anther cultures (culture of complete anther containing immature pollen microspores) (Edwin et al., 2008).

Preparation of tissue culture begins with preparing media. Plant materials will only grow *in vitro* when provided with specialized media consist of solutions of major and minor elements supplemented with vitamins, an energy source which is usually sucrose and plant growth regulators necessary for a plant growth. Successes achieved in tissue culture would be by the contribution of auxin and would be as incomplete without an understanding of the auxins involving the culture of isolated cells, root tips, stem tips and other explants ended in the development of callus. The function of cytokinins, on the other hand are used to induced divisions in cells of highly mature and differentiated tissues even in the presence of auxin in cultures. High concentration of auxin promoted rooting whereas proportionately more kinetin initiated bud or shoot formation. Unequal proportion of auxin and cytokinins will resulted in unorganized growth (Razdan, 2003). Before starting the tissue culture process, sterilization of whole plant or parts of plants need to be done thoroughly without damaging the plants. Sterilization process differ from one to another plant, so optimization of the process is essential.

Tissue culture process undergone either direct or indirect propagation. The first phase is the initiation of tissue culture by the excision of explants (small organ or pieces of tissue), then it will be isolated into a container generally use test tubes or flasks with securely closed and incubated with light sources with selected photoperiod and suitable temperature under sterile condition. The growth rate of a culture will be depending on the selected photoperiod, temperature and also humidity. The second phase is the maintenance and multiplication phase. Once a culture from organized or unorganized growth has been started *in vitro*, it will usually continue if callus or explant cultures were divided and maintenance need to be done to provide new explants for culture initiation on fresh medium. Subculturing often becomes imperative when density of cells, tissues or organs becomes excessive, increasing in volume, development of new organs or an increment of number of organs (e.g. shoots or somatic embryo) for micropropagation. Rapid rates of plant propagation depend on the ability to subculture shoots from proliferating shoot or node cultures, from cultures giving direct shoot regeneration, or callus capable of reliable shoot or embryo regeneration. A further reason for subculturing is that, the accumulation of toxic metabolites and the exhaustion or the drying of medium. Thus, maintaining a culture requires all or part of it must be transferred onto fresh medium (Edwin et al., 2008).

Tissue culture possess techniques and methodologies can be used to research into many botanical disciplines such as taxonomy and has several practical objectives to further in depth study of plant such as genomics, proteomics and metabolomics (Edwin et al., 2008). Tissue culture process able to enhance further qualitative study by improving the environment of one complete plant due to the aseptic condition throughout the development.

Plants contain sources for natural products which are highly beneficial in the field of medicine and biotechnology. Several plants had undergone preliminary screening to detect its phytochemical properties using mass spectrum analysis for identification of compounds, antimicrobial and antioxidant screening including remarkable bioactivity screening such as inhibition of anticancer activity (Weckwerth & Kahl, 2013).

Analytical strategies for plant metabolite analysis include metabolic profiling, metabolite targeted analysis and metabolic fingerprinting are important steps in characterizing metabolites and metabolic pathways. Metabolite profiling aims the detection of highest number of metabolites possible, contrasting to targeted metabolite analysis which function to determine absolute concentrations of metabolites involved and final approach by metabolic fingerprinting which generally not to identifying individual metabolites but rather provides a fingerprint of all chemicals measurable for sample comparison and discrimination analysis by nonspecific rapid analysis of crude metabolite mixtures (Weckwerth & Kahl, 2013).

Lippia nodiflora medicinal value has contributed in adenopathy which is an enlargement or swelling of lymph nodes treated with a poultice of fresh plants, chronic indolent ulcers, diuretic and aphrodisiac and is also used for the treatment of heart diseases, ulcers, bronchitis, fevers, and colds (Kirtikar & Basu, 1975). A mixture of *L. nodiflora* and the seeds of *Cuminum cyminum* are utilized as a cure for gonorrhea. A decoction of the plant with *Leucas aspera* and the roots of *Ocimum gratissimum* are known to possess anti-malarial properties. A mixture of the extracts of the leaves of *L. nodiflora* leaves, onion and ginger oil is used to treat Alopecia, a hair loss problem (Kirtikar, 1918; Chopra et al., 1956; Siddiqui et al., 2007; Siddiqui et al., 2009).

Flavonoids, sugars, sterols, essential oils, resins, tannins and other medicinally valuable constituents are present in *Lippia nodiflora* (Ahmed et al., 2011b). Ravikanth et al. (2002) reported that halleridone and hallerone, which are bioactive compounds, to have anti-cancer, anti- tumor, anti-malarial and anti-fungal properties with cytotoxic activities. Another bioactive compounds, stigmasterol and β- sitosterol possess anti-diabetic (Balamurugan et al., 2011) and anti- cancer properties were also found in the species (Woyengo et al., 2009). Forestieri et al. (1996) has reported various pharmacological properties of *L. nodiflora* including antispasmodic, hypotensive, anti-inflammatory, analgesic, and antipyretic. Furthermore, Agarwal (1997) reported that the aerial parts of *Lippia nodiflora* plant contains anodyne, antibacterial, diuretic, emmenagogue, parasiticide, refrigerant and febrifuge agents. *Lippia nodiflora* also contains various pharmacological properties including antibacterial, antinociceptive, antifungal, antioxidant and free radical scavenging activities (Shukla et al., 2009; Malathi et al., 2011; Zare et al., 2012). Abbasi et al. (2010) have found combination of *L. nodiflora* and black pepper water extracts have been used to cure multiple skin diseases such as pimples, carbuncles which were caused by bacteria, skin burns and bleeding gums. *L. nodiflora* extracts also possess eupafolin, a melanogenesis inhibitor for skin disease treatment as reported by Feng et al. (2012) and Horng et al. (2014).

Owing to many useful properties of *Lippia nodiflora*, this study was conducted to identify the bioactive compounds from organic solvent extract and analyse the medicinal values.

1.2 Problem Statement

Previously, several studies have been done in the phytochemical components extracted from *Lippia nodiflora* intact plant either from aerial parts or only parts of plants. Moreover, the study on tissue culture sample could provide an aseptic medium but has yet to be exploited especially on callus. However, the focus of this study is to identify the compound responsible for anticancer property. Stigmasterol has been recorded by Ghosh et al. (2011) and Ali et al. (2015), to reduce the volume of cancer cell and has chemopreventive activity in an experimental model of cancer. Kazlowska et al. (2013) mentioned that β-sitosterol and campesterol probably contribute to the anticancer property. Therefore, this study was done to determine the highest callus induction obtained from single and combination of plant growth regulators on Murashige and Skoog (MS) media, continuing to examine its phytochemical properties such as antimicrobial and antioxidation. A targeted High Performance Liquid Chromatography (HPLC) and High Performance Thin Layer Chromatography (HPTLC) analysis has to be done to detect desired compound, next is Gas Chromatography- Mass Spectometry (GC-MS) profiling and further investigation of liquid extracts along with stigmasterol and β-sitosterol to screen the antiproliferative property of samples properly of samples on HCT116 colon cancer using MTT assay.

The establishment of plant regeneration of *Lippia nodiflora*, the phytochemical studies and pharmacology studies seems to be growing. However, the micromorphological study of *Lippia nodiflora* is still considered inadequate. Until now, only a few micromorphological studies have focused on *Lippia nodiflora* (Pant & Kidwai, 1964; Yashvanth et al., 2012). Therefore, a research from a micromorphological point of view was done in this study to understand the morphology of *Lippia nodiflora.*

1.3 Research Objectives

The objectives of this study are:

1. To study the micro morphological characteristics of *Lippia nodiflora* (L.) Michx.
2. To develop the callus and plant regeneration from leaf and stem explants of *Lippia nodiflora* (L.) Michx.
3. To optimize the callus growth using biomass in *Lippia nodiflora* (L.) Michx.
4. To screen the presence of bioactive compound in leaf, stem and callus extracts of *Lippia nodiflora* (L.) Michx.
5. To analyse the anticancer property of *in vivo* leaf and stem extracts of *Lippia nodiflora* (L.) Michx. on HCT116 colon cancer cells.

1.4 Scope and Limitation of Study

This study was done to increase the productivity of *Lippia nodiflora* callus cultures and to slow down the reaction of browning effect on the callus. Eight concentrations of each auxins and three concentrations of each cytokinins used including six concentrations on combination of auxins and cytokinins chose based on the response and biomass from induction of callus. Leaf and stem of *L. nodiflora* were selected to be cultured on MS media supplemented with designated PGRs for callus induction while nodes were selected to be inoculated on MS media supplemented with designated PGRs for plant regeneration.

Different concentrations were examined to investigate high proliferation callus to continue the phytochemical study. *In vitro* callus growth curve was recorded and highest biomass of each PGRs were selected to be maintained for further studies. Leaf, stem and callus were extracted using methanolic extraction and the phytochemical constituents from the extracts were used on preliminary study on Thin Layer Chromatography (TLC), identified using GC-MS and targeted analysis using HPLC and HPTLC. In addition, only

leaf and stem of *L. nodiflora* were used for the micromorphological study and the analyzation of anticancer property on HCT116 colon cancer cells due to the nonsignificant quantity of desired compounds in the callus.

CHAPTER 2: LITERATURE REVIEW

2.1 *Lippia nodiflora* (L.) Michx.

Figure 2.1: *Lippia nodiflora*, leaves and inflorescence.

Taxonomic Hierarchy of *Lippia nodiflora* (L.) Michx. (Sankaranarayanan, 2008)

Kingdom	Plantae
Division	Spermatophyta
Class	Dicotyledonnae
Order	Lamiales
Family	Verbenaceae
Genus	*Lippia*
Species	*nodiflora*

Verbenaceae has a wide range of important species such as *Avicennia officinalis, Cleorodendron fragrans,* genus *Chitarexylum, Duranta repens, Lantana camara, Lippia nodiflora, Premna latifolia,* genus *Stachytarpheta, Tectona grandis, Verbena venosa, Vitex trifolia* and *Petrea volubilis.* They are mostly mesophytes. They comprises of

mostly shrubs, distributed into mangrove shrub (*Avicennia*), climbing or straggling shrub (*Lantana aculeata*), herbs (*Lippia*) and a large tree with big woody trunk such as *Tectona grandis*. They are of the usual tap root type, with simple, opposite and exstipulate structure of leaves. They consist of inflorescence either axillary or terminal and showed much variations. The inflorescence is penduculate, capitate spike in *Lantana* and *Lippia*. The flowers are bracteates with absence of bracteoles, colored or white, complete, hermaphrodite, hypogynous, clearly zygomorphic due to the corolla, and pentamonous reduction of numbers of stamens and carpels. Five sepals, gamosepalous and valvated calyx were recorded. The corolla showed a long or short tube with the upper lip formed by two petals and three sepals at the bottom. The fruit is either a drupe or a capsule, with each cell of the fruit contains one or two seeds (Sambamurthy, 2010). Further notes on *Lippia nodiflora* will be discussed on section 4.3, elaboration on its macromorphology and micromorphology structure.

2.2 Tissue culture on *Lippia nodiflora*

Ahmed et al. (2005, 2011a, 2011b) have conducted several studies on *Lippia nodiflora* from micropropagation, callus induction and organogenesis. For micropropagation, MS media supplemented with sucrose (3% w/v), myoinositol (100 mg/L, w/v), benzyl adenine (BA) (0.50- 3.00 mg/L BA) either individually or in combination with 6-furfurylaminopurine (KN) (0.50- 3.00 mg/L KN), indole acetic acid (IAA) (0.40- 1.40 mg/L IAA), indole butyric acid (IBA) (0.40- 1.40 mg/L IBA), naphthalene acetic acid (NAA) (0.50 mg/L NAA) was used for the experiment while Balaji and Ebenezer (2013) had used 0.30% activated charcoal on his experiment. The result showed that the axillary nodal explants cultured on Murashige and Skoog (MS) media without Plant Growth Regulators (PGRs) (control) has induced two shoots with 6.0 cm shoot length. All concentrations of BA and KN had successfully promoted shoot bud initiation in node explants of *L. nodiflora* where BA was the most efficient cytokinin for the axillary bud

initiation and subsequent proliferation of axillary buds with mean of 12.50 shoots from MS media supplemented with 2.50 mg/L BA (Ahmed et al., 2005). MS medium supplemented with BA and IBA was most effective combination PGRs for shoot regeneration where MS media supplemented with 2.50 mg/L BA + 0.50 mg/L IBA successfully obtained mean of 8.70 shoots per node explants (Ahmed et al., 2005). Full strength, half strength and quarter strength of MS media without auxins which acts as control and supplemented with 0.4- 1.4 mg/L concentration of IAA and IBA were used for root induction. Balaji and Ebenezer (2013) also mentioned that root induction had only occurred when a cut vein such as the midrib is in contact with the media. Root was not obtained on control from all different media however, highest root induction was observed on half-strength MS basal medium supplemented with IBA 1.00 mg/L resulted in 72.70% root initiation.

For callus induction, stem and shoot tip explants of *L. nodiflora* were grown in Murashige and Skoog (MS) medium (Murashige & Skoog, 1962), Schenk and Hildebrandt (SH) medium (Schenk & Hildebrandt, 1972), Gamborg B5 medium (Gamborg et al., 1968) and woody plant (WPM) medium (Lloyd & McCown, 1980) supplemented with 1.00- 2.50 mg/L of NAA, 2,4-D, IBA and IAA also 1.00- 3.00 mg/L of BA and KN were used. In all media tested, the callus initiation did not occur without PGRs (control) in stem and shoot tip explants. Callus induction was able to be obtained in PGRs 2,4-D and NAA on all media MS, SH, B5 and WPM which induced green compact callus, green friable callus, white friable callus, white watery callus and brown friable callus in stem and shoot tip explants. Successful callus induction among the various concentrations of auxins were 1.50 mg/L NAA and 2.00 mg/L 2,4-D significantly induced green compact natured callus with a maximum biomass at day 45 based on their fresh and dry weight. Callus biomass progressively increased with an increase in the NAA concentration (>0.50- 3.00 mg/L) and especially with MS media supplemented with 3.50-

5.00 mg/L NAA, callus nature with white friable callus and white watery callus were induced significantly while IAA and IBA drastically reduced the callus biomass resulting in white friable callus in all the media.

Organogenesis on *L. nodiflora* was performed on stem and shoot tip callus where they were placed in respective culture tubes containing MS media supplemented with different concentrations of 1.00- 3.00 mg/L BA and 1.00- 3.00 mg/L KN with 1.50 mg/L NAA and 2.00 mg/L 2,4-D. 100 mg/L of ascorbic acid was added into MS media which function to avoid phenolic excretion from the callus that cause browning of callus and it was subcultured every week up to callus regeneration. Successful callus regeneration was obtained from MS media supplemented with 2.00 mg/L BA and 1.50 mg/L NAA which increased the shoot number (mean of 12.30 shoots per node) with shoot length (4.20 cm) followed by shoot tip explants (mean of 9.60 shoots per shoot tip) with shoot length (3.50 cm) derived callus from stem of *L. nodiflora* after 60 days.

2.3 Phytochemical study on *Lippia nodiflora*

Shukla et al. (2009) recorded that the steroid compounds and essential oil can be obtained from petroleum ether extracts of *L. nodiflora* while flavonoids and phenolic compounds was able to be screened from methanolic and aqueous extracts of *L. nodiflora.* The author also mentioned that maceration process of *L. nodiflora* dried powder was done on petroleum ether while soxhlet apparatus was used for chloroform and methanol. On the other hand, Ravikumar and Sudha (2011) recorded that ethanol and aqueous extracts contain alkaloids, carbohydrates, glycosides, fixed oils, fats, tannin and phenolic compounds, proteins, amino acids, gums, mucilage, flavors and flavonoids while petroleum ether extracts contain alkaloids, steroids, fixed oils, fats, proteins and amino acids.

Halleridone and Halleron were isolated from dichloromethane and methanol extracts of *L. nodiflora* leaf (Ravikanth et al., 2002). Flavonoids, such as hispidulin, eupafolin, nodifloretin has been reported from methanolic aerial extracts of *L. nodiflora* (Barua et al., 1971; Ko et al., 2013). Ethanolic aerial extracts of *L. nodiflora* has been reported to contain lippiflorin, nodifloridin, jaceosidin, nepetin and batatifolin (Joshi, 1970; Nair et al., 1973) whereas 6-hydroxyleutolin and luteolin-7-O-glucoside were found to be present in the flowers of *L. nodiflora* (Barnabas et al., 1980). Mono and disulphates of nepetin, hispidulin, jacoesidin, 6-hydroxyleutolin and nodiflorentin were also isolated from the aerial parts of *L. nodiflora* (Tomas et al., 1987). Ecteoside has been isolated from ethanolic ectracts of whole plant of *L. nodiflora* (Khalil et al., 1995). Methyl salicylate, eugenol, α-copaene, β-bisabolene, β-sitosterol and stigmasterol have been identified from methanolic aerial extracts (Ko et al., 2013). Steam distillation of *L. nodiflora* extracts showed the presence of volatile constituents including mixtures of hydrocarbons and oxygenates. The major components β-carbolene, methyl salicylates, linalool and cymen-8-ol (Elakovich & Stevents, 1985).

It was discovered by Siddiqui et al. (2009) that by using methanolic extraction, a compound had been fractionated into two unknown steroids compound which concluded to be stigmasterol and β-sitosterol. Methanolic extracts was obtained from maceration process was done four times (in room temperature) and solvent were removed by reduced pressure to obtain a syrupy extracts. The extract was fractionated using ethyl acetate subjected into column chromatography over 70- 230 mm silica gel 60E (Merck) eluted with petroleum ether and petroleum ether: ethyl acetate in increasing the polarity order. Priya and Ravindhran (2015) screened the presence of proteins, amino acids, starch, alkaloids, flavonoids, saponins, tannins, steroids and terpenoids. They reported the presence of terpenoids in plant exhibited cytotoxicity activity against microorganism. Plants derived triterpenoids, steroids and saponin are natural anti-infecting agents reacted

against infection-causing agents while flavonoids responsible for the free radical scavenging activity.

Forty-three compounds were identified from Gas Chromatography- Flame Ionization Detector (GC-FID) and Gas Chromatography- Mass Spectrophotometer (GC-MS) reported by Argyropoulou et al. (2007) for essential oil of *Lippia citriodora* (Verbenaceae). The major components were geranial (38.70%), neral (24.50%) and limonene (5.80%) were found to be the main components, constituting 69% of the total oil, followed by geraniol (6.00%), germacrene D and α-curcumene (3.10%), bicyclogermacrene (2.40%), β-caryophyllene (1.80%), (Z)-β-ocimene (1.30%) and geranyl acetate (1.10%). However it was mentioned by Pascual et al. (2001) and Terblanche and Kornelius (1996), limonene is the component found to occur in higher quantities in essential oils of the genus *Lippia* (Verbenaceae), followed by: ρ-cymene, α-pinene, camphor, β-caryophyllene, linalool and thymol in a decreasing order.

Eight compounds were found in *L. nodiflora* as reported by Calvache et al. (2010) where limonene was found abundance (0.914 mg/kg) followed by carvone (0.493 mg/kg) as the two major compounds. The extraction of *L. nodiflora* essential oil was carried out by using hydrodistillation assisted by microwave irradiation (MWHD) using Clevenger (5L) capacity which was located inside microwave and run the essential oil on Shimadzu GC-MS.

Antimicrobial studies of *L. nodiflora* were done to ensure its source as an antimicrobial agent. Several species of bacteria (positive/negative) and fungi had been done according to previous study. Different solvents of extraction gave different yields of bacteria inhibition. According to Wink (1999), plant extracts was fractionated to obtain different secondary metabolites presence especially for exhibiting antimicrobial activities to be isolated. The antimicrobial activity that presence was mentioned might be due to

alkaloids, flavonoids, phenolic compounds, terpenoids and tannins. Table 2.1 and 2.2 show some of the antibacterial and antifungal studies, respectively.

Table 2.1: Antibacterial studies on selected species of *Lippia nodiflora*.

Species	Solvent of extraction	Reference
Bacillus subtilis	Hexane, chloroform, alcohol	Malathi et al. (2011)
	Methanol	Sumathi et al. (2012)
	Methanol	Zare et al. (2012)
	Aqueous, ethanol, ethyl acetate, chloroform, η-butanol, η- hexane	Ullah et al. (2013)
Bacillus cereus	Hexane, chloroform, alcohol	Malathi et al. (2011)
	Methanol	Zare et al. (2012)
	Methanol, chloroform, Hexane, Petroleum ether	Thamaraiselvi et al. (2013)
Enterococcus faecalis	Hexane, chloroform, ethyl acetate	Priya & Ravindhran (2015)
Escherichia coli	Methanol, chloroform	Sumathi et al. (2012)
	Methanol	Zare et al. (2012)
	Methanol	Salve & Bhuktar (2012)
	η- butanol	Ullah et al. (2013)
	Methanol, chloroform, Hexane, Petroleum ether	Thamaraiselvi et al. (2013)
	Ethyl acetate	Priya & Ravindhran (2015)
Haemophilus influenza	Methanol	Molina-Salinas et al. (2007)
Klebsiella pneumoniae	Methanol, chloroform	Sumathi et al. (2012)
	Methanol	Zare et al. (2012)
	Chloroform, ethyl acetate, methanol	Priya & Ravindhran (2015)
Kelebsiella oxytoca	Methanol	Zare et al. (2012)
Micrococcus luteus	Methanol	Zare et al. (2012)
	Ethyl acetate	Priya & Ravindhran (2015)
Proteus mirabilis	Methanol, chloroform, Hexane, Petroleum ether	Thamaraiselvi et al. (2013)
Proteus valgaris	Methanol	Sumathi et al. (2012)
Pseudomonas aeroginosa	Methanol	Salve & Bhuktar (2012)
	Methanol	Zare et al. (2012)
	Methanol, chloroform, Hexane, Petroleum ether	Thamaraiselvi et al. (2013)
	Hexane, chloroform, methanol	Priya & Ravindhran (2015)

Table 2.1, continued

Species	Solvent of extraction	Reference
Salmonella typhi	Aqueous, ethyl acetate, chloroform, η- butanol, η-hexane	Ullah et al. (2013)
	Methanol, chloroform, Hexane, Petroleum ether	Thamaraiselvi et al. (2013)
	Hexane, ethyl acetate, methanol	Priya & Ravindhran (2015)
Salmonella paratyphi A	Hexane, chloroform, alcohol	Malathi et al. (2011)
Salmonella paratyphi B	Hexane, ethyl acetate	Priya & Ravindhran (2015)
Shiegella flexneri	Hexane, chloroform, alcohol	Malathi et al. (2011)
	Chloroform, ethyl acetate	Priya & Ravindhran (2015)
Staphylococcus aureus (+)	Hexane, chloroform, alcohol	Malathi et al. (2011)
	Methanol	Salve & Bhuktar (2012)
	Methanol	Zare et al. (2012)
	Methanol, chloroform	Sumathi et al. (2012)
	Methanol, chloroform, Hexane, Petroleum ether	Thamaraiselvi et al. (2013)
	Ethyl acetate	Priya & Ravindhran (2015)
Staphylococcus aureus (Methicillin resistant)	Aqueous, ethanol, ethyl acetate, chloroform, η-butanol, η- hexane	Ullah et al. (2013)
	Ethyl acetate, methanol	Priya & Ravindhran (2015)
Staphylococcus epidermatitis (-)	Aqueous, ethanol, ethyl acetate, chloroform, η-hexane	Ullah et al. (2013)
	Hexane, chloroform, ethyl acetate	Priya & Ravindhran (2015)
Streptococcus pneumoniae	Methanol	Molina-Salinas et al. (2007)
Vibrio cholerae	Chloroform, ethyl acetate	Priya & Ravindhran (2015)

Table 2.2: Antifungal studies on selected species of *Lippia nodiflora*.

Species	Solvent of extraction	Reference
Aspergillus niger	Aqueous, ethanol, petroleum ether	Ravikumar & Sudha (2011)
	Methanol	Zare et al. (2012)
Candida albicans	Aqueous, ethanol, petroleum ether	Ravikumar & Sudha (2011)
	Methanol	Zare et al. (2012)
	Hexane, ethyl acetate	Priya & Ravindhran (2015)
Candida tropicalis	Ethyl acetate, methanol	Priya & Ravindhran (2015)
Microsporum gypseum	Ethyl acetate	Priya & Ravindhran (2015)
Malassezia pachydermatis	Hexane, ethyl acetate	Priya & Ravindhran (2015)
Trichophyton mentagrophytes	Ethyl acetate, methanol	Priya & Ravindhran (2015)

Antioxidant activity has been described to be associated with a lower incidence of cancer and cardiovascular diseases (Diplock, 1998). The cause of diseases occurred from several types of reactive species in the form of free radicals or non-radicals that are generated in human body as a result of metabolic reaction, in which the species can be called as prooxidants consisted from either oxygen-derived or nitrogen-derived. The prooxidants attack macromolecules including lipid, protein and DNA, which lead to cellular level and tissue level damages. The presence of endogenous and exogenous antioxidants are therefore essential to counter these effects. Equally important are minerals like Se, Mn, Cu and Zn, enzymes like superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, vitamins like vitamin A, C and E also compounds with antioxidant activity include glutathione, flavonoids, bilirubin and uric acid. Prooxidants and antioxidants need to maintain its ratio in a healthy body where a slight shift in this ratio towards prooxidants gives rise to oxidative stress (Irshad & Chaudhuri, 2002).

Oxidative stress may be either mild or severe, depending on the extent of shift of prooxidants over antioxidants. Monsen (2000) and Irshad and Chaudhuri (2002) recorded that, this oxidative stress is the cause of several diseases, which are cancer, cardiovascular diseases, cataracts, diabetes, inflammatory problems, neurological diseases, age-related macular degeneration malignancies, renal diseases, skin diseases, aging, respiratory diseases, liver diseases and different types of viral infections. Shukla et al. (2009) reported that methanolic extracts of aerial parts of *L. nodiflora* has shown to possess antioxidant property, nonetheless leaf extracts was recorded by Teoh et al. (2013) to possess higher antioxidant content than stem extracts and there are several antioxidant studies has been done on *L. nodiflora* as tabulated in Table 2.3.

Table 2.3: Antioxidant studies on *Lippia nodiflora* extracts.

Extracts	**Type of assay**	**Significant/ not significant (√/ X)**	**Part of plant**	**Reference**
Methanol (defatted by petroleum ether and chloroform)	DPPH	√	Aerial	Shukla et al. (2009)
	HP	√		
	NO	√		
	NBT	√		
	βCL	√		
	RP	√		
Crude ethanol	DPPH	√	Leaf	Teoh et al. (2013)
		X	Stem	
Crude methanol		√	Leaf	
		√	Stem	
Crude methanol	DPPH	√	Leaf	Thamaraiselvi et al. (2013)
	HP	√		
Ethyl acetate fraction	DPPH	√	Aerial	Sudha and Srinivasan (2014)
	SO	√		
	-H	√		
	FRAP	√		
	LP	√		
Methanol (purified with η-hexane)	DPPH	√	Aerial	Fang et al. (2014)
	ABTS	√		
	SO	√		
	MT	√		

*DPPH= DPPH Radical Scavenging Activity, HP= Scavenging Of Hydrogen Peroxide, NO= Nitric Oxide Radical (NO) Scavenging Activity, NBT= Nitro blue Tetrazolium (NBT) Reduction Assay, βCL= β -Carotene Linoleate Bleaching Assay, RP= Reducing Power Assay, SO= Superoxide Radical-Scavenging Assay, -H= Hydroxyl Radical Scavenging Assay, FRAP= Ferric Reducing Antioxidant Power) Assay, LP=Lipid Peroxidation Assay, ABTS= ABTS Cation Radical Scavenging Assay, MT= Mushroom Tyrosinase Inhibition Assay

2.4 Anticancer studies on *Lippia nodiflora*

Cancer can be defined as an abnormal growth of cells caused by multiple changes in gene expressions leading to dysregulated balance of cell proliferation and cell death. This will ultimately evolve into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host Ruddon (2007). Cancer is a condition that affects people all over the world and a leading cause of morbidity and mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related death in 2012 as reported in World Health Organization (WHO). It is expected that incident of new cancer cases will rise 70% within two decades with five common cancers diagnosed among women are breast, colorectum, lung, cervix and stomach cancer. The undesirable side effects of chemotherapy and surgery have triggered the search of new compounds from plant to fight cancer. Plants have become primary sources for cancer prevention and treatment strategies because they are relatively safer than synthetic drugs (Teoh et al., 2013).

The extracted compounds from *L. nodiflora* leaf which are Halleridone and Halleron are known to possess anticancer, antitumor and cytotoxic activities (Ravikanth et al., 2002). The methanol and ethyl acetate extracts of leaf and stem of *L. nodiflora* exert anti-prolifeative effect on human breast adenocarcinoma (MCF-7) cell within the range of 90-120 µg/ml (Teoh et al., 2013). In comparison, the IC_{50} values obtained from Teoh et al. (2013) has been found much higher than as recorded by Vanajothi et al. (2012), whom conducted anti- proliferative study on lung cancer. The anti- proliferative activity of *L. nodiflora* was associated with its antioxidant activity, where extract with lower EC_{50} values will have higher antioxidant activity, thus resulting in high IC_{50} values of anti-proliferative activity.

Extensive research has been carried out from last decades to discover potential constituents from plant sources. Kaur et al. (2011) have found that stigmasterol is an important constituent and has been successfully isolated from plants. Stigmasterol has involved in many of the synthesis of hormones such as progesterone, androgens, estrogens and corticoids. It also has many of its derivatives which are spinasterol, fucosterol, cyasterone, stigmasterol glucoside, fucosterol epoxide, stigma-4en-3one, 29-fluorostigmasterol and others. Effective in pharmacological studies, stigmasterol possess anti-osteoarthritic, anti-hypercholestrolemic, anti-tumor, antioxidant, antimutagenic, anti-inflammatory, cytotoxic, showed significant analgesic activity and also contributes to hypoglycemic activity. Stigmasterol has been found in *L. nodiflora* in several studies (Zare et al., 2012; Sharma & Singh, 2013; Sudha & Srinivasan, 2013; Jabeen et al., 2016). The summary of anticancer studies on *L. nodiflora* has been simplified into Table 2.4.

Table 2.4: Anticancer studies on *Lippia nodiflora* extracts.

Cell line	Extraction	Assay	Significant/ not significant (√/ X)	Part of plant	Reference
Human lung cancer cell line, NCI-H460	Methanol	MTT assay	√	Aerial	Vanajothi et al. (2012)
		Apoptasis	√		
Human breast adenocarcinoma, MCF-7	Methanol	MTT assay	√	Leaf	Teoh et al. (2013)
			X	Stem	
	Ethyl acetate		√	Leaf	
			X	Stem	
	Methanol	Apoptasis	√	Leaf	
			X	Stem	
	Ethyl acetate		√	Leaf	

2.5 Other studies on *Lippia nodiflora*

L. nodiflora has been used to treat many diseases in past years and has been used as traditional medicine for various skin diseases and cosmetics among the tribal communities (Sharma & Singh, 2013). β-sitosterol isolated from methanolic extracts of *L. nodiflora* has been screened for antidiabetic potential by Rangachari and Savarimuthu (2011). The compound was administered to streptozotocin induced diabetic rats and glycosylated hemoglobin was decreased along with blood glucose level with significant increment of plasma insulin, body weight and food intake. β-sitosterol also had been evaluated to possess antihyperlipidemic effect. Other than anticancer properties, it also contains a number of potential compounds to inhibit or cure other diseases as listed in Table 2.5.

Table 2.5: Other studies on aerial part of *Lippia nodiflora* extracts.

Target study	Procedure	Reference
Skin disease (Melanogenesis inhibitor)	• Methanolic extracts • MTT assay on B16F10 melanoma and human keratinocyte (HaCaT) cells and 3T3 (mouse embryonic fibroblast) cells • Determination of cellular melanin content and cellular tyrosinase activity • Analysis of the expression of proteins regulating melanogenesis by Western Blotting	Yen et al. (2012)
Melanogenesis inhibitor- Eupafolin	• Methanolic extracts • MTT assay on B16F10 melanoma and human keratinocyte (HaCaT) cells • Determination of cellular melanin content and cellular tyrosinase activity • Analysis of the expression of proteins regulating melanogenesis by Western Blotting	Chiang et al. (2014)

Table 2.5, continued

Target study	Procedure	Reference
Antihyperuricemic agents (flavonoids and phenylethanoid glycosides)	• Methanolic extracts • Chemically-induced hyperuricemic animal model - Male Sprague-Dawley rats (160–300g) • Evaluation of uricosuric effect • Molecular docking of 6-hydroxyluteolin on xanthine oxidase • Acute toxicity study	Cheng et al. (2015)
Anti- hepatotoxic activity	• Methanolic extracts • Fractionation of plant extract • Biochemical analysis on treated liver of male albino Wistar rats (150-170g)	Sudha et al. (2013)
Anti- diuretic	• Methanol and aqueous extracts • Aerial part of *L. nodiflora* • Assessed in albino rats using *in vivo* Lipschitz test model	Shukla et al. (2009)
Anti- diabetic and hypolipidemic effect	• Methanol extract • Successfully isolated β-sitosterol • Assessed in streptozotocin induced diabetic rats	Rangachari & Savarimuthu (2011)

2.6 Research Gap

Previous reviews of tissue culture on callus induction on media supplemented with PGRs has experienced the browning of callus. Ahmed et al. (2011b) has observed brown friable callus in the callus induction of *Lippia nodiflora.* Gupta et al. (2001) recorded a dark-brown calli after two to three weeks of initiation on *Lippia alba* (Verbenaceae). However, JibinaBai et al. (2014) reported *Lippia alba* induced brown friable callus and profuse callusing after 9 days of inoculation. In the present study, the browning of callus was generated as fast as eight days of inoculation. Rapid and consistent subculturing were needed to maintain the green, viable culture for harness. This situation has demanded substantial amount of media, space, labour and time, thus an effective solution is desperately needed for the culturing of this important species.

Callus was induced in MS media supplemented with PGRs, investigating several treatments using single and combination PGRs. Single treatments auxins (2,4-D, NAA, IAA and IBA) and cytokinins (BA and KN) showed similar outcome of previous study while combination PGRs auxin and cytokinins have shown positive development of callus. As the present investigation has provided a longer maintenance duration after inoculation of callus, a cost- effective, less labour intensive and time saving *Lippia nodiflora* culturing can be achieved to ensure sufficient time for callus harvesting and optimum nutrient absorption by the callus of *L. nodiflora.* Data on phytochemical properties will further contribute to medical and pharmaceutical industry for cancer treatment along with antidiabetic and antimelogenesis properties of *L. nodiflora* cultures.

In morphology aspect, *Phyla canescens* was found to have almost similar characteristics to *P. nodiflora,* however it can be distinguished by its canescent stems and leaves, and blunt short teeth on its leaf tips (Munir, 1993). Taxonomic revision of both species must be interpreted in the current order of taxonomic classification as there may

be an overlap in the morphology and habitat of these two species (Leigh & Walton, 2004). Mashile and Tshisikhame (2017) mentioned the importance of separation and linkage in higher level of taxonomic hierarchy provided through leaf epidermal features in some cases, and its properties could be associated in functional aspects of leaf venation. Furthermore, additional taxonomic data based on its foliar micromorphology can improve the identification of this two species. Based on the research discussed above, there are lack of taxonomic reports on *P. nodiflora* in Malaysia. Therefore, this study aims to carry out taxonomic study particularly on the micromorphology to add more taxonomic information to the species.

CHAPTER 3: METHODOLOGY

3.1 Introduction

The following part explicates the basic principle of analytical methods applied in this study to provide general understanding on the flow and employment of the present experimental design. Sections represent the field of all studies elaborated in method steps by steps of each sub-section conducted throughout the experiments.

3.2 Plant Materials and Identification

Healthy, young leaf and stem explants of *Lippia nodiflora* were collected from Taman Rimba Ampang, Selangor, Malaysia (Latitudes 3° 8' 57", longitudes 101° 47' 28"), and maintained at the Institute of Biological Sciences (IBS) Garden, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. Voucher specimen (Figure 3.1) was also prepared (KLU47924) and kept at the University of Malaya herbarium (KLU). *L. nodiflora* can be found in MARDI Station, Klang, Selangor; Taman Rimba Ampang, Selangor; Penang Botanic Garden, Penang; Pantai Peranginan Kelulut, Terengganu and Singapore Botanic Garden, Singapore which are near the river and the sea (Figure 3.2). It is also widely cultivated by farmers at Jelebu, Negeri Sembilan. The collected plants were mostly from Taman Rimba Ampang, Selangor. The plants were maintained in 10 pots at Institute Biological Sciences Garden, Faculty of Science, University of Malaya for tissue culture work. *L. nodiflora* was collected from the field and had been dried for phytochemical work.

Figure 3.1: Herbarium specimen of *Lippia nodiflora* at University of Malaya Herbarium (KLU47924).

Figure 3.2: Distribution of *Lippia nodiflora* in Peninsular Malaysia.

3.3 Morphology of *Lippia nodiflora*

3.3.1 Macro-morphology of *Lippia nodiflora*

L. nodiflora was collected from the field and washed thoroughly using tap water. Dirt on the sample was removed to obtain quality image for viewing. The sample was cut into parts to be recorded and identified. The collected seed was viewed under Optika ST-30-2LF, binocular dissecting microscope with 20x- 40x magnification with additional aid light source from Nikon Inverted Microscope Lamp and Transformer.

3.3.2 Micromorphology of *Lippia nodiflora*

3.3.2.1 Field Emission Scanning Electron Microscopy (FESEM)

Small fresh leaves of *L. nodiflora*, 4- 6 mm in length, were collected and were fixed in 8% glutaraldehyde with Sorensen's Phosphate Buffer solution (1: 1) for one hour and were rinsed using distilled water: Sorensen's Phosphate Buffer solution (1: 1). Samples were soaked in 4% Osmium: distilled water (1: 3) for 14 hours in low temperature (5°C) or for one hour in room temperature inside the fume chamber. Samples were dehydrated in a graded series of ethanol (20- 100% X3) for 15 minutes per rinse. Then the samples were dehydrated in three times degradation of 100% ethanol: 100% acetone for 20 minutes with ratio of 3: 1, 1: 1 and 1: 3 and 4 times degrade in 100% acetone for 20 minutes. This was followed by critical point drying with liquid carbon dioxide (CO_2) in an Auto sampler 810 critical point dryer and sputter-coating with gold-palladium in a LEICA EM SCD005 sputter coater. Both the adaxial and abaxial surfaces were observed under JEOL- 7500F Field Emission Scanning Electron Microscope. Structures such as stomata, trichomes and oil gland were observed and recorded.

3.3.2.2 Leaf Epidermal Peel

Leaves were soaked in 10% nitric acid (HNO_3) solution overnight until the laminas of the leaves were soft and bloated. The leaves were then washed with distilled water. The epidermal layers of the abaxial and adaxial surfaces of the leaves were peeled using sharp forceps and then placed into separate Petri-dishes containing distilled water. Every epidermal of the leaves that were peeled were then immersed in a solution containing 5% sodium hypochlorite, or also known as Clorox, for five minutes. Next, dehydration steps were done 15 minutes for Safranin 'O' (Merck, Germany), followed by 30, 50, 75, 90, 95, 100% ethanol and 100% ethanol: 100% xylene for 2 minutes each, then with 100% xylene for one minute. Slides were observed under a binocular microscope. The epidermal peel was done to study the presence of stomata and trichomes on the adaxial and abaxial surfaces, anticlinal wall, and types of stomata. Stomata index can be calculated as shown;

$$\textbf{Stomata index: } \frac{\text{Total number of stomata}}{\text{Total number of epidermal cell + number of stomata}} \times 100\%$$

3.4 Tissue Culture Studies

3.4.1 Sterilization Method

Leaf and stem explants of *L. nodiflora* were washed thoroughly for 30 minutes, then rinsed with sterile distilled water including 2% (v/v) Teepol (Labchem, Malaysia) for 10 minutes; then washed with 70% ethanol for three minutes followed by 20% sodium hypochlorite (Clorox, Malaysia) for three minutes and lastly washed with 0.10% mercury (II) chloride, $HgCl_2$ (Merck, Germany) for two minutes. Prior to inoculation, explants were washed three times with sterile distilled water.

3.4.2 Callus Induction of *Lippia nodiflora*

L. nodiflora was cultured in aseptic conditions to obtain callus induction. The leaf and stem explants were cultured in MS media (Murashige & Skoog, 1962) supplemented with plant growth regulators (PGRs) such as auxin, naphthaleneacetic acid (NAA, Duchefa Biochemie, Netherlands), 2, 4- dichlorophenoxyacetic acid (2, 4-D, Duchefa Biochemie, Netherlands), indole-3-butyric acid (IBA, Duchefa Biochemie, Netherlands) and indole-3-acetic acid (IAA, Duchefa Biochemie, Netherlands), also cytokinin of benzyl adenine (BA, Duchefa Biochemie, Netherlands) and kinetin (KN, Duchefa Biochemie, Netherlands) which will be tested in many concentrations (0.50- 4.00 mg/L). Number of treatments on callus induction for single PGRs was shown in Table 3.1 and combination PGRs was shown in Table 3.2. The leaf and stem explants were surface injured using scalpel to give a few slight cuts and it was faced the media abaxially. They were maintained at 25 ± 2°C, 16/8 hours (light/dark) of photoperiod with 25 μmol $m^{-2}s^{-1}$ of light intensity.

Table 3.1: Number of treatments on callus induction for single PGRs.

No.	Plant Growth Regulators (PGRs)					
	Auxin				Cytokinin	
	NAA	2,4-D	IAA	IBA	BA	KN
1	-	-	-	-	-	-
2	0.50					
3	1.00					
4	1.50					
5	2.00					
6	2.50					
7	3.00					
8	3.50					
9	4.00					
10		0.50				
11		1.00				
12		1.50				
13		2.00				
14		2.50				
15		3.00				
16		3.50				
17		4.00				
18			0.50			
19			1.00			
20			1.50			
21			2.00			
22			2.50			
23			3.00			
24			3.50			
25			4.00			
26				0.50		
27				1.00		
28				1.50		
29				2.00		
30				2.50		
31				3.00		
32				3.50		
33				4.00		
34					1.00	
35					2.00	
36					3.00	
37						1.00
38						2.00
39						3.00

Table 3.2: Number of treatments on callus induction for combination PGRs.

No.	Plant Growth Regulators (PGRs) (mg/L)					
	Auxin				Cytokinin	
	NAA	2,4-D	IAA	IBA	BA	KN
1	2.00				1.00	
2	2.50				1.00	
3	3.00				1.00	
4	2.00				2.00	
5	2.50				2.00	
6	3.00				2.00	
7		2.00			1.00	
8		2.50			1.00	
9		3.00			1.00	
10		2.00			2.00	
11		2.50			2.00	
12		3.00			2.00	
13			2.00		1.00	
14			2.50		1.00	
15			3.00		1.00	
16			2.00		2.00	
17			2.50		2.00	
18			3.00		2.00	
19				2.00	1.00	
20				2.50	1.00	
21				3.00	1.00	
22				2.00	2.00	
23				2.50	2.00	
24				3.00	2.00	
25	2.00					1.00
26	2.50					1.00
27	3.00					1.00
28	2.00					2.00
29	2.50					2.00
30	3.00					2.00
31		2.00				1.00
32		2.50				1.00
33		3.00				1.00
34		2.00				2.00
35		2.50				2.00
36		3.00				2.00
37			2.00			1.00
38			2.50			1.00
39			3.00			1.00
40			2.00			2.00
41			2.50			2.00
42			3.00			2.00
43				2.00		1.00
44				2.50		1.00
45				3.00		1.00
46				2.00		2.00
47				2.50		2.00
48				3.00		2.00

3.4.3 *In vitro* callus growth curve

Callus cultures were optimized and evaluated quantitatively for their nature, biomass and bioactive compound content at the end of their respective growth cycle based on a previous protocol (Ahmed et al., 2011a). By treating with various concentrations of auxin, fresh and dry weights of the callus were determined at 7, 14, 21 and 28 days. At regular interval for all the treatments, each callus was harvested by careful separation from media using scalpel, and fresh and dry weight were recorded (Figure 3.3).

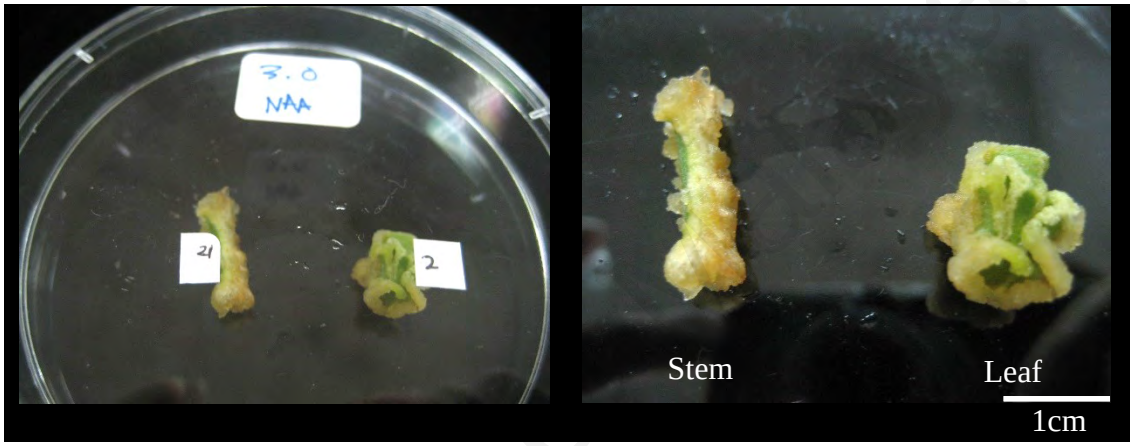


Figure 3.3: Induced callus from leaf and stem explants.

3.4.4 Shoot Regeneration of *Lippia nodiflora*

L. nodiflora was cultured in aseptic conditions to regenerate the shoots. The leaf and stem explants were cultured vertically in MS media (Murashige & Skoog, 1962) supplemented with plant growth regulators such as auxin, NAA, 2, 4-D, IBA and IAA with its combinations concentrations (2.00 mg/L, 2.50 mg/L and 3.00 mg/L) also cytokinin of BA and KN with its combinations which will be tested in many concentrations (1.00- 3.00 mg/L). The number of treatments for shoot regeneration is shown in Table 3.3. The regenerated shoots were transferred to the MS media with supplemented PGRs to provide mass plant production. They were maintained at 25±2°C, 16/8 hours (light/dark) of photoperiod with 25 μmol $m^{-2}s^{-1}$ of light intensity.

Table 3.3: The number of treatments for shoot regeneration of stem explants of *L. nodiflora.*

No.	Plant Growth Regulators (PGRs) (mg/L)					
	NAA	**2,4-D**	**IAA**	**IBA**	**BA**	**KN**
1	-	-	-	-	-	-
2	2.00					
3	2.50					
4	3.00					
5		2.00				
6		2.50				
7		3.00				
8			2.00			
9			2.50			
10			3.00			
11				2.00		
12				2.50		
13				3.00		
14					1.00	
15					2.00	
16					3.00	
17						1.00
18						2.00
19						3.00
20			2.00		1.0	
21			2.50		1.0	
22			3.00		1.0	
23			2.00		2.0	
24			2.50		2.0	
25			3.00		2.0	
26			2.00			1.0
27			2.50			1.0
28			3.00			1.0
29			2.00			2.0
30			2.50			2.0
31			3.00			2.0
32				2.00	1.0	
33				2.50	1.0	
34				3.00	1.0	
35				2.00	2.0	
36				2.50	2.0	
37				3.00	2.0	
38				2.00		1.0
39				2.50		1.0
40				3.00		1.0
41				2.00		2.0
42				2.50		2.0
43				3.00		2.0

3.4.5 Root Induction and Acclimatization

The minimal length of shoots were developed up to 4.00 cm and above, then shoots were cut and transferred to rooting MS media supplemented with 1.00- 3.00 mg/L 2,4-D, IBA, IAA and NAA. After 20 days, the successful root induction was observed, the number of roots as per shoot was recorded and tabulated.

Plantlets were successfully developed from rooted plantlets. The roots were washed in running tap water to remove the media. Rooted shoots were planted in Plantaflor® soil in plastic pots covered with a plastic bag that had holes for about four weeks duration. The plantlets were removed from the pots and transferred into the field. The soil used was Plantaflor® Young Plant Substrate, which consisted of 50% white peat + 50% black peat + 1.00 kg NPK fertilizer, including trace elements and the structure of the soil was fine.

3.5 Phytochemical studies on *in vivo* leaf and stem and *in vitro* callus

3.5.1 Sample extractions

15 g powdered *L. nodiflora* dried leaves and stems of intact plant and 5 g of callus that was dried using oven at 35˚C for 48 hours were used.

Decoction of *L. nodiflora*

The samples were boiled in 100 mL of distilled water using boiling water bath for 15-20 minutes while stirring occasionally. The beaker was removed from the heat and allowed to cool for 15 minutes. The extracts were filtered through regular filter paper to remove any solid particles.

Maceration of *L. nodiflora*

The samples were placed in a closed flasks containing 100 mL of methanol, ethanol (HPLC grade, Merck, Germany) and distilled water respectively. The flasks were shaken

occasionally for seven days using shake culture at 80 rpm. The extracts were filtered through regular filter paper to remove any solid particles.

Percolation of *L. nodiflora*

The samples including 15 g of fresh leaf and stem explants were extracted using Soxhlet apparatus with 250 mL of methanol (HPLC grade, Merck, Germany) for 24 hours. The extracts were filtered through regular filter paper to remove any solid particles.

The collected extracts from the three protocols were centrifuged at 10,000 x g for about 10 minutes in room temperature. Then, the methanol supernatant of dried leaf, stem and callus went through the rotary evaporator (IKA) and the crystallized extracts were kept in -4°C, until it wants to be used, they were dissolved in methanol. The dried extracts were screened through TLC to optimize the method, then followed by metabolite profiling using GCMS and targeted analysis through HPLC and HPTLC with standard of the bioactive compounds for the quantification of bioactive compounds (Seal, 2016). The fresh and dried extracts of *L. nodiflora* were used in anticancer screening on colon cancer cells.

3.5.2 Thin Layer Chromatography (TLC)

Methanolic extracts of leaf, stem and callus explants was collected and was analyzed by TLC. TLC was performed using 10 x 20 cm (width x height) silica gel 60 F_{254} (Merck, Germany) with hexane: acetone (4:1) and (3:1) as solvent system. After loading the extracts onto the TLC plate, the experiment was run for one hour long and next it was viewed under 254 nm and 365 nm UV light then the 10% sulphuric reagent was applied to the TLC plate for the determination of steroid compounds with standard stigmasterol and β- sitosterol. Distance of solvent and distance of each bands were measured to calculate retardation factor (R_f) value using following formula;

$$R_f = \frac{\text{Migration distance of substance}}{\text{Migration distance of solvent front}}$$

3.5.3 Gas Chromatography- Mass Spectrometry (GC- MS)

GC- MS analysis of leaf and stem extracts of *L. nodiflora* was performed using the equipment Shimadzu GC- MS QP2010 with with a CPB-capillary column (mm i.d. × 50 m length). The initial oven temperature was 40ºC held for one minute which then it was increase from 40ºC to 280ºC at the rate of 4ºC/minute. The injector temperature was 280ºC, the transfer line 280ºC and the ion source 200ºC. The carrier gas used was helium with a flow rate of 20 psi. The sample volume injected was 10µL as sample was dissolved in methanol with the ratio of 1: 10. The identification of components was based on NIST library on comparison of their retention indices. The constituents were identified after comparison with those available in the computer library (NIST 8.0) attached to the GC-MS instrument and the results obtained have been tabulated. Quantitative analyses of each bioactive component (expressed as area percentage) were carried out by a peak area normalization measurement, calculated as mean values of three injections from each sample.

3.5.4 High Performance Thin Layer Chromatography (HPTLC)

The methanolic extracts of *L. nodiflora* leaf, stem, and callus explants (5 µL) was applied in the Camag HPTLC system assisted with sample applicator Linomat V for quantification of the desired bioactive compound standards (stigmasterol and β-sitosterol). HPTLC plate Silica gel 60 F_{254}, 20 x 20 cm was used, the mobile phase used were hexane: acetone (4:1; 3:1) according to TLC and chloroform: ethanol (98:2) was adopted from Kamboj and Saluja (2013) for 20 minutes with saturation pads. Mobile phase chloroform: ethanol (98:2) were continued for analysis as it has given good separation of bands especially better view under white light, UV 254 nm (green) and UV 365 nm (purple). Then, it was scanned at 254 and 365 nm by the use of TLC visualizer

and the supporting software winCAT 4.0. After the data was recorded, the plate was sprayed with 10% sulphuric acid (H_2SO_4) in methanol and heated with CAMAG TLC Plate Heater III at 110°C for 10 minutes.

3.5.5 High Performance Liquid Chromatography (HPLC)

Stigmasterol and β-sitosterol were screened in *in vitro* callus, leaf and stem (1 mg/mL) extracts of *L. nodiflora*. A JASCO HPLC model with UV detector was used with Chromolith® High Resolution Reserve Phase (RP)-18 column (with column dimension: 250mm x 4mm and particle size of 5 µm) (Merck, Germany). The flow rate was set at 1.50 mL/minute with injection volume of 10 µL. The detection wavelength for all dyes was set at 506 nm. The mobile phase used was acetonitrile: methanol with 80:20 ratio. The solvents and other chemicals used were of HPLC grade (Merck, Germany). The data obtained was recorded. The quantification of stigmasterol and β-sitosterol in *L. nodiflora* extracts has been done. Chromatographic peaks were identified on the basis of retention time. The concentrations of stigmasterol and β-sitosterol were calculated by comparing the integrated peak areas of stigmasterol and β-sitosterol in the extract chromatograms with that of a standard curve prepared for the corresponding standard solution. The conventional flow standard curves (area versus concentration) were constructed for the external standard stigmasterol and β-sitosterol, freshly prepared. The external standard curves constructed by six concentration points (100, 200, 300, 400, 500 and 600 mg/mL). Data for the standard curve were generated using Microsoft Excel. For analytical recovery studies, sample extract was mixed with known amounts of standards and re-chromatographed to ascertain whether the peak of interest increased in height and area. Spiked peaks were also checked for peak symmetry and peak purity.

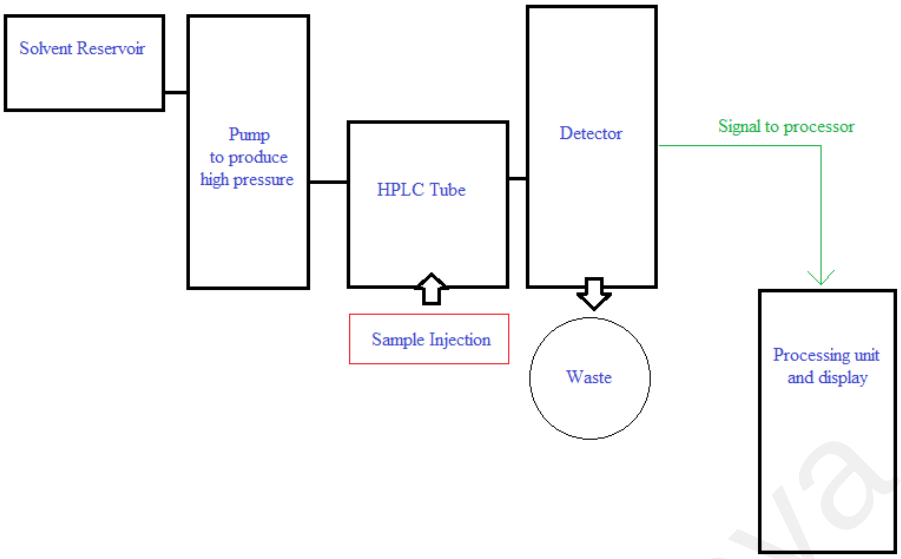


Figure 3.4: HPLC system unit.

3.5.6 Antimicrobial Activity Assay (Disk Diffusion Method) (Adopted from Bauer et al., 1966)

The agar media was prepared for bacterial and fungal culture. Nutrient agar was used to culture two species of bacteria which were *Escherichia coli* and *Bacillus subtilis*. While, potato dextrose agar was prepared to culture two species of fungi which were *Candida albicans* and *Aspergillus niger*. The plant extracts were tested for antimicrobial activity by using disc diffusion method. The antibacterial activities were tested against methanolic extracts of leaf, stem and callus. About 15- 20 mL of nutrient and potato dextrose agar media was poured in the sterilized petri dish and allowed to solidify. Disc for extracts were prepared by using filter paper with 5 mm diameter. Extracts with different concentrations (0.50, 1.00 and 1.50 mg/mL) were used to test antimicrobial activity and compared with positive control which were standard ampicillin (20 mg/mL) for bacteria and fluconazole (20 mg/mL) for fungi with 0.02 mg/mL as final concentration. 20 µL of the test strain ($1.00x10^5$ CFU/mL) was inoculated into the media in laminar flow under sterile condition. 5 µL of plant extracts or control were dropped on top of the disc with different concentrations were placed on the agar with different types of microbes. Plates were incubated in air at 37°C (bacteria) and 25°C (fungal) for 24

hours. Antimicrobial activities were evaluated by measuring inhibition zone diameters and the data was observed and recorded.

3.5.7 Radical Scavenging Activity (DPPH Assay)

Lippia nodiflora extracts of different concentrations (2000, 4000, 6000, 8000 and 10000 µg/mL) were used to test the antioxidant activity. 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) and ascorbic acid that were used was obtained from R&M Marketing (Essex, UK). Stock solutions of DPPH were prepared in methanol, and methanol buffered with acetic acid buffer (0.10 M, pH 5.5), respectively. Buffered methanol was prepared by mixing 40 mL of 0.10 M acetate buffer (pH 5.5) with 60 mL methanol. The solvents and other chemicals used were of analytical grade. The reaction tubes, in triplicates, were wrapped in aluminium foil and kept at 30°C for 30 minutes in dark. All measurements were done under dim light. Spectrophotometric measurements were done at 517 nm using spectrophotometer. Antioxidant level was calculated using the following formula:

$$\text{DPPH free radical scavenging (\%)} = 1 - \frac{\text{(Absorbance of sample)}}{\text{(Absorbance of control)}} \times 100$$

These values were plotted against concentration of samples to obtain amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (IC_{50}). The kinetics of the antioxidant reaction in the presence of *Lippia nodiflora* extracts was compared with vitamin C (ascorbic acid) as antioxidant reference. All the assays were performed in triplicate.

3.6 HCT 116 Cell Line Anticancer Study

3.6.1 Media Preparation

Basic Media Preparation

McCoy's 5A media was used for HCT116 colon cancer cells maintenance. Firstly, the distilled water was filled into 1 L beaker and 10.40 g of the media powder was poured in. 2 g of sodium bicarbonate ($NaHCO_3$) and 0.5206 g HEPES was weighed and added into mixture. The mixture then was adjusted to pH 7.4 with HCl and the mixture was filtered into sterile bottle using sterile swinnex.

Supplemented 10% Media

The basic media was used to make the media supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, United States). Fresh made basic media was then used to be added with 10% FBS. If the basic media was refrigerated, it was thawed to 37ºC along with FBS, Penicillin/ Streptomycin (Sigma, United States) and Amphotericin B (Sigma, United States). 90 mL of basic media was transferred into a sterile beaker and 10 mL of FBS was also added along with 2 mL of Penicillin/ Streptomycin and 1 mL of Amphotericin B. Then the mixture was filtered into sterile bottle using sterile millipore filter, 0.22 µm (Whatman).

Supplemented 20% Media

Fresh made supplemented 10% media was used along with thawed FBS. 50 mL of supplemented 10% media was added with 5 mL of FBS into a sterile beaker. If the media and FBS was refrigerated, it was thawed to 37ºC. Then the mixture was filtered into sterile bottle using sterile millipore filter, 0.22 µm and labelled.

3.6.2 Reviving Cell and Cell Inoculation

Supplemented 20% media was thawed to 37ºC and was filled 1 mL into centrifuge tube. HCT116 cryovial was took out from -80ºC and placed in the ice. The cryovial was gently swirled in 37ºC water bath and the cell collected in the cryovial was transferred into the centrifuge tube with media. The centrifuge tube was centrifuged at 1000 rpm for 5 minutes and after that, the supernatant collected was discarded. 1 mL of the supplemented 20% media was then added into the cell pellet and mixed thoroughly. Another 9 mL of the supplemented 20% media was transferred into 75 cm^2 culture flask before the HCT116 cell obtained was transferred into the same culture flask.

3.6.3 Subculturing Cell Line

Subculturing the cell line was done in order to propagate so that the assay can be done on a few replicates. Firstly, the old media was discarded and 10 mL of Phosphate Buffer Saline (PBS) (Sigma, United States) was added into the flask for washing the previous media thoroughly and later it was discarded. Then, 3 to 5 mL of accutase (Sigma, United States) was filled into the flask and incubated in carbon dioxide (CO_2) incubator for 5 to 10 minutes. After it was took out, it was gently tapped to ensure the detachment of the cells and then was transferred into centrifuge tube. It was centrifuged at 1000 rpm for 5 minutes. 10 mL of supplemented 10% media was transferred into two new culture flask and one current flask each respectively. After centrifuged, the supernatant was slowly and carefully discarded and another 3 mL of media added into the tube and mixed thoroughly. The cell suspension was distributed 1 mL into each flask used. The first step of subculturing cell line was used to change the media if the cell was around 30- 50% confluent and the media was turning orange. After the old media and PBS were discarded, the media was transferred so that to provide sufficient nutrients for the cells.

3.6.4 MTT Cell Proliferation Assay

Day 1: The Plating of Cells

A fully confluent culture was observed to be done the assay. Cell detachment was performed using accutase. 1 mL of supplemented 10% media was added into pellet containing the detached cells and it was mixed well. 90 µL of tryphan blue was added and mixed well along with 10 µL cell suspension. Then, 20 µL of the obtained mixture was dropped on haemocytometer and was calculated its count cells using $M_1V_{1=}M_2V_{2.}$ After the calculation, the supplemented 10% media (V_2) was added into a sterile beaker with cell suspension (V_1). 200 µL of the cell suspension mixture obtained was transferred into each wells of the 96 well plates (Figure 3.5). Lastly it was incubated in the CO_2 incubator.

Day 2: Treatment on HCT116 Cells

Fresh and dried extracts of *L. nodiflora* went through the rotary evaporator (IKA) and the crystallized extracts were obtained. 0.02 g of the crystallized extracts of each samples were used in the producing the extract stock for HCT116 cells treatments.

DA: Supplemented 10% media + DMSO

DB: Supplemented 10% media

Extract Stock: Crude extract (0.02 g) + DB

Substock: 2 µL extract stock + 998 µL DB

Serial dilution was done with adding 150 µL of DA into well B to G while 300 µL of substock was added into well A. Then, 150 µL of mixture in well A was aspirated till well G using multichannel micropipette. The last 150 µL was discarded. The seeded plate from day 1 was taken from incubator then the old media was decanted. 100 µL of supplemented

10% media was then transferred into each well. All the well from A to G was transferred accordingly 100 µL from the substock to the seeded plate into each well and 100 µL of DB was transferred into well H as control. The treated 96 well plate was then incubated in the CO_2 incubator.

Day 3: MTT Assay

Preparation of MTT had been done in dark room and any apparatus used was covered using aluminium foil. Firstly, 0.005 g formazan dyes (reduction of tetrazolium salt) MTT was added into 10 mL PBS in centrifuge tube and mixed well. The solution was filtered into reagent reservoir. 20 µL of MTT reagent was added to each well including the controls and was kept incubated for 2 to 4 hours. After 4 hours, the plate was taken out and the old media was discarded. 100 µL of dimethyl sulfoxide (DMSO) (Sigma, United States) was added into all well and left shaken for about 15 minutes. The plate was measured on 570 nm and 690 nm and the results was recorded.

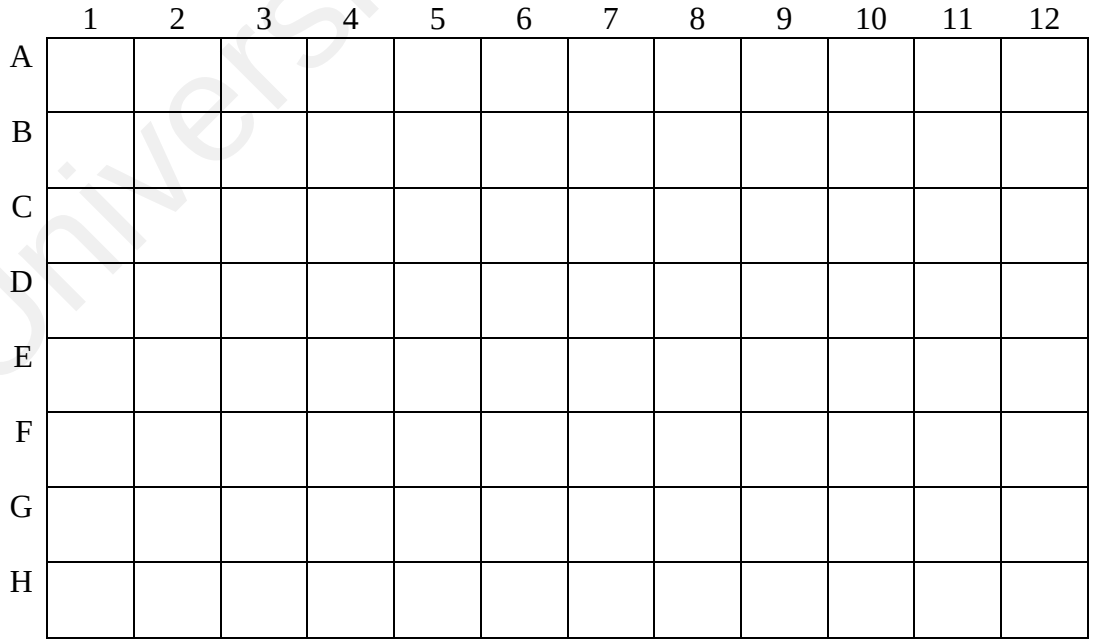


Figure 3.5: 96 well plate A to H arrangement for MTT Assay.

The absorbance reading of the blank must be subtracted from all samples. Absorbance readings from test samples must then be divided by those of the control and multiplied by 100 to give percentage of inhibition (POI) (as shown in formula below). Absorbance values greater than the control indicate cell proliferation, while lower values suggest cell death or inhibition of proliferation. The half maximal inhibitory concentration (IC_{50}) was determined from the graph of samples concentration versus POI.

$$\text{POI:} \quad \frac{\text{(Absorbance of control – Absorbance of sample)}}{\text{Absorbance of control}} \times 100$$

3.7 Statistical Analysis

All the experiments were repeated three times and the data pertaining for all study was subjected to mean separation and standard errors were carried out. Analysis of variance (ANOVA) was performed to test if there were significant differences in callus induction, plant regeneration and root induction among the samples tested on MS media supplemented with single and combination PGRs, using Minitab statistical computer software version 17.0 (Minitab Inc., Pennsylvania, USA). Means were separated using Tukey's Honest Significant Difference test at a confidence level of 95% ($p \leq 0.05$). Correlation analysis was analyzed using SPSS Version 21.0 (IBM, USA) at a confidence level of 95% ($p \leq 0.05$) and 99% ($p \leq 0.01$).

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

This chapter includes discussion on the results based on four sub-studies done throughout this research work. First, the macro morphology and micromorphology of *Lippia nodiflora* will be discussed. Secondly, the tissue culture studies of callus induction and micropropagation will be discussed. Thirdly, the methanolic extraction of *L. nodiflora* and its major compounds were analysed by GC-MS analysis and tested for antimicrobial and antioxidant activities. The present research also analysed stigmasterol and β-sitosterol compounds, using HPLC and HPTLC instruments. Lastly, the crude extracts of the plant are examined for its anticancer activities using MTT Assay on HCT116 Colon cancer cells.

4.2 *Lippia nodiflora* Identification

Lippia nodiflora had been identified by Dr. Sugumaran A/L Manickam from the University of Malaya herbarium (KLU). The herbarium voucher specimen (KLU47924) was deposited at University of Malaya Herbarium.

The collected plant and the plant maintained in greenhouse, IBS Garden exhibited different sizes. It was shown that the environmental condition; affect the plant growth even in the same climate condition. The soil condition at Taman Rimba Ampang was a mixture of peat soil and sand with a running river on the side. The collected plant was maintained in several pots filled with peat soil complemented with NPK fertilizer in a greenhouse. The soil condition and moisture of the environment based on water retention might be the factors affecting the plant development.

4.3 Morphology of *Lippia nodiflora*

4.3.1 Macro-morphology of *Lippia nodiflora*

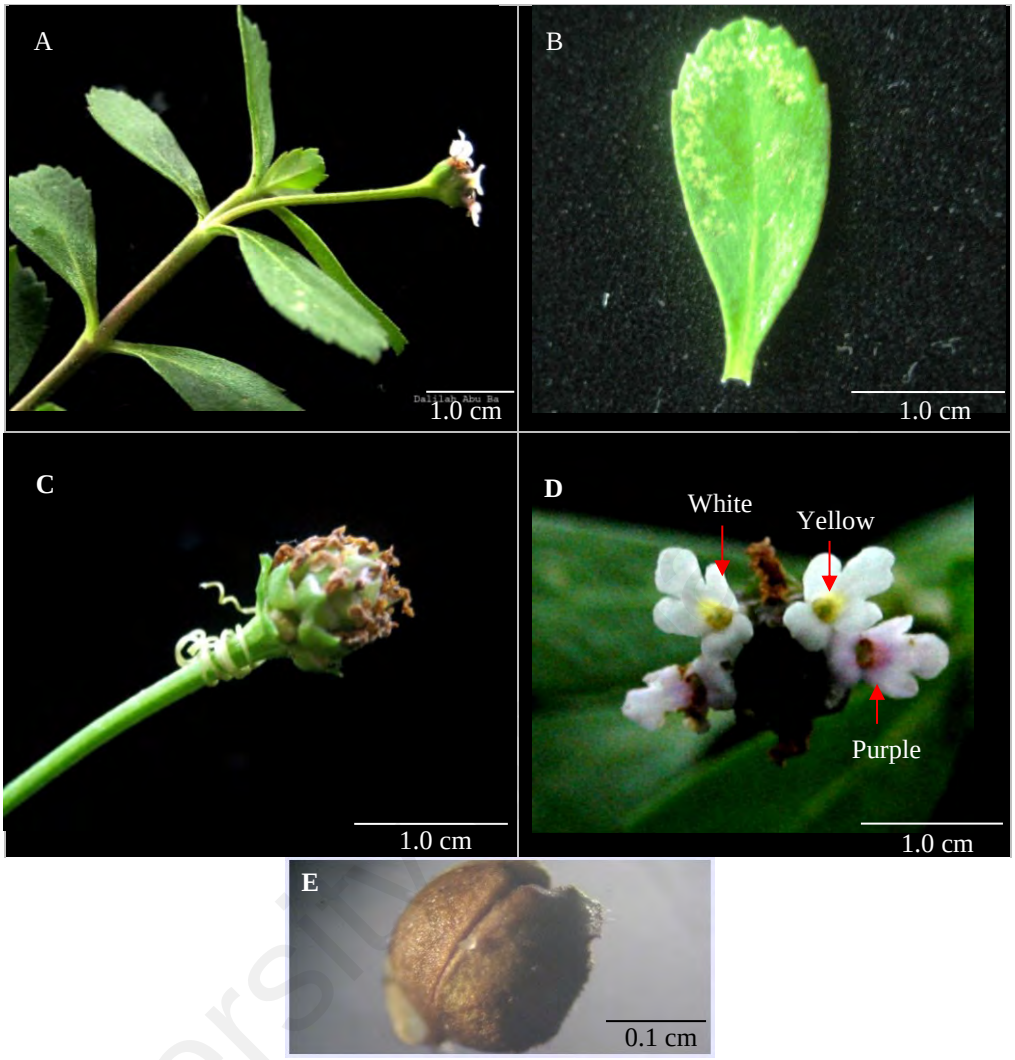


Figure 4.1: *Lippia nodiflora* plant A. The leaf is in opposite arrangement B. Obovate shaped leaf C. Oblong shaped inflorescence D. The petal outer color is white while the inner color is either yellow or purple E. Capsule.

L. nodiflora has opposite leaf arrangement as shown in Figure 4.1A. The plant features slender branches, nodal roots, leaves are numerous and nearly without stalk. *L. nodiflora* is a creeping plant and because of this, it can be used as a replacement for ground covering grass. *L. nodiflora* is easily propagated from seeds and due to its property of sending out roots that shoots from the nodes (Dole, 2003). Figure 4.1B shows the leaf shape which is obovate and dentate on upper one third of the margin. The leaf of *L. nodiflora* may have three to seven teeth on each margin above the middle part, contradictory to *Phyla cuneifolia* which has one to two teeth while *Phyla lanceolata* has five to 11 teeth with

some extending to below margin (Robert, 2010). The leaf of *L. nodiflora* is thicker and smaller (Figure 4.1B) in Taman Rimba, Ampang which the size was 16 mm (10 to 25 mm) compared to the plants that was maintained in the greenhouse where the size was about 20 to 35 mm in length. Leaf size and thickness differed as the soil condition and nutrients varies. The morphology of *L. nodiflora* is almost similar to *Phyla lanceolata* but *L. nodiflora* has narrower leaf and it is wider in the middle part (Clarke, 2007). *L. nodiflora* has oblong spike and the collected *L. nodiflora* spike ranges from 5 to 10 mm long (Figure 4.1C) while Sujanapal and Sankaran (2016) recorded the size of 10 to 25 mm long and about 6 to 9 mm in diameter. The flower of *L. nodiflora* was shown in Figure 4.1D. The flower has four fused petals and its outer color is white while the inner color is either yellow or purple, with calyx that is membranous and bilobed. Corolla consists of a slender and cylindric tube, about 3 mm long, with a limb 2.50 mm wide, opening at the apex as it lengthens. Almost all flowers in Verbenaceae family are hermaphroditic which have both functional stamens and stigma, however insect pollination still can occur as a result of its nectar production, flower color and scent (Kadereit & Kubitzki, 2004).

Figure 4.1E showed the seed of *L. nodiflora*. The calyx or sepal is flattened, it is shorter than the bracts, hyaline-membranous layer, and deeply dissected with lanceolate lobes. Figure 4.1F showed the seed formation in which it is ovate- shaped, separating at maturity into two with one-seeded pyrenes (Roy & Shukla, 1992).

4.3.2 Micromorphoology of *Lippia nodiflora*

4.3.2.1 Field Emission Scanning Electron Microscope of Leaf Surface of *Lippia nodiflora*

The micromorphology of the leaf surface of *Lippia nodiflora* is presented in Figure 4.2-4.4. Firstly, the plant surfaces that was observed on *L. nodiflora* showed epidermal cells with convex shape and covered with cuticular folds. The existence of epicuticular wax crystals or cuticular foldings on plant surfaces may influence the reduction of insect attachment (Prüm et al., 2012). Secondly, *L. nodiflora* leaves are amphistomatous where the stomata are present on adaxial and abaxial sides of leaf surfaces. It might be an adaptive measure of the plant to the environmental condition of tropical and subtropical countries. The leaves are characterized by diacytic stomata which are abundant on both adaxial and abaxial surfaces (Figure 4.2A and 4.2B). Diacytic stomata consist of a single ring of two cells enclosing the guard cells at right angles to the long axis of the guard cells.

The leaves of *L. nodiflora* possess simple unicellular trichomes which are densely distributed on the entire leaf, but denser on abaxial than the adaxial surface (Figure 4.3A and 4.3B). Within the abaxial surface, the trichomes are found more on the midrib compared to the edge of the leaf. The trichomes have rough surface and vary in length as shown in Figure 4.3C (175.4μm, 222.6μm and 376.7μm) and it is attached to the epidermal at the center of the trichomes (Figure 4.3D). The trichomes development from the epidermis is usually resulted from the degree of difference enlargement and subsequent divisions of the epidermal cells and their derivatives (Carlquist, 1958). The function of having abundance distribution of trichomes on the surface of the leaves is as a mechanism of defence against foraging insects and airborne propagules of fungi (Pendota et al., 2008) as well as to prevent water loss by trapping air over the stomata especially on abaxial surface (Naidoo et al., 2009). The correlation between trichomes

density over insect resistance were positively demonstrated in various plant species (Levin, 1973).

L. nodiflora was found to have essential oil (Shukla et al., 2009) in which oil glands were spotted throughout the leaf surfaces (Figure 4.4). The abaxial surface view shows the pore of an oil gland where the fragrance was emitted. The surfaces show different oil gland characteristics, adaxial surface shows smooth exterior (Figure 4.4A) while abaxial surface shows shrivel exterior with an opening (Figure 4.4B). Loughrim and Kasperbauer (2003) reported a distint aroma and flavor from herb leaf, where usually, the main source of aroma is usually the glandular epidermis of the petals, but the fragrant compounds can be emitted by stamens, nectaries and many other glands as well (Roshchina & Roshchina, 2012).

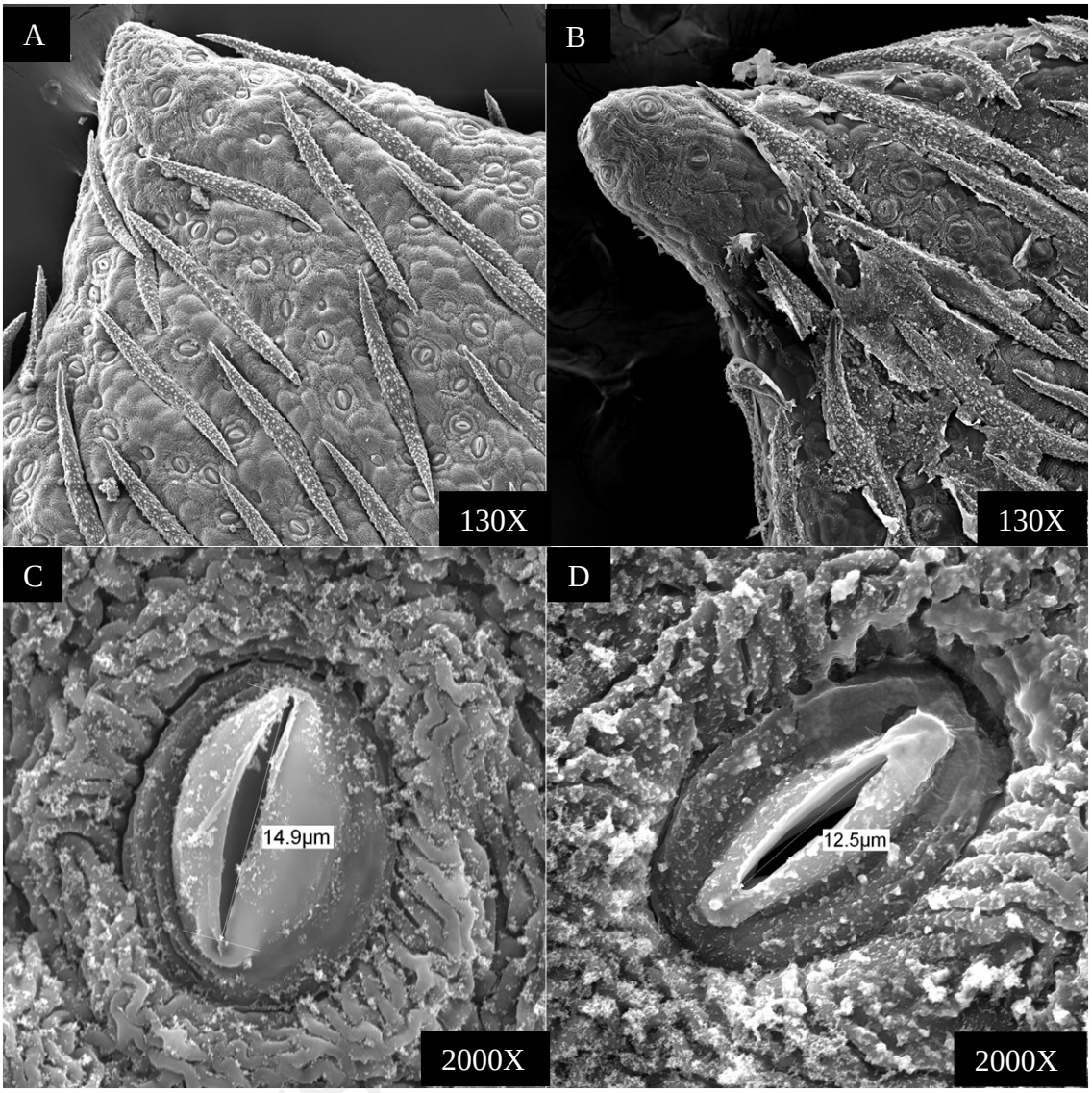


Figure 4.2: Stomata distribution in *Lippia nodiflora* A. Adaxial surface B. Abaxial surface C & D diacytic stomata from adaxial and abaxial surface, respectively.

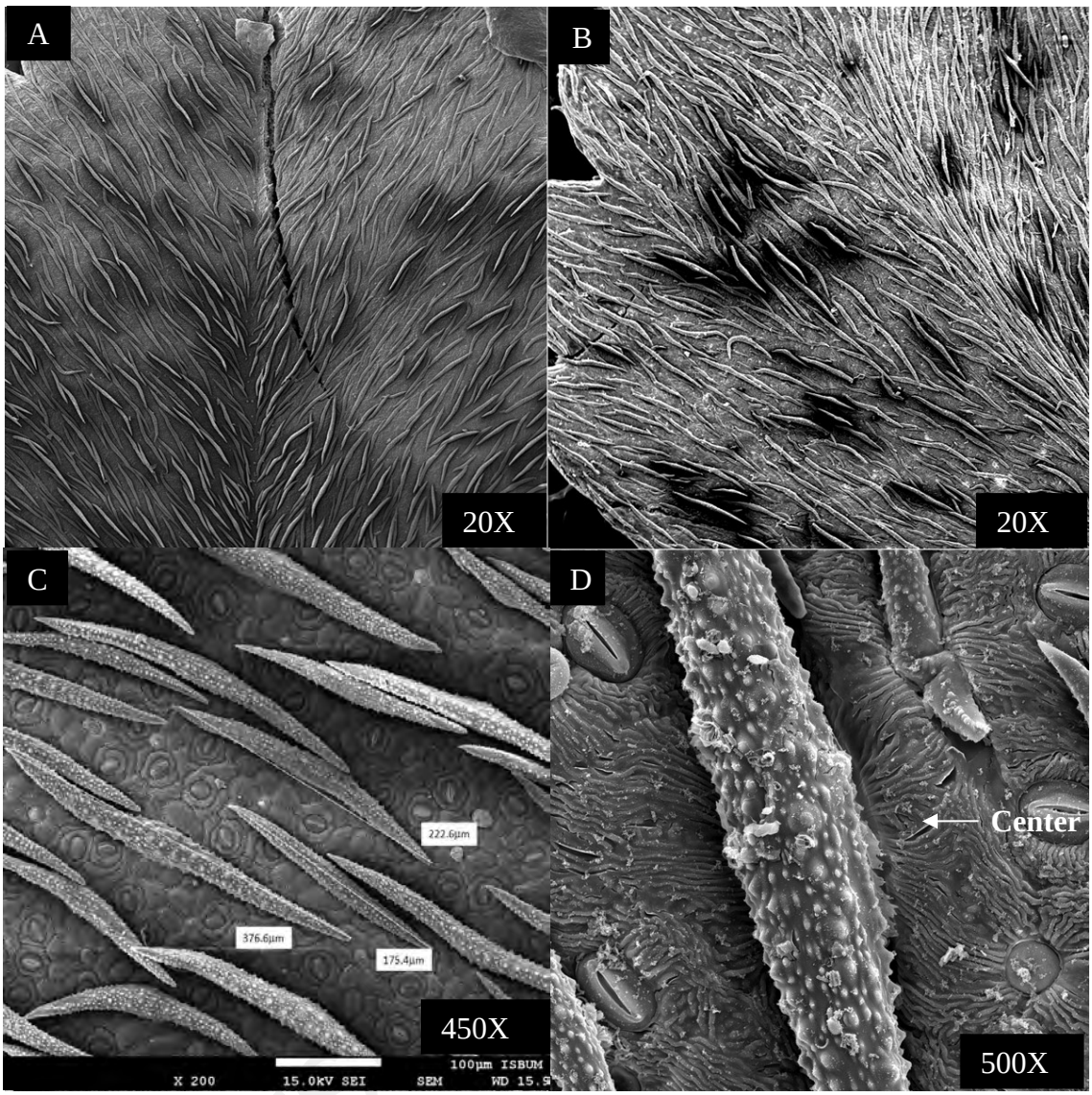


Figure 4.3: Micromorphology of simple unicellular trichomes A. Adaxial surface B. Abaxial surface C. Simple unicellular trichomes D. Trichomes showing the middle part attached to the epidermal surface.

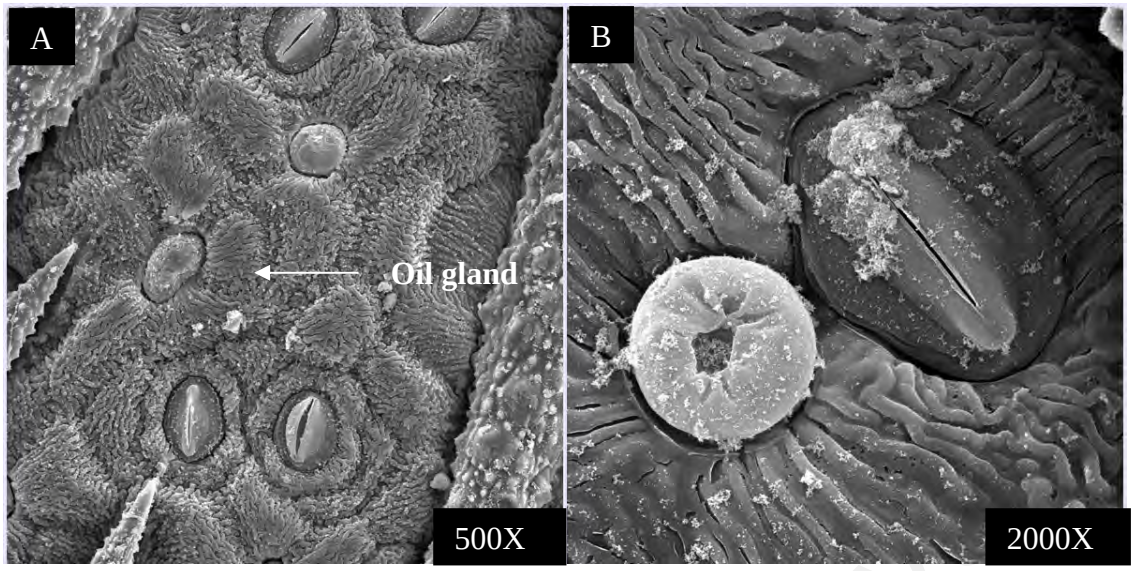


Figure 4.4: The oil gland on epidermis A. Oil gland structure from adaxial surface B. Oil gland near stomata on abaxial surface

4.3.2.2 Leaf Epidermal Peel

A clear diacytic stomata can be seen under light microscope by staining using leaf epidermal peel method. Based on the test and FESEM, stomata were present on both abaxial and adaxial surfaces of the leaf of *L. nodiflora*. FESEM gave an actual image under 100 to 2000 times magnification (Figure 4.5). In this Figure, a guard cell with two subsidiary cells surrounding it can be seen clearly and the guard cell size can be estimated. Paracytic stomata also give two or more subsidiary cells surrounding it but parallel from the stomata while subsidiary cells on diacytic stomata is at the right angle of the stomata. Most of the guard cells sizes obtained are 130.00 µm average in length during the opening of the guard cell, which compared from FESEM, it was an average of 13.6 µm. This might be due to the efficiency of the microscope. An oil gland can also be seen in this figure, showing the oil gland surrounded by six subsidiary cells. The arrangement of stomata and oil gland of *L. nodiflora* was close to each other and abundant along the surface on adaxial and abaxial surfaces.

Stomata index obtained from data collection and calculation was 19.52% from abaxial surface and 19.86% on adaxial surface of the leaf. There were a small amount of different

in the stomata index where it can be suggested that the abundance of stomata was equivalent on both leaf surfaces.

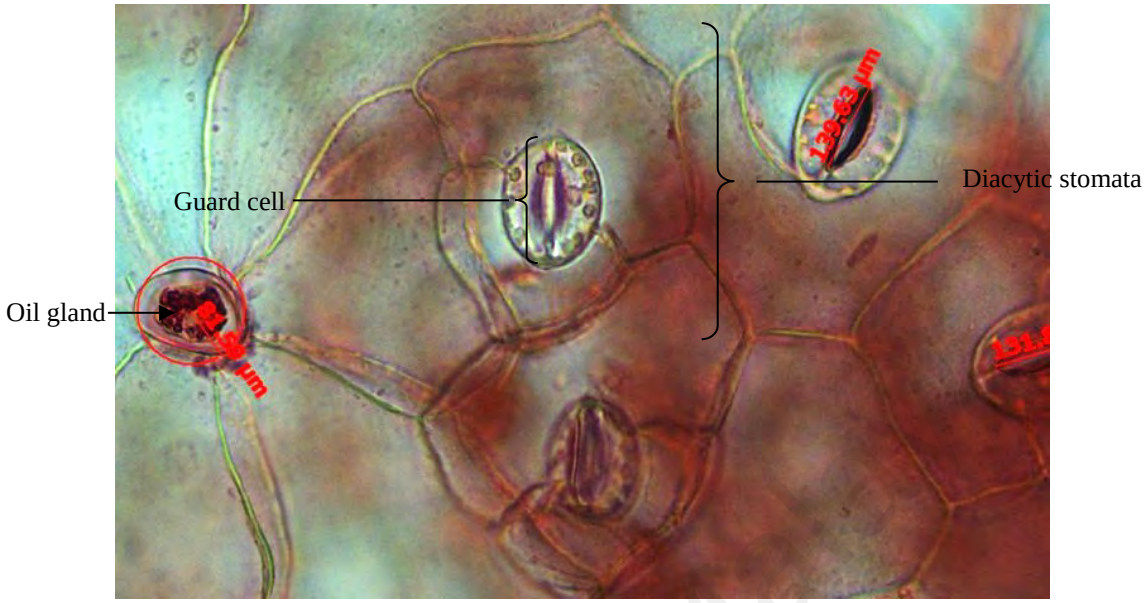


Figure 4.5: Diacytic stomata under 100x magnification

4.4 Tissue Culture Studies

4.4.1 Surface Sterilization of *Lippia nodiflora*

L. nodiflora sterilization protocol was optimized with minor modifications from Ahmed et al. (2005). Two to three different sets of adjustment were done due to high contamination rate of 30- 40%. Similar chemicals were used to sterilize the intact plants with varying sterilising duration but the duration of time was tried and adjusted up until 80- 90% successful rate of callus induction and about 10% of contamination only. The source of come from the contamination may be used of unsterilized apparatus or human-error.

4.4.2 Callus Induction of *Lippia nodiflora*

Lippia nodiflora plant was tested with different auxin and cytokinin concentrations and in combinations. The most common callus obtained was green compact callus, followed by light green compact callus and a few white colored callus. Explants from both leaf and stem showed high percentage of response as recorded on the 21st day of

inoculation, however, stem explants gave higher biomass and more compact callus. Table 4.1 shows nature of callus on *L. nodiflora* plant. As shown, it varied on the media supplemented with plant growth regulators NAA and 2,4-D, while in the presence of other PGRs, the callus was mainly green compact callus. Ahmed et al. (2011a, 2011b), reported the success of obtaining white watery callus, white friable callus, brown friable callus, green friable callus and green compact callus and also able to test the presence of somatic embryogenic callus. Figure 4.6 shows the callus that was obtained. Many varieties of callus can be seen on a single PGRs such as NAA and 2,4-D (Figure 4.6A- C). Other than these two PGRs, IAA, IBA, BA and KN and their combinations all gave green compact callus. The texture of the callus was rough and hard as shown in Figure 4.6D and E. Browning effect can occur as early as the second week to some friable callus. For instance, Figure 4.6F shows the third week of callus that was induced on MS media supplemented with 4.00 mg/L 2,4-D.

Table 4.1: *Lippia nodiflora* callus nature for single and combination plant growth regulators (PGRs)

PGRs	**Nature**
Single	
NAA	Light green friable callus Light green compact callus Whitish greenish compact callus
2,4-D	Light green friable callus Light green compact callus Whitish greenish compact callus
IAA	Green compact callus
IBA	
BA	
KN	
Combination	
NAA + BA	Green compact callus
NAA + KN	
2,4-D + BA	
2,4-D + KN	
IAA + BA	
IAA + KN	
IBA + BA	
IBA + KN	

Most callus can be classified into two categories namely friable and compact callus. The cells of compact callus are densely aggregated, whereas the cells of friable callus are loosely associated with each other. The friable callus are also soft and easily disintergrated. The friability of a callus can sometimes be improved by manipulating the media components or by subculturing (Slater & Wand-Fowler, 2003). Gürel et al. (2001) described two types of callus occurrence, the first type occurs regardless of the presence of auxins, green and compact callus will be produced in any medium containing high concentrations of BA (1.00 mg/L) alone or in combinations with auxins while secondly, whitish and friable callus normally be initiated through media containing 0.50 mg/L BA or 1.00 mg/L KN for various beets explants. In this study, starting from IAA and IBA for auxin, BA and KN for cytokinin, to any combination of auxin and cytokinin exhibits green compact callus. However, previous studies have suggested that the organogenic potential is related to callus structure. Large cells of white and friable callus produce more roots and shoots (Saunders & Daub, 1984; Konwar & Coutts, 1990; Shimamoto et al., 1993), while small cells of green and compact callus has less organogenic capacity (Ritchie et al., 1989; Tetu et al., 1987).

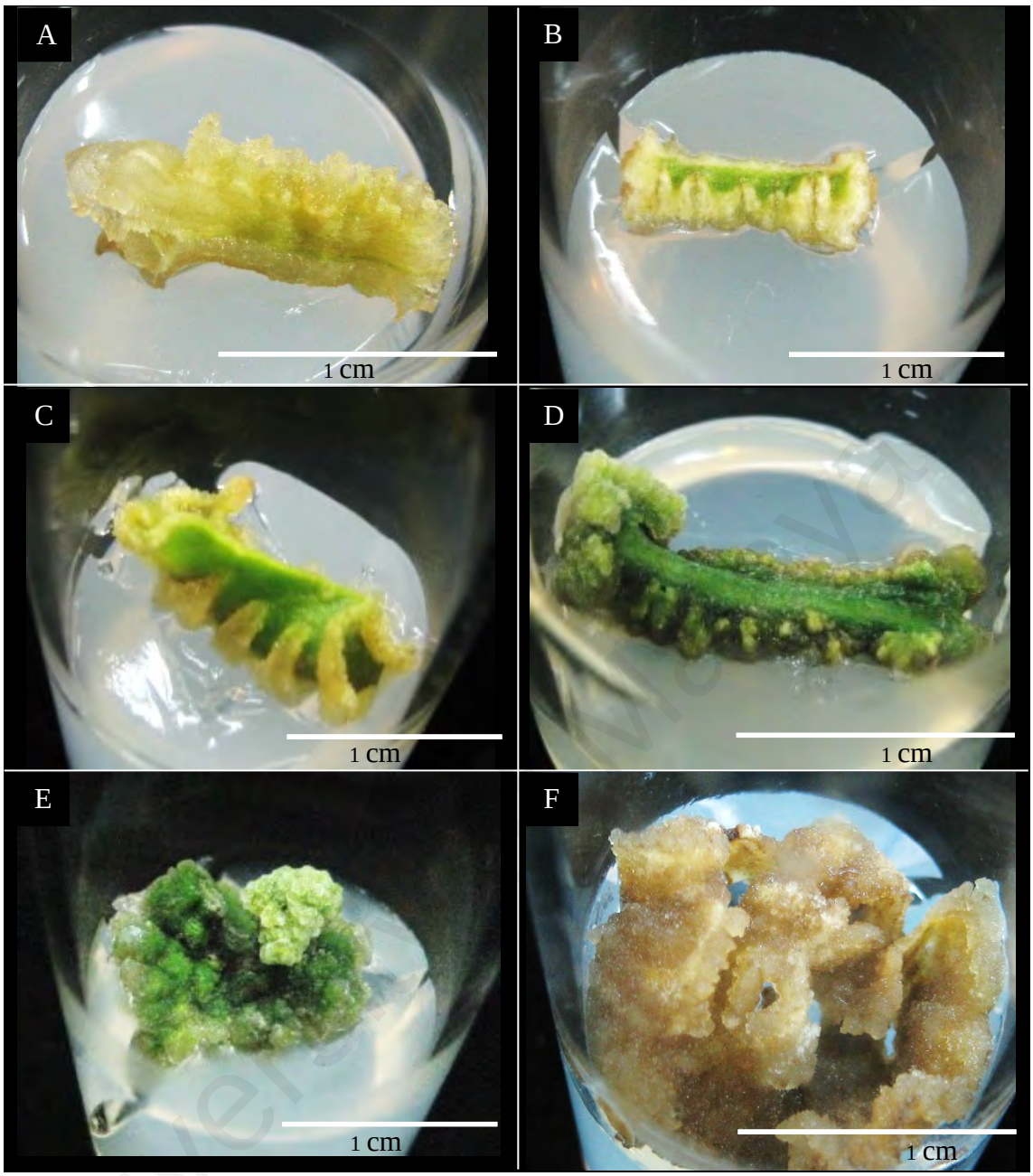


Figure 4.6: The nature of callus obtained induced from *Lippia nodiflora* on MS media supplemented with different plant growth regulators A. Light green friable callus - 1.00 mg/L NAA (2nd week) B. Milky greenish compact callus - 4.00 mg/L NAA (2nd week) C. Light green compact callus - 2.00 mg/L NAA (3rd week) D. Green compact callus - 3.00 mg/L IAA (3rd week) E. Green compact callus - 2.50 mg/L IBA + 1.00 mg/L BA (3rd week) F. Browning effect on callus supplemented with 4.00 mg/L 2,4-D at 4th week.

According to Ahmed et al. (2011b), stem explants was able to obtain higher frequency of callus induction compared to shoot tips explants by 92% to 83% respectively. Similarly, stem explants induced higher percentage of callus compared to leaf explants as shown in Table 4.2 and 4.3 by 92.50% and 90% for single PGRs while 89.20% and 88.50% for combination of PGRs respectively. Also, shown in Table 4.2, MS media

without PGRs, which acted as a control, was not able to produce callus, instead only shoot regeneration has been observed.

Table 4.2: Frequency of callus induction (%) for single PGRs.

Plant Growth Regulators (PGRs)						Frequency of Callus Induction (%)	
Auxin				Cytokinin		Leaf	Stem
NAA	2,4-D	IAA	IBA	BA	KN		
-	-	-	-	-	-	0.00±0.00^{h}	0.00±0.00^{d}
0.50						80.00±0.58^{fg}	85.00±0.58^{abc}
1.00						80.76±0.23^{efg}	85.00±0.58^{abc}
1.50						85.00±0.58^{bcdefg}	88.33±0.92^{abc}
2.00						87.50±0.87^{abc}	86.67±0.83^{abc}
2.50						92.50±1.00^{a}	85.83±0.49^{abc}
3.00						85.83±0.68^{bcdef}	87.50±0.86^{abc}
3.50						84.17±1.29^{bcdefg}	86.73±1.85^{abc}
4.00						79.23±1.03^{g}	85.83±0.69^{abc}
	0.50					86.67±1.84^{abcde}	85.00±0.98^{abc}
	1.00					84.17±1.28^{bcdefg}	87.50±1.44^{abc}
	1.50					81.67±0.83^{cdefg}	87.50±0.87^{abc}
	2.00					85.00±0.98^{bcdefg}	85.83±0.68^{abc}
	2.50					83.33±1.23^{bcdefg}	88.33±0.92^{abc}
	3.00					84.17±0.88^{bcdefg}	85.83±0.83^{abc}
	3.50					84.17±1.29^{bcdefg}	88.33±0.92^{abc}
	4.00					85.83±0.49^{bcdef}	88.33±0.92^{abc}
		0.50				80.00±0.58^{fg}	83.33±1.23^{abc}
		1.00				85.79±0.69^{bcdef}	87.50±0.87^{abc}
		1.50				84.17±0.88^{bcdefg}	85.83±0.49^{abc}
		2.00				85.00±0.98^{bcdefg}	89.17±1.29^{b}
		2.50				81.67±0.83^{cdefg}	88.33±0.92^{abc}
		3.00				84.17±1.29^{bcdefg}	83.33±1.93^{abc}
		3.50				87.50±1.44^{abc}	89.23±1.29^{b}
		4.00				82.50±1.44^{cdefg}	85.83±0.49^{abc}
			0.50			86.73±1.12^{abcd}	83.33±0.92^{bc}
			1.00			82.50±1.44^{cdefg}	89.23±1.29^{b}
			1.50			81.67±0.95^{cdefg}	84.17±0.88^{abc}
			2.00			83.33±1.93^{bcdefg}	82.50±1.44^{c}
			2.50			86.73±0.55^{abcd}	88.33±0.92^{abc}
			3.00			81.67±0.83^{cdefg}	85.00±0.98^{abc}
			3.50			85.00±0.58^{bcdefg}	83.33±1.93^{bc}
			4.00			89.17±0.43^{ab}	87.50±1.44^{abc}
				1.00		81.67±1.39^{cdefg}	87.50±1.44^{abc}
				2.00		84.17±0.88^{bcdefg}	90.00±1.15^{a}
				3.00		84.17±0.88^{bcdefg}	87.50±1.44^{abc}
					1.00	82.50±1.44^{cdefg}	85.00±0.58^{abc}
					2.00	81.67±0.95^{cdefg}	84.17±0.88^{abc}
					3.00	80.83±1.01^{defg}	85.00±0.58^{abc}

Table 4.3: Frequency of callus induction (%) for combination PGRs.

No.	Plant Growth Regulators (PGRs) (mg/L)						Frequency of Callus Induction (%)	
	Auxin				Cytokinin		Leaf	Stem
	NAA	2,4-D	IAA	IBA	BA	KN		
1	2.00				1.00		80.80±0.40^{abcdef}	87.50±1.44^{abc}
2	2.50				1.00		76.30±2.76^{ef}	87.50±0.50^{abc}
3	3.00				1.00		81.70 ±2.11^{abcdef}	85.77± 1.27^{abc}
4	2.00				2.00		75.00±1.44^{f}	80.00±1.15^{c}
5	2.50				2.00		80.00±0.58^{abcdef}	87.50±0.87^{abc}
6	3.00				2.00		76.30±1.40^{ef}	89.20±0.22^{a}
7		2.00			1.00		77.50±1.44^{cdef}	80.00±0.58^{c}
8		2.50			1.00		85.00±0.20^{abc}	87.50±0.50^{abc}
9		3.00			1.00		80.00±0.58^{abcdef}	87.50±1.44^{abc}
10		2.00			2.00		80.00±2.52^{abcdef}	88.33±2.20^{ab}
11		2.50			2.00		75.00±1.61^{f}	80.00±1.15^{c}
12		3.00			2.00		83.80±0.60^{abcde}	80.00±0.58^{c}
13			2.00		1.00		80.00±1.44^{abcdef}	88.33±2.20^{ab}
14			2.50		1.00		76.30±1.88^{ef}	88.30±0.80^{ab}
15			3.00		1.00		79.20±0.85^{bcdef}	80.80±0.80^{bc}
16			2.00		2.00		80.00±0.58^{abcdef}	87.50±1.44^{abc}
17			2.50		2.00		80.00±1.44^{abcdef}	87.50±0.29^{abc}
18			3.00		2.00		80.00±1.84^{abcdef}	88.30±1.50^{ab}
19				2.00	1.00		77.50±1.29^{cdef}	87.50±0.50^{abc}
20				2.50	1.00		85.00±0.58^{abc}	89.20±0.80^{a}
21				3.00	1.00		77.50±0.60^{cdef}	85.77±1.27^{abc}
22				2.00	2.00		83.27±1.63^{abcde}	85.83±1.29^{abc}
23				2.50	2.00		85.0±0.88^{abcd}	88.33±2.20^{ab}
24				3.00	2.00		76.70±0.90^{def}	88.33±2.20^{ab}
25	2.00					1.00	83.30±1.68^{abcde}	89.20±0.75^{a}
26	2.50					1.00	82.50±0.40^{abcdef}	85.00±2.50^{abc}
27	3.00					1.00	86.70±0.85^{ab}	80.00±1.53^{c}
28	2.00					2.00	83.30±0.69^{abcde}	86.70±2.17^{abc}
29	2.50					2.00	78.30±1.94^{cdef}	86.67±1.09^{abc}
30	3.00					2.00	84.20±1.91^{abcde}	89.20±1.29^{a}
31		2.00				1.00	79.20±0.85^{bcdef}	87.50±1.44^{abc}
32		2.50				1.00	87.50±0.63^{a}	82.50±1.33^{abc}
33		3.00				1.00	80.00±0.58^{abcdef}	86.70±2.17^{abc}
34		2.00				2.00	77.50±1.73^{cdef}	80.00±1.53^{c}
35		2.50				2.00	79.20±0.85^{bcdef}	80.00±0.58^{c}
36		3.00				2.00	83.30±0.69^{abcde}	80.00±0.58^{c}
37			2.00			1.00	80.00±1.00^{abcdef}	88.50±1.60^{ab}
38			2.50			1.00	85.00±2.11^{abc}	87.50±0.64^{abc}
39			3.00			1.00	81.70±1.22^{abcdef}	86.70±2.17^{abc}
40			2.00			2.00	80.00±0.58^{abcdef}	85.83±1.29^{abc}
41			2.50			2.00	82.50±1.33^{abcdef}	80.00±1.00^{c}
42			3.00			2.00	81.70±1.22^{abcdef}	85.80±1.32^{abc}
43				2.00		1.00	79.20±0.80^{bcdef}	80.00±0.58^{c}
44				2.50		1.00	79.20±0.85^{bcdef}	87.50±1.44^{abc}
45				3.00		1.00	78.30±2.75^{bcdef}	80.00±0.58^{c}
46				2.00		2.00	79.20±0.85^{bcdef}	87.50±0.29^{abc}
47				2.50		2.00	79.20± 2.23^{bcdef}	87.50±0.64^{abc}
48				3.00		2.00	78.30±2.86^{cdef}	85.77±1.27^{abc}

Stem explants also induced green compact callus, the characteristic was observed in nodular showing the cells are in big clumps that does not present on leaf explants. Callus induction explants were incised by similar length and width for leaf and length for stem however, the stem diameter should be constant too but there was slight differences between the stem explants collected from the greenhouse. This is due to the maturity of the plant collected, as mentioned earlier, *L. nodiflora* is a creeping plant so the continuity of the elongation of the plant not all can be observed. As the stem was acquired in the internodes, the apical meristems were used for shoot induction and usually left to propagate.

Based on Figure 4.7, even though some callus induced with MS media supplemented with auxin may gave leaf explants a better results, overall results showed that stem explants has higher biomass. Data was examined based on Figure 4.8- 4.11. On each leaf and stem explants that has been placed on PGRs supplemented media, they had length around 1.00 to 1.50 cm and were all injured around four to five times. Some variables were ensured to be constant so that the results obtained can be applicable. The function of giving injured to the explants was because callus can be formed from the results of wounding where a callus consists of an amorphous mass of loosely arranged of thin-walled parenchyma cells arising from the proliferating cells of the parent tissue (Dodds & Roberts, 1987). Figure 4.7 shows callus obtained from third week of inoculation, where there were huge differences between the nature of explants on different plant growth regulators and also leaf and stem explants biomass.

The MS media consistency also plays a huge role for nutrients transmission, usually the media will be put on pH 5.7 to 5.8 and the quantity of agar used was 7 g for 1 L of media instead of 8 g from the MS media guidelines. The reason is to make the agar softer, that the explants were able to consume the nutrients 100% before subculturing was done.

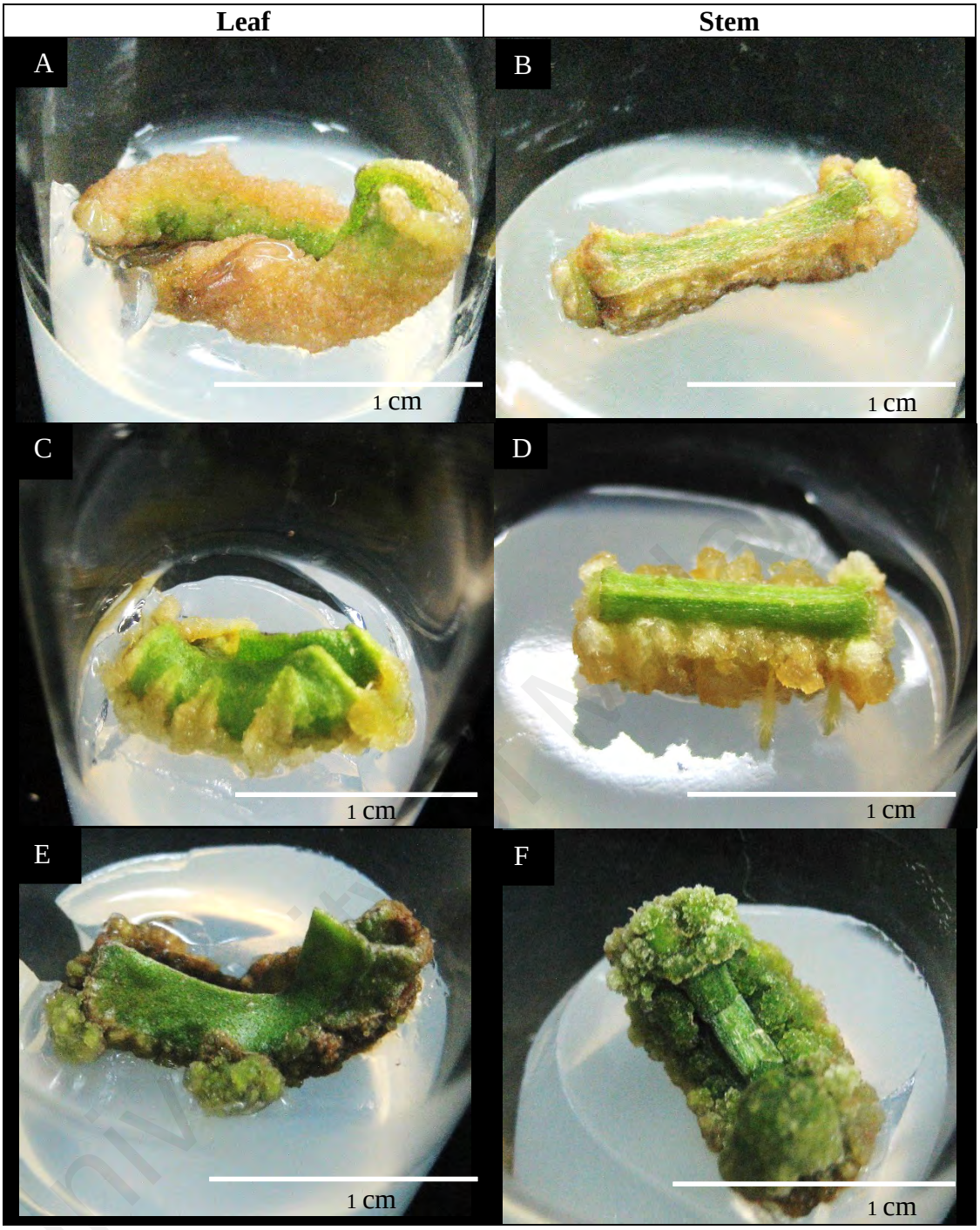
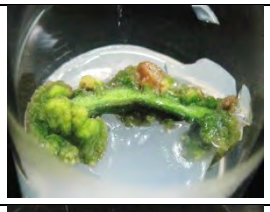


Figure 4.7: The differences of nature and biomass between leaf and stem explants of *Lippia nodiflora* on MS media supplemented with different plant growth regulators at the third week of induction A & B. Leaf and stem of 1.00 mg/L IAA C & D. Leaf and stem of 1.00 mg/L NAA E & F. Leaf and stem of 2.50 mg/L IBA+1.00 mg/L BA.

Ahmed et al. (2011b) recorded brown callus on *L. nodiflora* callus. Gupta et al. (2001) and JibinaBai et al. (2014) reported *Lippia alba* (Verbenaceae) induced brown callus in the second week of inoculation. Table 4.4 shows the browning effect that has occurred during the callus induction on *L. nodiflora* which was as early as second week onwards for single PGRs and third week onwards for combination PGRs. On the first week, the callus on stem explants that was seen on MS media supplemented with 2.00 mg/L NAA is light green friable callus however, when it reached the second week the callus has started to be colored in brown similarly reported by Hussain et al. (2013) on a different species.

Table 4.4: Browning effect on *L. nodiflora* callus induction.

Duration	**Single PGRs MS media + 2.00 mg/L NAA**	**Combination PGRs MS media + 2.00 mg/L IAA + 2.00 mg/L KN**	**Notes**
Week 1			Callus was induced.
Week 2			Browning of *L. nodiflora* callus started at the 2nd week can be seen clear on single PGRs.
Week 3			Browning of *L. nodiflora* callus started at the 3rd week but very low and not really visible on combination PGRs.
Week 4			Single PGRs browning effect has increased and biomass has started to decline.
Week 5			Combination PGRs browning effect has been more visible.

Oxidation of polyphenols and formation of quinones results in browning of explants tissue and tissue culture media are highly reactive and toxic to the tissues. The inhibitory effect may be resulted from the bonding of phenols with protein and their subsequent oxidations to the quinones (Raj Bhansali & Singh 1982; Jain 2000). Previous research was done to minimize browning effect such as pre-soaking explants in ascorbic acid and citric acid prior to the sterilization method (Murashige, 1974). In recent studies of Naz and Khatoon (2014), to overcome the problem of callus browning which was investigated on *Achyranthes aspera*, 0.20% charcoal was used on 2.00 mg/L 2,4-D but the problem was not solved and also higher sucrose level was tested but resulted in decrease of callus fresh weight as the sucrose concentration was increased from 3% to 6% and 9%.

In present study, the combination of auxin and cytokinin were examined to enhance biomass furthermore at the same time, it have found to decrease the browning effect that was occurring throughout the experiments on single PGRs especially on NAA and 2,4-D which can be seen on Table 4.4, combination PGRs with the example of 2.00 mg/L IAA + 2.00 mg/L KN. From the Table, we can differentiate the duration that had occurred and also the ratio of browning effect on single PGRs and combination PGRs. At week 4, the callus induced on 2.00 mg/L NAA has started to decline significantly though for 2.00 mg/L IAA + 2.00 mg/L KN, from week 5 onwards the declining phase slowly took place.

4.4.3 *In vitro* callus growth curve

The results showed callus induction was successful with all the PGRs treated. Data was analyzed in the third week from induction where the highest biomass and best nature (as shown in Figure 4.8- 4.11) obtained for single PGRs were 2.50 mg/L NAA (118.60 mg/L) with light green friable callus for auxins and next, 1.00 mg/L BA (42.70 mg/L) with green compact callus for cytokinin while as for combination PGRs were 3.00 mg/L NAA + 2.00 mg/L KN (165 mg/L) with green compact callus and 2.50 mg/L IBA + 1.00 mg/L BA (139.60 mg/L) with green compact callus.

All of the graphs on *in vitro* growth curve of *L. nodiflora* (including other single and combination PGRs that was not shown) were mostly sigmoid type and can be identified through four phases based on the duration of the callus fresh and dry weight that was collected and recorded (7, 14, 21 and 28 days). The *in vitro* growth curve graphs showed that the optimum biomass were observed at 3rd week for single PGRs and 3rd to 4th week for combination PGRs. Firstly, for single PGRs the lag phase can be identified at 7- 14 days, Leticia et al. (2010) reported that this is the phase where callus was induced and proliferated as observed by profound cell division. Secondly, the second phase which is the exponential phase was detected at 14- 18 days of inoculation while the stationary phase of the callus induction was at 18- 25 days. Even though it was exponentially increasing in biomass, the browning effect had occur early and causing early declined phase at the 25 days onwards. It took place due to the inability to replace new callus with the dead callus. As for combination PGRs, the lag phase was also identified at 7- 14 days with an exponential growth at about 14- 22 days. The stationary phase took place on the 22- 30 days of inoculation. On day 31 onwards, the declined phase had occur. However, the callus that was inoculated on MS media supplemented with combination PGRs had browning only on the third week but in small amount. Ahmed (2011b) was able to sustain the callus duration at 55 days which may be due to different environment such culture

condition, temperature, light intensity and others. The highest biomass results on the stationary phase were mass produced so that further analysis can be done through extraction to undergo TLC, GC- MS, HPTLC and HPLC analysis.

However, the maturity of the callus also plays a part to determine the presence of desired compounds as it may take longer duration to be produced nevertheless for it to be accumulated. It does not mean that the highest biomass is considered as the best time to be collected and performed as mentioned analysis previously but this investigation is required to examine the life span of the callus. So, the callus at third and fourth week were collected and subcultured into a new MS media supplemented with similar plant growth regulators. Then, the subcultured callus was examined with TLC analysis to check the presence of desired compounds and the process had gone towards four times. Mass production for all *L. nodiflora* callus was also done with the same method. The function of mass production of *L. nodiflora* callus was to increase yield to be collected to perform extraction so that the chances of gaining high amount of desired compounds would be increased as the concentration of extraction was also increased. Then, the high concentration of desired compounds inside the extraction leads to visible results obtained. However, the concentration of the extracts can be adjusted with the ratio used to either a more concentrated extracts or a more diluted extracts. As for GC- MS, a serial dilutions of *L. nodiflora* extracts were recommended as it is a very sensitive instrument and also the function is to identify the possible compounds. As for HPLC, a more concentrated extracts was used because of the identification, confirmation and quantification of the desired compounds.

Callus growth on MS media supplemented with single PGRs

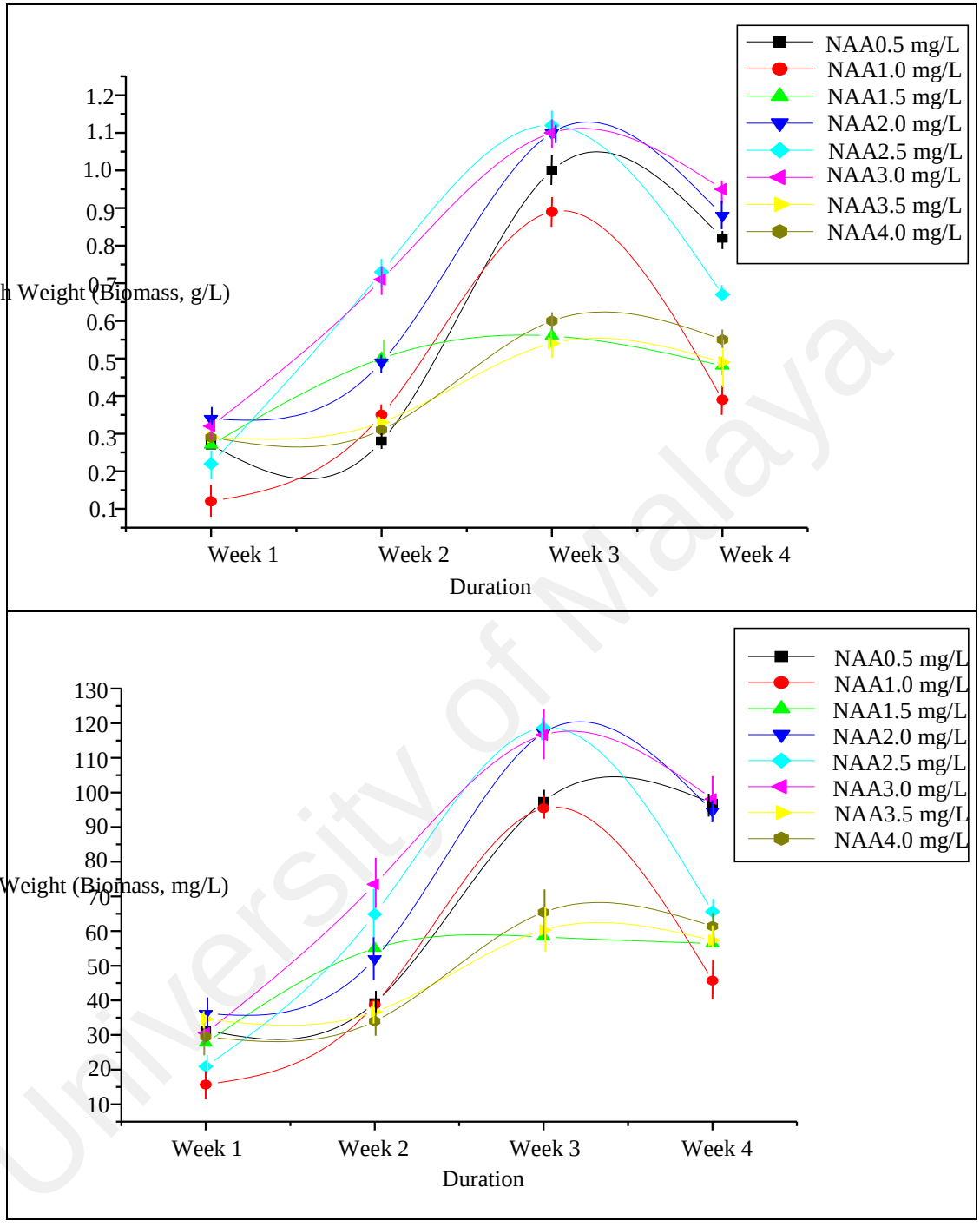


Figure 4.8: Fresh Weight (g/L) and Dry Weight (mg/L) for Leaf Explants of *Lippia nodiflora* Callus on MS media with NAA Treatments.

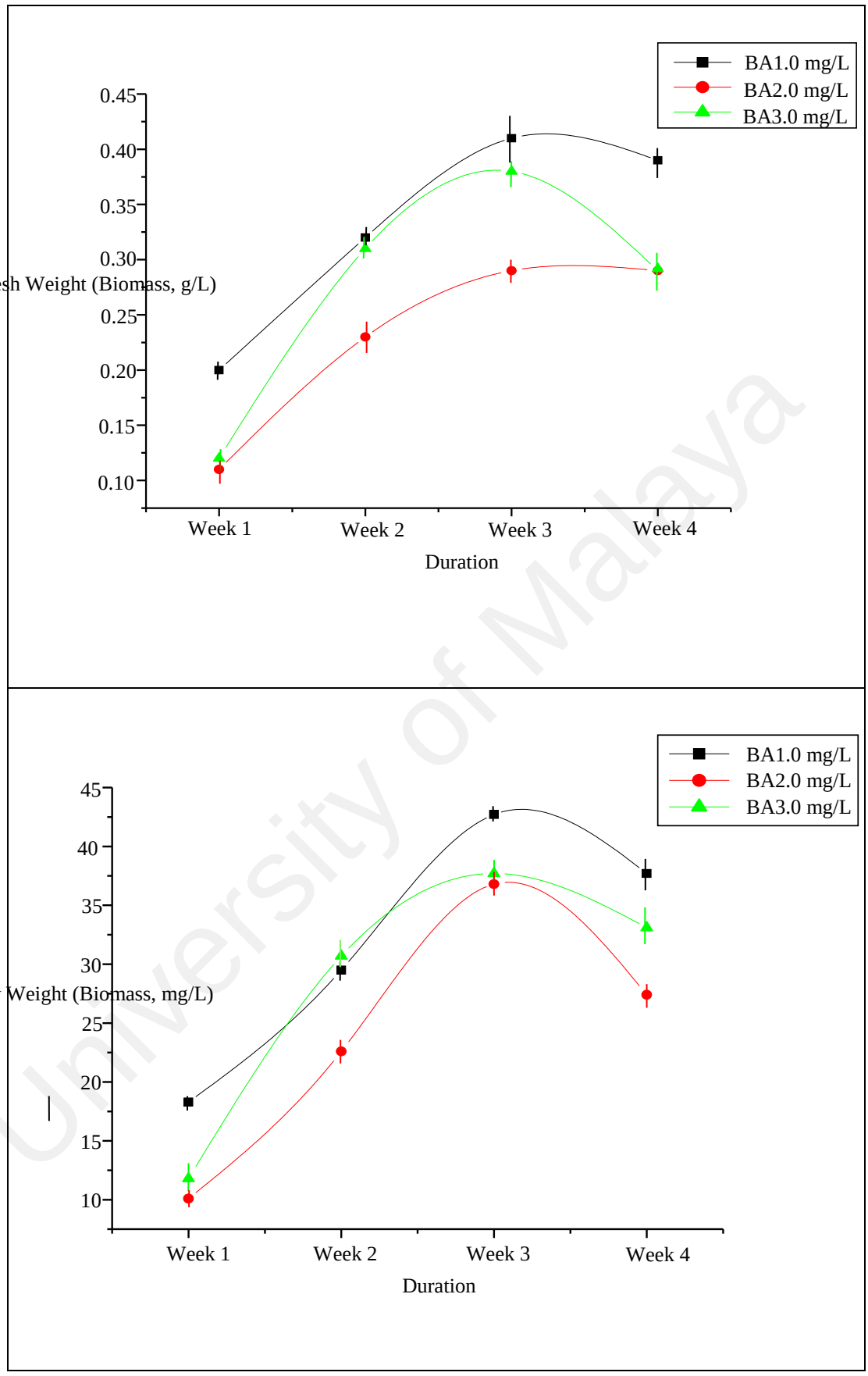


Figure 4.9: Fresh Weight (g/L) and Dry Weight (mg/L) for Stem Explants of *Lippia nodiflora* Callus on MS media with BA Treatments.

Callus growth on MS Media with Combinations PGRs

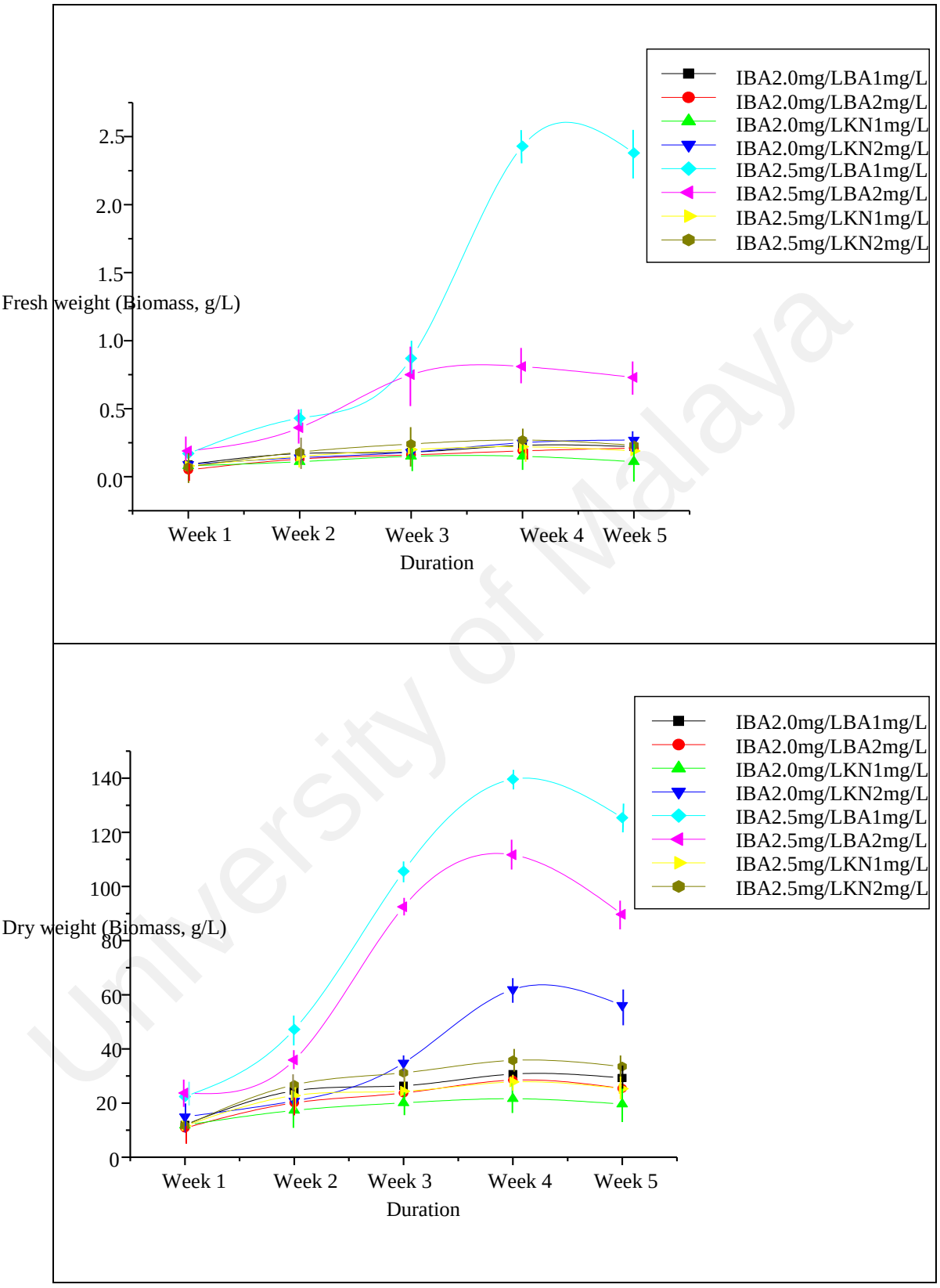


Figure 4.10: Fresh Weight (g/L) and Dry Weight (mg/L) for Stem Explants of *Lippia nodiflora* Callus on MS media with IBA + BA & KN Treatments.

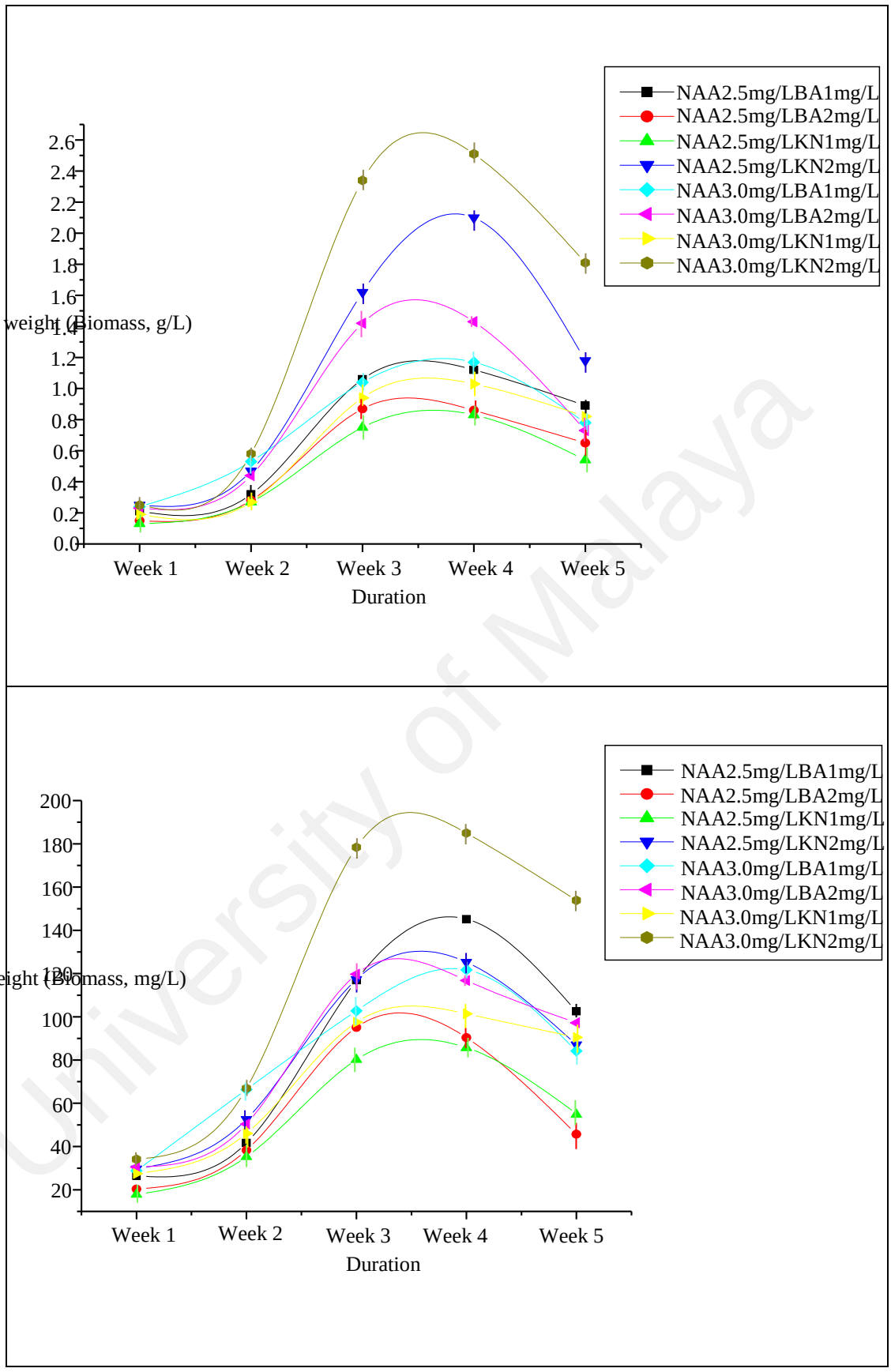


Figure 4.11: Fresh Weight (g/L) and Dry Weight (mg/L) for Stem Explants of *Lippia nodiflora* Callus on MS media with NAA + BA & KN Treatments.

4.4.4 Plant Regeneration on *Lippia nodiflora*

The percentage of plant regeneration was very low in selected concentrations on single and combination PGRS, Table 4.5 showed the average of PGRs concentrations except BA and KN as each concentrations gave out good response. Figure 4.12 shows shoot regeneration on stem explants that was inoculated vertically on MS media supplemented with PGRs and control. Based on the experiment, MS media without PGRs (MSO) was used as control and any explants that were inoculated has been successfully regenerated as seen in Figure 4.12A and B with highest percentage of 90% from other treatments as shown in Table 4.5. Explants that were inoculated on MS media supplemented with auxin especially NAA and 2,4-D induced more callus than the plant regeneration itself. Even if it was successful, the regenerated plant was stunted at the induction of callus and resulted for short length of the plant. This might be due to the division of nutrients absorption by proliferation of callus and elongation of shoots. PGRs IAA and IBA on the other hand, gave positive responsive of plant regeneration inclusive of a few roots along with minimal shoots and the length. However, some of it reached 4 cm onwards that it was able to be acclimatized. PGRs cytokinin BA and KN were more responsive than auxins where from each of the concentrations tested, more than one over four responded unlike auxins especially 2,4-D, minimal response from one batch was about one over eight. So, for auxins were the compilation results of all tested concentrations. As the control had given high response and high in length of shoots, it might be suitable for *in vitro* propagation however, 2.00 mg/L KN gave highest shoot number recorded with five shoots but with no consistency. In average, plant regeneration on MS media supplemented with combination PGRs IAA + BA gave the highest shoot numbers with 2.70 with mostly three to five shoots per explants with a minimum of one shoot per explants.

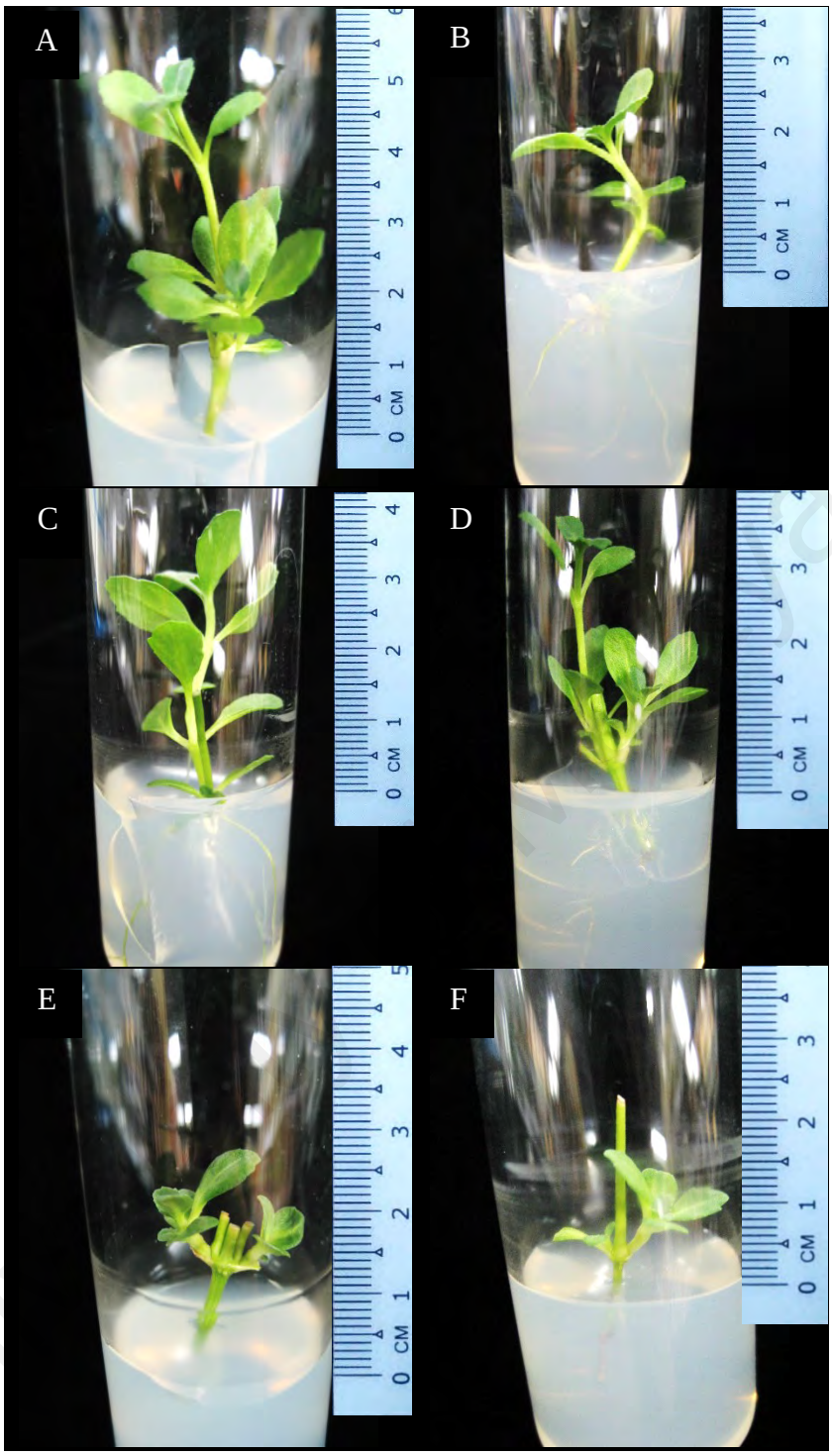


Figure 4.12: Shoot regeneration of stem on MS media supplemented with plant growth regulators (PGRs) and control. A & B. Control C. MS media supplemented with 2.00 mg/L BA D. 2.00 mg/L KN E. 2.50 mg/L IAA F. 2.50 mg/L IBA.

Ahmed et al. (2005) reported that the axillary nodal explants used inoculated in MS media supplemented with cytokinin promoted shoot buds initiation and BA (2.50 mg/L) was proven to be most efficient with 6 cm in length and high number of shoots with mean of 12.50 shoots per node. The *L. nodiflora* nodes were able to give good response too with combination PGRs of 2.00 mg/L BA + 2.50 mg/L IBA with mean 8.70 shoots per node. Comparing with this study, two to three out of ten regenerated shoots had one responsive shoots while highest was five shoots. This however, may resulted low mean number in total as shown on Table 4.5. Priya and Ravindhran (2011) also had successfully micropropagate *L. nodiflora* and it was found that the most efficient media was MS media supplemented with 3.00 mg/L BA (mean 10.50 shoots per node) where it was mentioned shoot multiplication was induced better with PGRs BA than KN which produce less shoots.

Table 4.5: Shoot regeneration of stem explants of *Lippia nodiflora*.

Plant Growth Regulators (PGRs)						Response (%)	No. of Shoots	Length of Shoots (cm)
NAA	2,4-D	IAA	IBA	BA	KN			
-	-	-	-	-	-	90.00±2.89^{a}	1.00±0.00^{d}	6.40±0.45^{a}
√						18.00±3.63^{ef}	1.20±0.13^{cd}	0.50±0.08^{e}
	√					8.00±3.00^{f}	1.10±0.10^{d}	0.40±0.04^{e}
		√				38.83±4.33^{cde}	1.70±0.20^{abcd}	2.90±0.26^{d}
			√			33.33±4.33^{def}	1.60±0.15^{abcd}	3.33±0.15^{cd}
				1		63.67±6.82^{abc}	2.00±0.20^{abcd}	2.60±0.22^{d}
				2		73.33±4.64^{ab}	2.33±0.38^{abc}	3.33±0.35^{cd}
				3		73.33±6.29^{ab}	1.90±0.26^{abcd}	4.60±0.19^{b}
					1	60.00±1.44^{bcd}	1.40±0.15^{bcd}	4.33±0.19^{bc}
					2	62.00±0.83^{abc}	1.80±0.19^{abcd}	2.80±0.33^{d}
					3	68.00±3.00^{ab}	1.90±0.26^{abcd}	2.90±0.35^{d}
		√		√		78.00±0.83^{ab}	2.70±0.14^{a}	3.50±0.13^{bcd}
		√			√	71.00±4.41^{ab}	2.00±0.24^{abcd}	3.70±0.15^{bcd}
			√	√		79.00±2.20^{ab}	2.50±0.29^{ab}	3.60±0.32^{bcd}
			√		√	76.67±2.20^{ab}	2.10±0.26^{abcd}	3.30±0.11^{cd}

√ indicates all the selected concentration of each single and combination PGRs (refer to Table 3.3). Means ± SE indicated with the same letter were not significantly different (HSD Tukey test: P<0.05)

Based on Figure 4.13, shoot regeneration with combination PGRs was successful. However, the length of the shoots was only about 3 to 5 cm long. This might be due to the interruption of callus proliferation as seen on the Figure 4.13. Figure 4.13B was recorded at week 6 where the stem that was initially planted vertically had fallen and dead but the shoot was there and it did not grow much. Callus that was induced together with shoot regeneration causing inhibition or slowing down the growth of the regenerated shoots. Figure 4.13D shows the shoot regenerated from callus induction replicates, proves that any favorable condition of the plant to be generated can be occurred as long as the parts of the nodes was present.

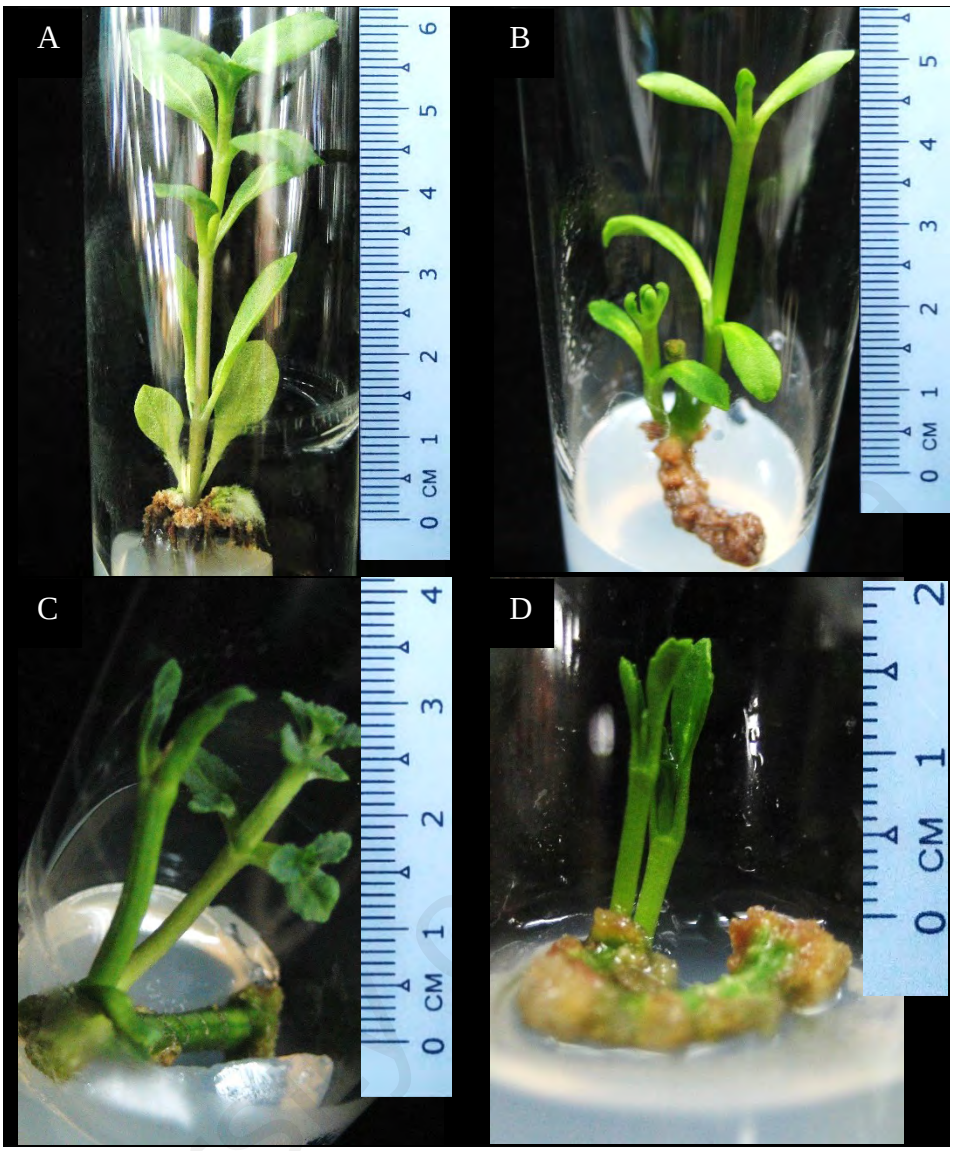


Figure 4.13: Shoot regeneration of *L. nodiflora* on MS media supplemented with combination PGRs A. 2.00 mg/L IBA + 2.00 mg/L KN B. 2.50 mg/L IBA + 1.00 mg/L BA C. 2.00 mg/L IAA + 1.00 mg/L KN D. 2.00 mg/L IAA + 1.00 mg/L BA**.**

4.4.5 Root Induction and Acclimatization

Regenerated plantlets from stem explants inoculated in MS media without PGRs (control) were able to produce roots aside from shoot regeneration. Shoot regeneration plantletfrom single and combination of auxin and cytokinin with differential lengths (3-8 cm) were cut and transferred to MS media supplemented with 2,4-D, IAA, IBA and NAA with different concentrations (1.00, 2.00 and 3.00 mg/L) as root induction was observed and recorded.

Table 4.6 shows that MS media supplemented with 3.00 mg/L IAA had most responsive number of roots (4.00 ± 0.33 roots) with maximum of eight roots per plantlet and the distribution of all three concentrations were similar. There were no root induction that has occurred on MS media supplemented with 2,4-D and NAA (concentration 1.00, 2.00 and 3.00 mg/L respectively for all PGRs tested) but only to observe the callus were forming at the end of the plantlet on the cut surface area. Roots of *L. nodiflora* were present on most of its nodes with at least one long root (Figure 4.14A) or two short roots minimum and can be up to 5- 8 roots per nodes but can be really high in number on its *in vivo* plant's main root. *L. nodiflora* was mentioned as a creeping plant where developing roots on its nodes is essential to give good nutrient supply to its elongating stems and leaves.

Table 4.6: Observation of root induction on *Lippia nodiflora* regenerated shoot.

Plant growth regulators (PGRs) IAA IBA Concentration (mg/L)	Number of roots
IAA	
1.00	3.10 ± 0.18^{b}
2.00	3.30 ± 0.15^{ab}
3.00	4.00 ± 0.33^{a}
IBA	
1.00	3.00 ± 0.21^{b}
2.00	3.30 ± 0.21^{ab}
3.00	3.70 ± 0.26^{ab}

Values are mean of 10 replicates per treatment and repeated thrice. Means ± SE indicated with the same letter were not significantly different (HSD Tukey test: $P<0.05$).

As can be seen on Figure 4.14, *L. nodiflora* plantlets that was obtained after the root induction and also its control has been acclimatized in tissue culture room 25 ± 2°C under 16/8 h photoperiod. Then, the hardened plantlets were successfully acclimatized to the natural environment. About 90% of the plantlets has survived in the garden soil and on the 30th day of acclimatization (Figure 4.14C), *L. nodiflora* has elongated and propagated in the small pot where then they were transferred into a longer square pot (24" × 6"). The

plantlets were compared with the existing plants that was already maintained on the long pot and shows not much different. They were watered regularly and keep on growing.

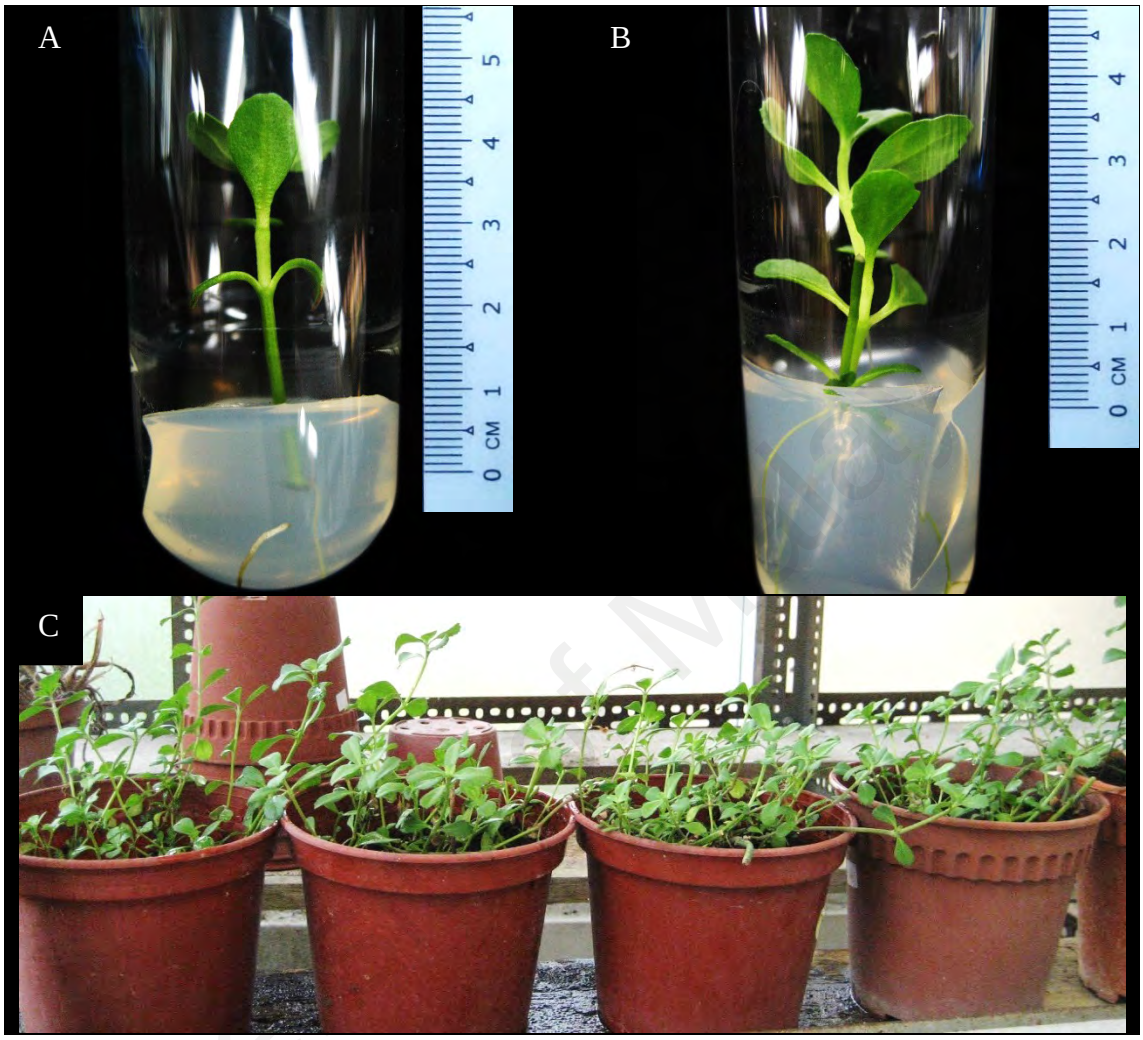


Figure 4.14: Hardening of *L. nodiflora* and its acclimatization A & B Roots formation on regenerated shoots of *L. nodiflora* C *L. nodiflora* plant has propagate and increased in number after 30 days acclimatization.

4.5 Phytochemical studies on leaf and stem of intact plants and *in vitro* callus

4.5.1 Thin Layer Chromatography (TLC) Analysis on Leaf, Stem and Callus Explants of *Lippia nodiflora*

The TLC plate of *L. nodiflora* extracts on Figure 4.15A was visualized under UV lamp (365 nm) and two of the spots gave fluorescence characteristics and their R_f values were comparable to respective standard compound (stigmasterol- R_f 0.83; β- sitosterol- R_f 0.90) based on Sharma et al. (2011). Both of the standard emitted greyish pink band under

white light as labelled in Figure 4.15B after sprayed with 10% sulphuric acid. Table 4.7 shows that the possibility of stigmasterol and β- sitosterol were present in stem extracts (R_f value 0.84; 0.92 respectively) while only stigmasterol for leaf extracts (R_f value 0.82).

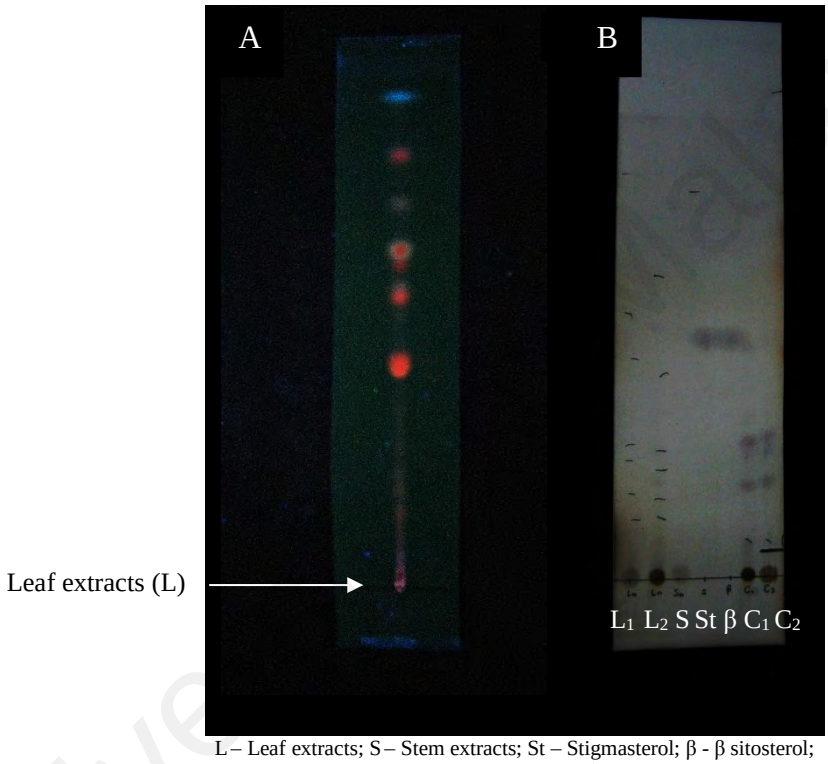
Figure 4.16 shows TLC plate using mobile phase hexane: acetone (3:1). Table 4.8 shows that the possibility of β-sitosterol and lupeol present on leaf extracts (R_f value 0.56, 0.71 respectively) and callus extracts (R_f value 0.57, 0.73 respectively), while β-sitosterol, stigmasterol and lupeol present on stem extracts (R_f value 0.56, 0.64 and 0.71 respectively). The mobile phase and methods of the experiment were done based on Sandhya et al. (2012) which the R_f value for standard compounds are 0.60 for β- sitosterol, 0.62 for stigmasterol and 0.71 for lupeol. Stigmasterol was observed only in stem extracts while both desired compounds were found using hexane: acetone with ratio 4:1. However, lupeol was also discovered based on the R_f value recorded by Sandhya et al. (2012).

A few methods were done to view the separation process of desired compounds. However, these two methods from Sharma et al. (2011) and Sandhya et al. (2012) were able to separate the bands clearly and also to view similarity of bands desired R_f value. Both methods were run for one hour on 10 x 20 cm TLC plate (Merck, Germany), although the different was only the ratio of hexane, the end of the solution was quite a different due to the polarity of the mobile phase that was prepared which resulted a totally different R_f values for stigmasterol and β-sitosterol. A smaller economical width size of TLC plate were used during optimatization.

However, if the bands or spots identified to be stigmasterol and β-sitosterol, where it was labelled in Figure 4.16B, lane one, two and three stands for leaf and stem explants of *L. nodiflora* respectively, while lane four and five representing stigmasterol and β-sitosterol respectively and lastly lane six and seven stands for callus initiated from stem explants in MS media supplemented with 3.00 mg/L NAA + 2.00 mg/L KN and 2.50

mg/L IBA + 1.00 mg/L BA respectively. In this Figure, the R_f value for stigmasterol and β-sitosterol was 0.56 (mobile phase hexane: acetone, 4:1), where similarly to lane two, four, five and six which had spots at the same R_f value. So, based on this statement can be concluded that there were high possibility that stigmasterol and β-sitosterol were present in leaf and callus of *L. nodiflora.*

Steroids



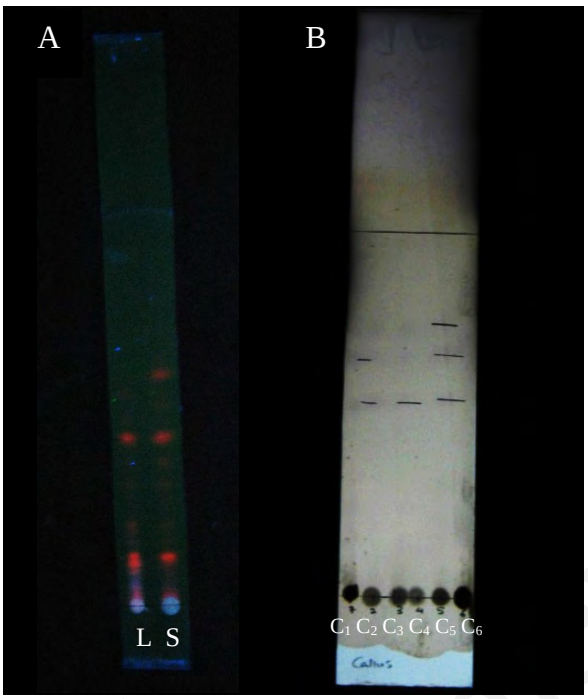
L – Leaf extracts; S – Stem extracts; St – Stigmasterol; β - β sitosterol;
C – Callus extracts; numbers referred to replicates

Figure 4.15: Thin Layer Chromatography for leaf, stem and callus extracts with mobile phase hexane: acetone (4: 1) A. TLC plate viewed under 365 nm UV lamp B. TLC plate viewed under white light after sprayed with 10% sulphuric acid, H_2SO_4 in ethanol.

Table 4.7: R_f value readings for leaf, stem and callus extracts of *L. nodiflora* on thin layer chromatography with hexane: acetone (4:1) mobile phase.

Leaf extracts		**Stem extracts**		**Callus extract**	
Distance (cm)	**R_f value**	**Distance (cm)**	**R_f value**	**Distance (cm)**	**R_f value**
6.40	0.37	2.50	0.16	5.70	0.53
9.10	0.52	5.30	0.34	7.10	0.66
10.30	0.59	5.90	0.38	8.00	0.75
10.60	0.61	13.20	0.84		
12.70	0.73	14.40	0.92		
14.40	0.82	14.90	0.96		

L – Leaf extracts S – Stem extracts; St – Stigmasterol; β - β sitosterol; C – Callus extracts



L – Leaf extracts; S – Stem extracts; C – Callus extracts;
numbers referred to replicates

Figure 4.16: Thin Layer Chromatography for leaf, stem and callus extracts with mobile phase hexane: acetone (3: 1) A. TLC plate viewed under 365 nm UV lamp B. TLC plate viewed under white light after sprayed with 10% Sulphuric acid, H_2SO_4 in ethanol.

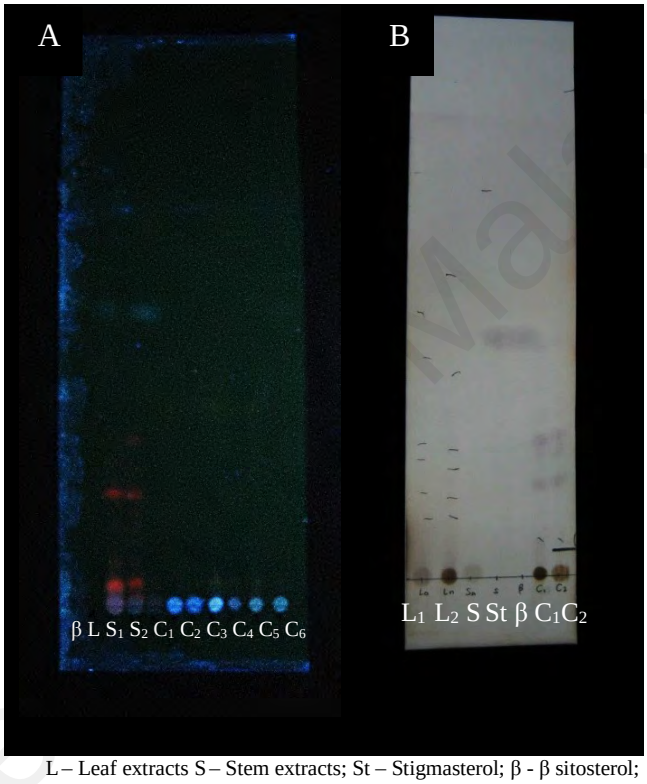
Table 4.8: R_f value readings for leaf, stem and callus extracts of *L. nodiflora* on thin layer chromatography with hexane: acetone (3: 1) mobile phase.

Leaf extracts		Stem extracts		Callus extract	
Distance (cm)	**R_f value**	**Distance (cm)**	**R_f value**	**Distance (cm)**	**R_f value**
4.40	0.36	4.40	0.36	8.00	0.57
5.70	0.47	5.70	0.47	10.20	0.73
6.80	0.56	6.80	0.56	11.80	0.84
8.50	0.71	7.70	0.64		
9.80	0.80	8.50	0.71		
10.60	0.87	9.80	0.80		
12.00	0.98	10.60	0.87		
		12.00	0.98		

L – Leaf extracts S – Stem extracts; St – Stigmasterol; β - β sitosterol; C – Callus extracts

On the other hand, leaf and stem extracts separated bands can be seen only under purple UV light (365 nm) while for callus extracts can only be seen under white light after 10% sulphuric acid was sprayed and heated as shown in Figure 4.17. All TLC plates were also observed under green UV light (254 nm) however, no bands or spots were observed.

Based on Figure 4.17A, lane 1 and 4 were β-sitosterol and stigmasterol standard compounds respectively, which was also shown on figure 4.17B on lane 4 and 5. The experiment was repeated several times but, the two standard compounds cannot be seen under both green and purple UV light (254 and 365 nm respectively) conversely for TLC plate that was sprayed using 10% sulphuric acid, a greyish pink spot was identified similarly reported by Sandhya et al. (2012) but do not possess the same R_f value.



L – Leaf extracts S – Stem extracts; St – Stigmasterol; β - β sitosterol;
C – Callus extracts; numbers referred to replicates

Figure 4.17: Thin Layer Chromatography for leaf, stem and callus extracts A. TLC plate viewed under 365 nm UV lamp using mobile phase hexane: acetone (3: 1) B. TLC plate for leaf, stem and callus extracts viewed under white light after sprayed with 10% sulphuric acid, H_2SO_4 in ethanol**.**

Other than investigating on mobile phase and methods of observations, three protocols of extractions were done to ensure which protocol gave the best results so that it can be proceeded with other analysis. Firstly, boiling of *L. nodiflora* in water; secondly, maceration of *L. nodiflora* in water, methanol and ethanol; and thirdly, methanolic extraction of *L. nodiflora* using soxhlet apparatus (percolation technique). Upon all three protocols, the results obtained for boiling in water on TLC plate was there were no bands

or spot observed or identified. The method percolation of water resulted in no bands or spot observed or identified, while percolation on methanol and ethanol, there were a few visible bands or spots that observed and identified. As for extraction using soxhlet apparatus, there were a few visible bands or spots observed and identified. The extraction of *L. nodiflora* using soxhlet apparatus gave the highest number and clear bands or spots.

4.5.2 Gas Chromatography Mass Spectrophotometer Analysis on *Lippia nodiflora* Leaf and Stem Explants

GC-MS is the best technique for bioactive compound identification of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino acid and nitro compounds (Muthulakshmi et al., 2012). Essential oil extracted from *L. nodiflora* was reported by Calvache et al. (2010) to contain eight major compounds classified as monoterpenes, sesquiterpenes and a saturated hydrocarbon. In the present study, 15 compounds have been identified from methanol extracts from leaf and stem explants of *L. nodiflora.* Among the identified compounds, hexylresorcinol has anaesthetic, antiseptic and anthelmintic properties (Wilson et al., 1966) while tetradecanoic acid has the property of antioxidant, 5-alpha-reductase inhibitor, antifibrinolytic, hemolytic and antimicrobial activities (Rosemeyer & Bodoprost, 2007; Abirami & Rajendran, 2011; Kala et al., 2011). Stearic acid contributes the highest percentage of compound found in the leaf extract, which is an inactive ingredients, a saturated, wax-like, fatty acid commonly used in the production of pharmaceutical tablets and capsules. Stearic acid functions as emulsifying agent, solubilizing agent, tablet and capsule lubricant in pharmaceutical industry. It is commonly found in lotions, detergents, soaps, and shampoos, and is a natural component of cocoa butter and shea butter. Based on Habib et al. (1987), stearic acid (octadeconoic acid) was found to inhibit tumor development. Esters were also present in the extracts and are important organic compounds with increasing number of commercial applications (Foresti et al., 2005).

These compounds are largely used in fragrances, cosmetics detergents, flavors and pharmaceuticals. Due to the presence of above mentioned compounds in the leaf and stem methanolic extracts of *Lippia nodiflora*, it has potential usage in various pharmaceutical and industrial applications.

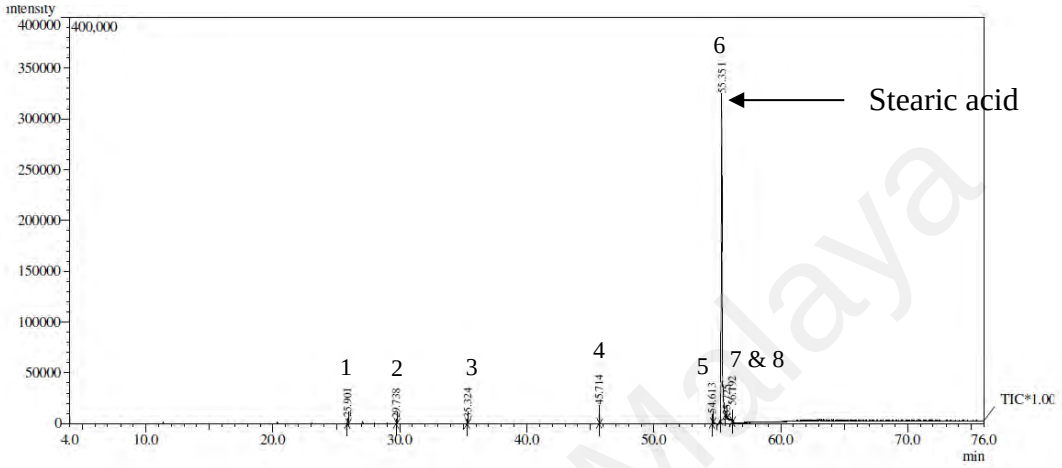


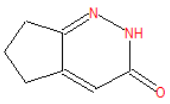
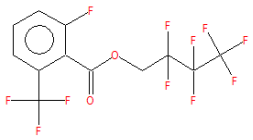
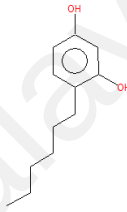
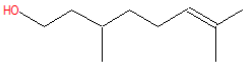
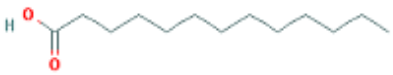
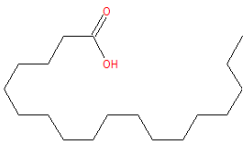
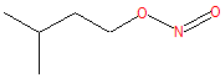
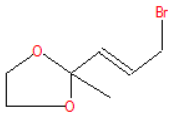
Figure 4.18: GC-MS chromatogram for leaf explants methanolic extract of *L. nodiflora.*

Table 4.9: GCMS analysis of methanolic extract of *L. nodiflora* leaf explants.

No.	RT (min)	Name of Compound	Molecular Formula	Molecular Weight	Peak Area %
1	25.901	3H-Cyclopenta[c]pyridazin-3-one, 2,5,6,7-tetrahydro	$C_7H_8N_2O$	136	0.85
2	29.738	6-Fluoro-2-trifluoromethylbenzoic acid, 3-fluorophenyl ester	$C_{14}H_7F_5O_2$	302	0.68
3	35.324	Hexylresorcinol	$C_{12}H_{18}O_2$	194	0.66
4	45.714	6-Octen-1-ol, 3,7-dimethyl-, propanoate	$C_{13}H_{24}O_2$	212	2.44
5	54.613	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	1.66
6	55.351	Stearic acid	$C_{18}H_{36}O_2$	284	93.45
7	55.725	Isoamyl nitrite	$C_5H_{11}NO_2$	117	0.15
8	56.192	1,3-Dioxolane, 2-(3-bromo-3-buten-1-yl)-	$C_7H_{11}BrO_2$	206	0.12

RT= Retention Time

Table 4.10: Molecular structure of compounds found from GCMS analysis of *L. nodiflora* leaf explants.

No.	Name of Compound	Molecular Structure
1	3H-Cyclopenta[c]pyridazin-3-one, 2,5,6,7-tetrahydro	
2	6-Fluoro-2-trifluoromethylbenzoic acid, 3-fluorophenyl ester	
3	Hexylresorcinol	
4	6-Octen-1-ol, 3,7-dimethyl-, propanoate	
5	Tridecanoic acid,	
6	Stearic acid	
7	Isoamyl nitrite	
8	1,3-Dioxolane, 2-(3-bromo-3-buten-1-yl)-	

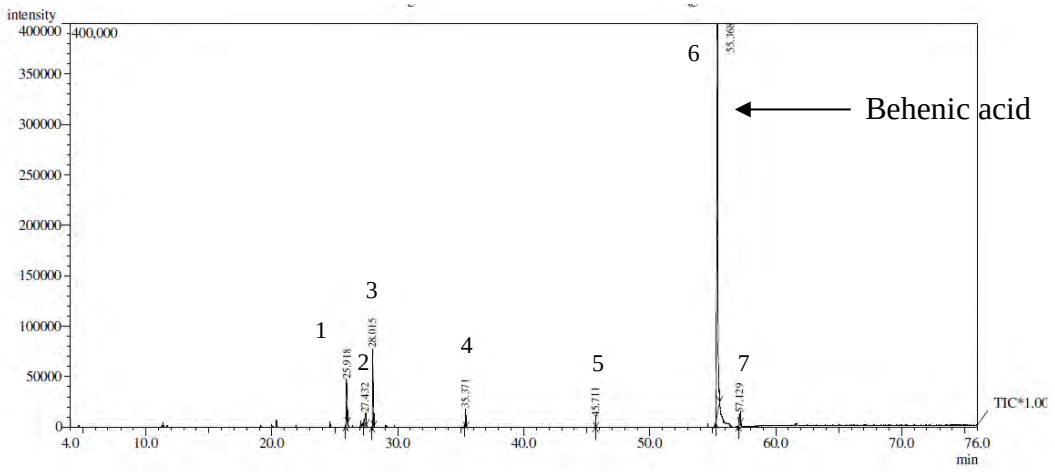
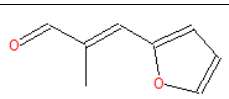
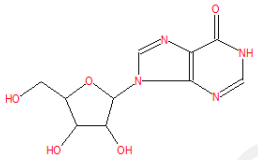
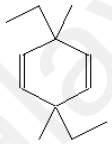
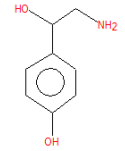
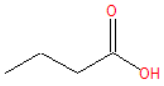
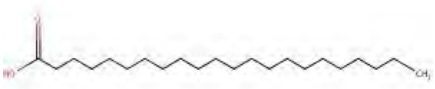
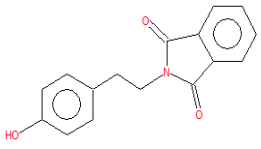


Figure 4.19: GC-MS chromatogram for stem explants methanolic extract of *L. nodiflora.*

Table 4.11: GCMS analysis of methanolic extract of *L. nodiflora* stem explants.

No.	RT (min)	Name of Compound	Molecular Formula	Molecular Weight	Peak Area %
1	25.918	2-Methyl-3(2-furyl)acrolein	$C_8H_8O_2$	136	5.69
2	27.432	6H-Purin-6-one, 9-.beta.-D-arabinofuranosyl-1,9-dihydro-	$C_{10}H_{12}N_4O_5$	268	1.97
3	28.015	2,5-Cyclohexadiene-1,4-dione, 2,3-dimethyl-	$C_8H_8O_2$	136	8.33
4	35.371	Octopamine	$C_8H_{11}NO_2$	153	1.41
5	45.711	Butanoic acid	$C_{12}H_{22}O_2$	198	0.46
6	55.368	Behenic acid	$C_{22}H_{44}O$	340	80.17
7	57.129	N-Phthaloyltyramine	$C_{16}H_{13}NO_3$	267	1.97

Table 4.12: Molecular structure of compounds found from GCMS analysis of *L. nodiflora* stem explants.

No.	Name of Compound	Molecular Structure
1	2-Methyl-3(2-furyl) acrolein	
2	6H-Purin-6-one, 9-.beta.-D-arabinofuranosyl-1,9-dihydro-	
3	2,5-Cyclohexadiene-1,4-dione, 2,3-dimethyl-	
4	Octopamine	
5	Butanoic acid	
6	Behenic acid	
7	N-Phthaloyltyramine	

4.5.3 High Performance Thin Layer Chromatography (HPTLC) Analysis on Leaf, Stem and Callus of *Lippia nodiflora* explants

High performance thin layer chromatography (HPTLC) analysis has been examined, the automated calculation of R_f value were 0.46 and 0.45 for stigmasterol and β-sitosterol respectively. The method used was adopted from Kamboj and Saluja (2013), the R_f value reported for stigmasterol compound was 0.45 which was 0.01 deviated from R_f value obtained. However, it may differ due to extraction process that was slightly not similar or any other handling error. Supposedly HPTLC is a continuation from TLC that has been done, in which it means the TLC was a point in the determination of which mobile phase should be used. The mobile phase has been investigated through the polarity of solvent to improve the separation of the two compounds bands. Chemical solvents and TLC plate characteristics determine the ratio is by polarity. As the silica gel plate is very high in polarity, polar compounds will tend to stick more to the plate, and will move more slowly. Lower polarity compounds will tend to dissolve in the solvent, and will move faster. If two different samples run the same height on a plate, this is good evidence that they are the same compound. If they move differently, they are different. The polarity of the solvent used to carry the spots up the plate will also affect the distance the spots travel. A more polar solvent will be able to compete with the silica gel on the plate, and all spots will be carried correspondingly higher than they would with a less polar solvent. So, after a few trials and adjusts that has been done, the mobile phase used which was chloroform: ethanol (98:2) that had gave the best definitions of bands and its fluorescence. Other than that, the trials and adjusts were mainly to try separating the two steroids desired which are stigmasterol and β-sitosterol. Based from Sharma et al. (2011) and Sandhya et al. (2012), both had a near R_f value for stigmasterol and β-sitosterol. In spite of this, the only difference between stigmasterol ($C_{29}H_{48}O$, 412.69 g/mol) and β-sitosterol ($C_{29}H_{50}O$, 414.71 g/mol) is the presence of double bond C_{22}=C_{23} in stigmasterol and single bond

C_{22}-C_{23} in β-sitosterol hence, the lack of practical difference in their R_f despite the use of several solvent systems (Kamboj & Saloja, 2010).

From the HPTLC analysis that has been done based on the extracts shown on Table 4.13, the R_f values were compared and we can conclude that most likely sample of lane 3, 4, 6, 7, 10 and 11 consist of the desired steroid compounds. However, for lane 1, 2 and 5, there were no band showing on R_f 0.45 and R_f 0.46 which we might conclude that the steroid compounds might be absence in these samples. The leaf extracts sample of *Lippia nodiflora* have similar profile (Lane 7 & 11), where it has a significant red band occur at R_f 0.72, whereas this red band is absence in all the other stems samples and the non-fresh leaf samples as shown on figure 4.21.

Table 4.13: Sample Application Pattern of *L. nodiflora* for HPTLC.

Lane	Sample	Application Volume (µL)
1	Stem callus (3.00 mg/L NAA)	5
2	Leaf callus (3.00 mg/L NAA)	5
3	Stem callus (3.00 mg/L NAA + 2 mg/L KN)	5
4	Leaf callus (3.00 mg/L NAA + 2 mg/L KN)	5
5	Stem callus (2.50 mg/L IBA + 1 mg/L BA)	5
6	Leaf callus (2.50 mg/L IBA + 1 mg/L BA)	5
7	Leaf explants 1	5
8	Stigmasterol 200 ppm	4
9	β-sitosterol 100 ppm	15
10	Stem explants	5
11	Leaf explants 2	5

*All callus were inoculated in MS media supplemented with PGRs mentioned

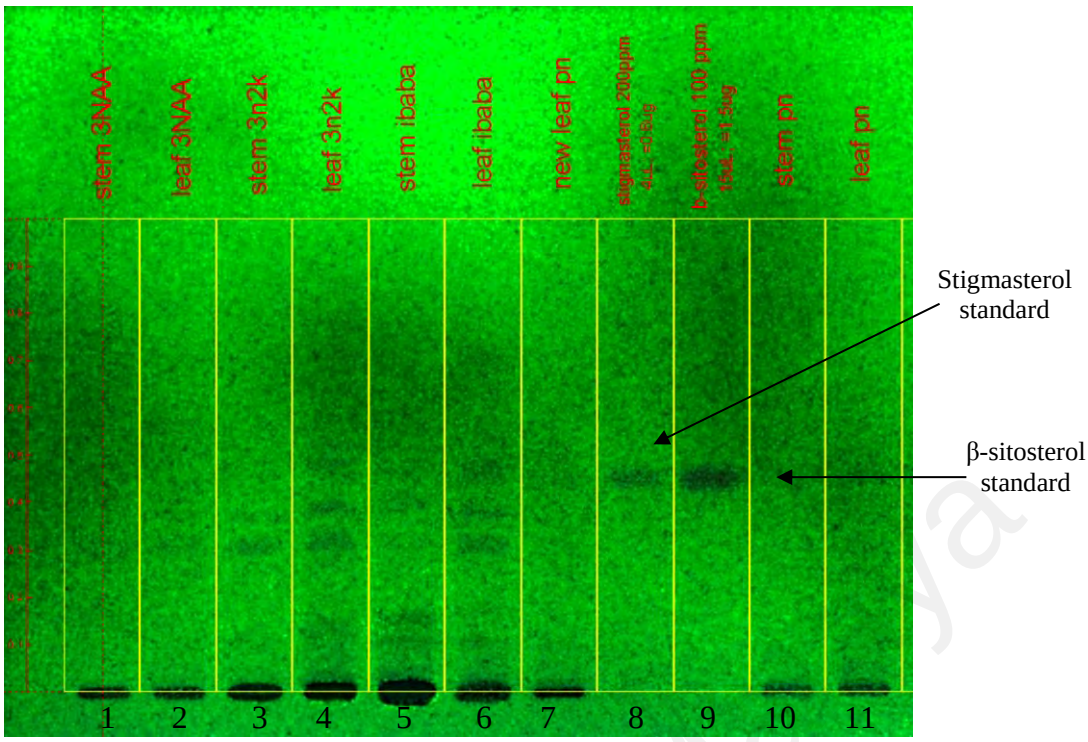


Figure 4.20: HPTLC analysis for *L. nodiflora* extracts with stigmasterol and β-sitosterol standard at 254 nm.

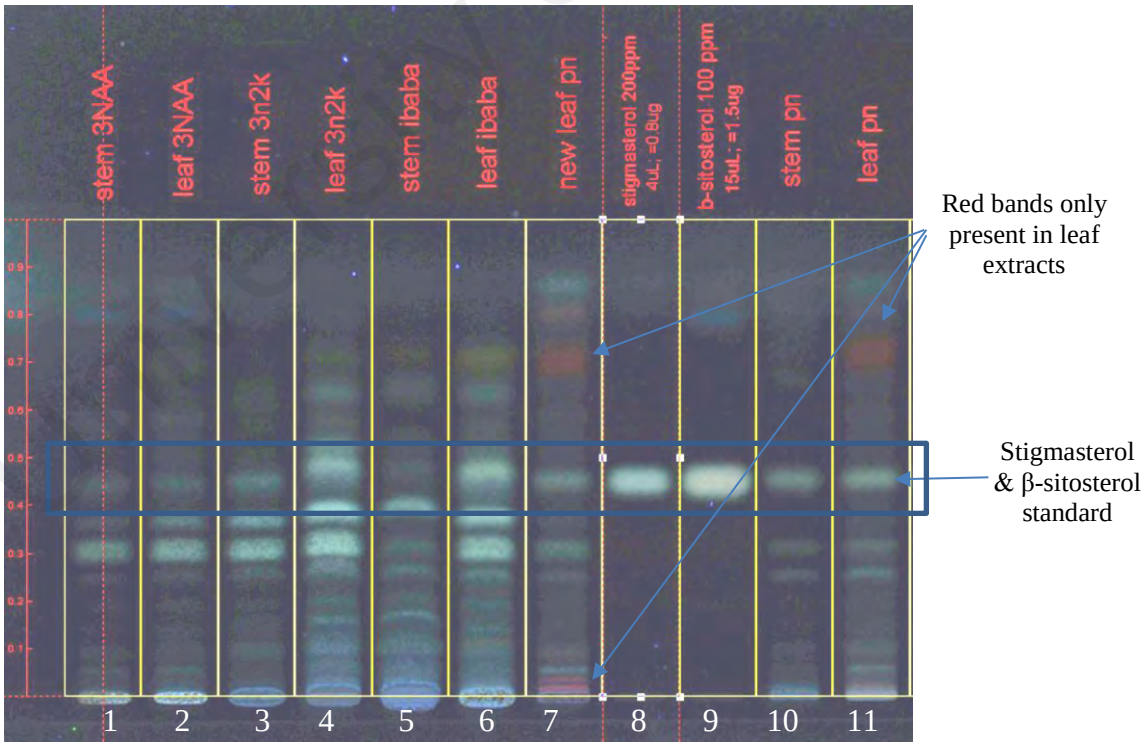


Figure 4.21: HPTLC analysis for *L. nodiflora* extracts with stigmasterol and β-sitosterol standard at 365 nm.

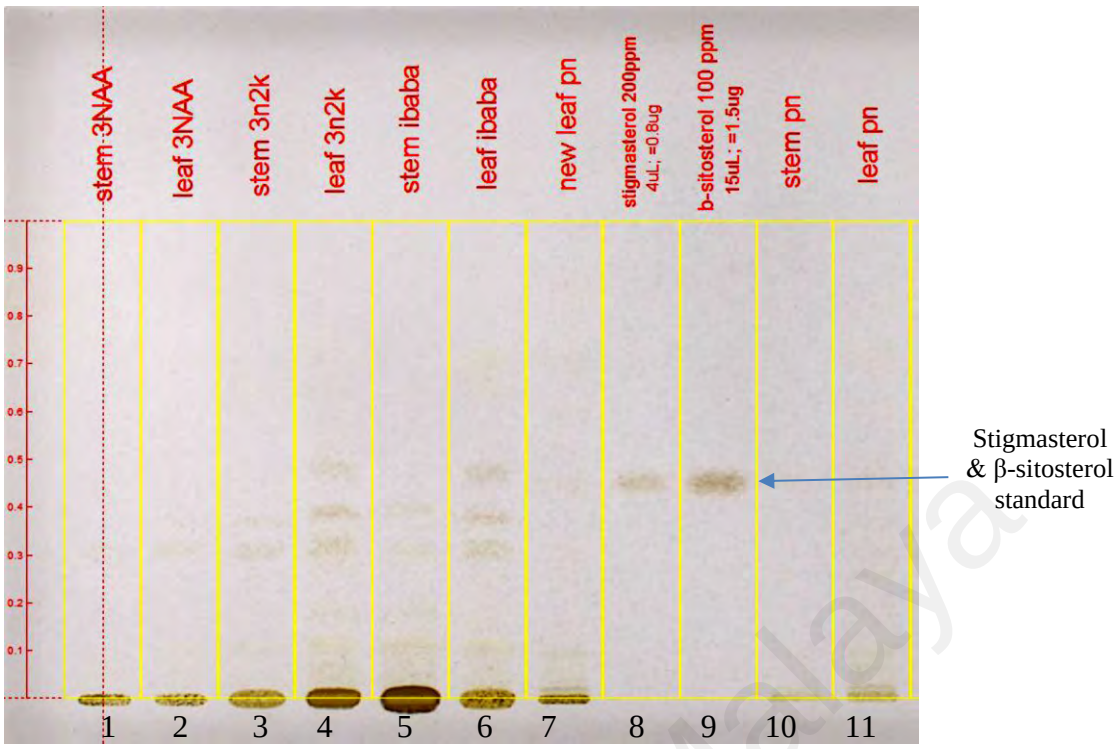


Figure 4.22: HPTLC analysis for *L. nodiflora* extracts with stigmasterol and β-sitosterol standard on with light after sprayed with 10% sulphuric acid, H_2SO_4 in ethanol.

4.5.4 High Performance Liquid Chromatography (HPLC) on Leaf, Stem and Callus Explants of *Lippia nodiflora*

HPLC was run to investigate the presence of desired compounds which are stigmasterol and β-sitosterol on *L. nodiflora* plant as previous analysis had mentioned. Standard compounds were used to confirm its presence, to ensure the statement that had discuss on TLC and HPTLC analysis.

Figures 4.23 to 4.28 showed the standard curve for β-sitosterol and stigmasterol and its peak on HPLC chromatogram with retention time. Table 4.14 explains retention time, peak area and height of injected standards using 1000 mg/L concentration. Standard curve was obtained from the serial dilution of 1000 mg/L which were, 100 mg/L, 200 mg/L, 300 mg/L, 400 mg/L and 500 mg/L of each standards including blank. From the standard curve, both β-sitosterol and stigmasterol has obtained its equation; y= mx + c which will lead us to calculate the concentration of compounds in *L. nodiflora* extracts with y= 6831.9x – 9207.3 (Figure 4.24) and y= 7648.8x + 121188 (Figure 4.26) respectively. The

concentration of compounds found in explants extracts were calculated by inserting its area number to y and calculate the x. Table 4.15 shows the concentration of stigmasterol and β-sitosterol present in 1.50 g/mL explant extracts.

There were 22 peaks recorded for both leaf and stem extracts (Figure 4.27A) while there were five to ten peaks recorded from callus extracts (Figure 4.28A). The age of explants also contributes as one of the factors in the extraction of compounds. Also, the methodology used for this experiment were designated to isolate the stigmasterol and β-sitosterol compounds. Thus, this might cause unfavorable condition especially in callus extracts. Optimization of methods are crucial factor in obtaining good results especially in age profile of explants, method to dry explants, extraction method, HPLC method and mobile phase used. However, this study was a targeted analysis, and the desired compounds were successfully separated and identified in the explants extracts.

In 10 µL of injection from 1.50 g/mL leaf and stem extract, it was found that there might be some appearance of β-sitosterol however, it was not significant as seen on the record. Similarly, it was also not significant for stigmasterol compound in callus extracts and the absence in stem explants but as for leaf extracts, the volumetric concentration 91.40 mg/L and pure concentration of 6.09×10^{-2} mg/g obtained, considered significant (highlighted in green) compare to callus. Its retention time was 8.71 min while stigmasterol standard was 8.70 min shows that it has high similarity with 0.01 deviation. As seen on Figure 4.25, leaf extracts peak was right under stigmasterol peak (as labelled) showed an indication of the same compound. The previous discussion on HPTLC analysis also mentioned that from the R_f value obtained, leaf extracts contained the steroid compounds. However the HPLC analysis, β-sitosterol and stigmasterol was separated and the observation of the peak shows that on stigmasterol is considerably significant in leaf extracts compare to other explants.

Other than that, Figure 4.27B shows the no significant peaks of stigmasterol and β-sitosterol on stem extracts. Equally for callus extracts in Figure 4.28B, callus initiated on MS media supplemented with 3.00 mg/L NAA + 2.00 mg/L KN showed no significant result. Previously discussed on TLC, callus extracts has were no bands aligned or even close to the standards bands. In HPTLC analysis, callus extracts from MS media supplemented with 3.00 mg/L NAA + 2.00 mg/L KN did not show significant result too, with only traces of band compared to another callus extracts from MS media supplemented with 2.50 mg/L IBA + 1.00 mg/L BA that has solid band even though both has no significant appearance on the analysis. There were peaks under stigmasterol and β-sitosterol chromatogram but in a very small amount (4.20 and 11.67 mg/L respectively) in callus extract. The peaks however, might represent other compounds which possess molecular weight 412 g/mol (stigmasterol) and 414 g/mol (β-sitosterol). As observed in Figure 4.28A, there were only five to ten peaks observed on the callus extracts. The extractions were prepared at callus 3rd to 4th week of its lifespan, secondary metabolites might have not developed at this stage but only primary metabolites. Figures 4.29 to 4.32 showed chromatogram of leaf, stem, callus induced on leaf explants and callus induced on stem explants extracts respectively.

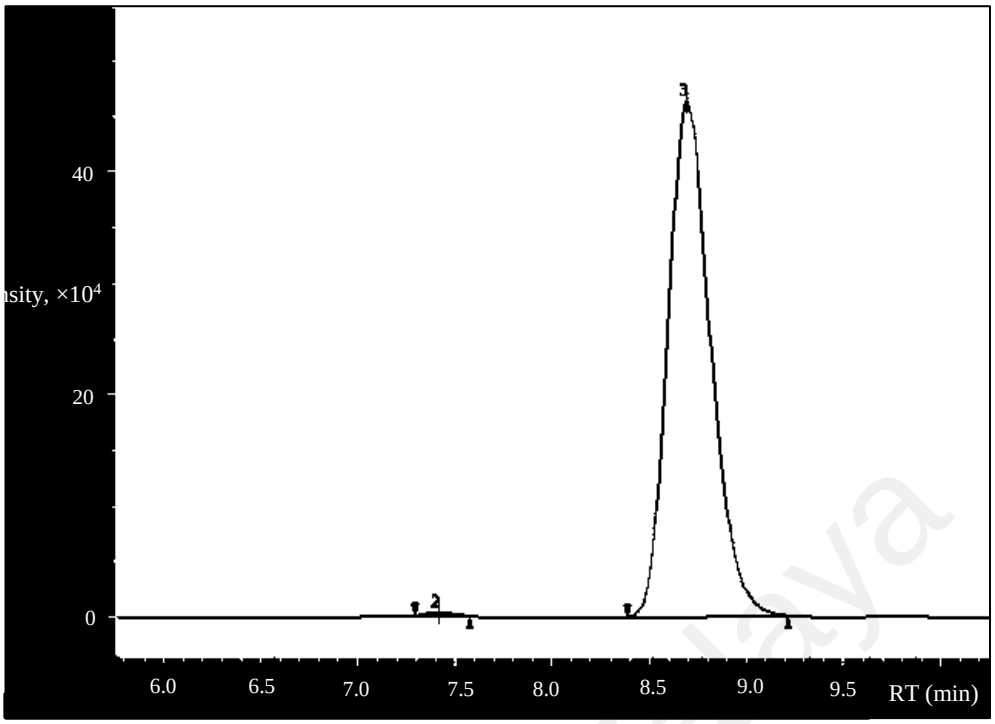


Figure 4.23: Peak of stigmasterol standard compound at retention time (RT) 8.70 min.

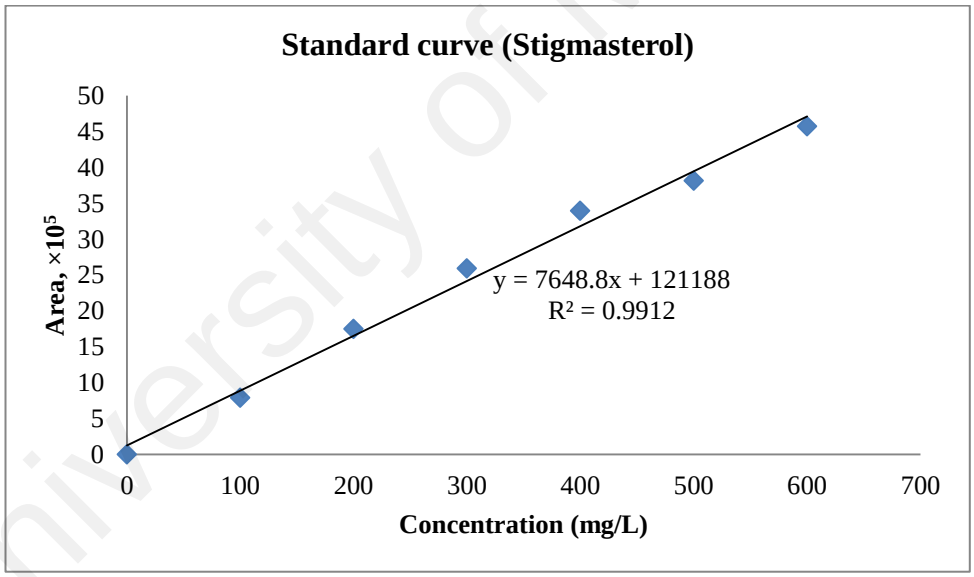


Figure 4.24: Standard curve for stigmasterol.

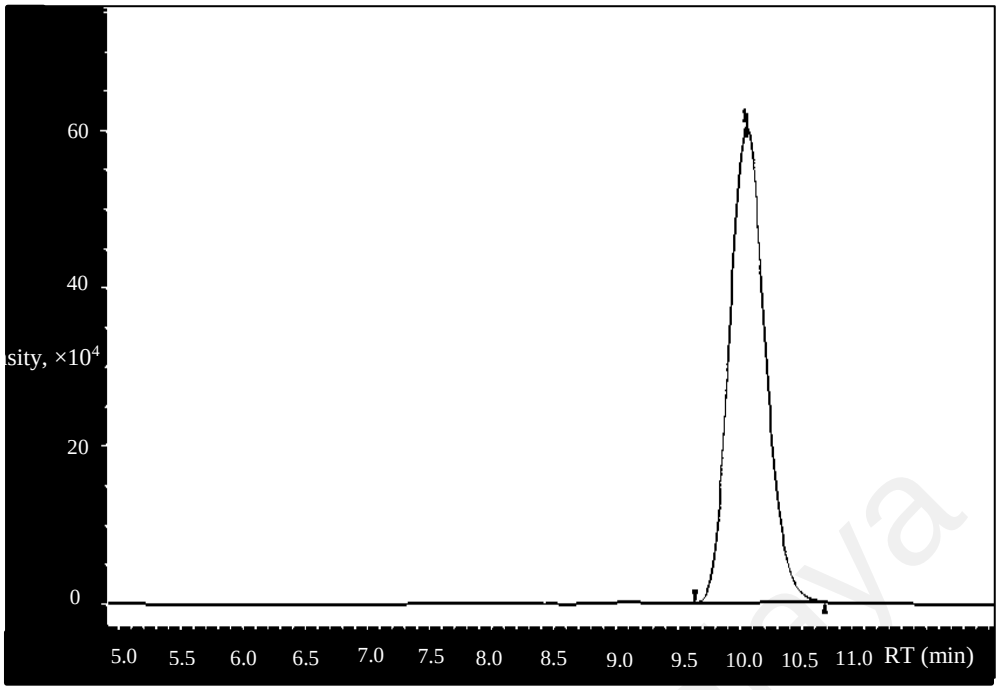


Figure 4.25: Peak of β- sitosterol standard compound at retention time (RT) 10.05 min.

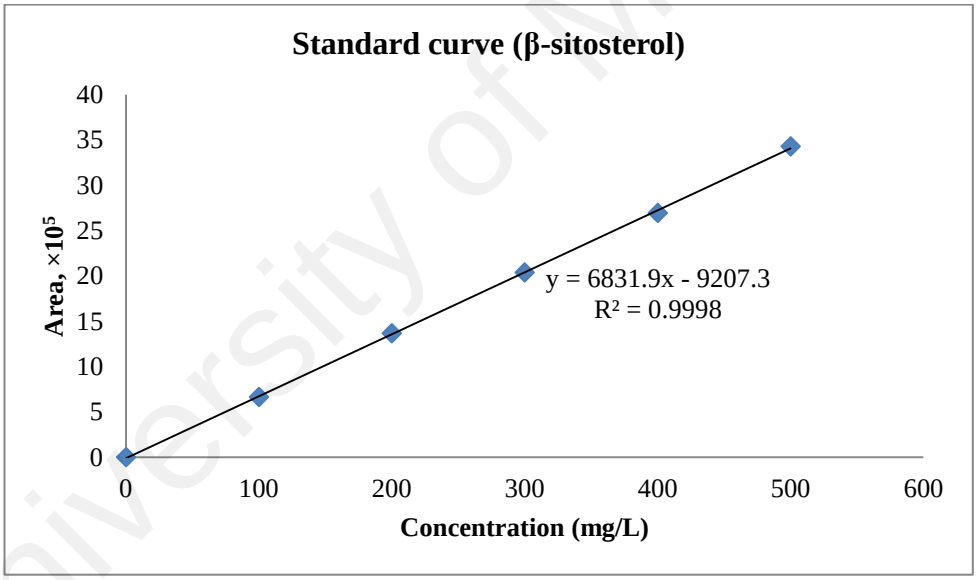


Figure 4.26: Standard curve for β- sitosterol.

Table 4.14: Standard β-sitosterol and stigmasterol HPLC analysis.

Standard	Retention Time (RT), min	Area, $\times 10^5$	Height, $\times 10^4$
β- sitosterol	10.05	12.06	60.13
Stigmasterol	8.70	7.03	46.18

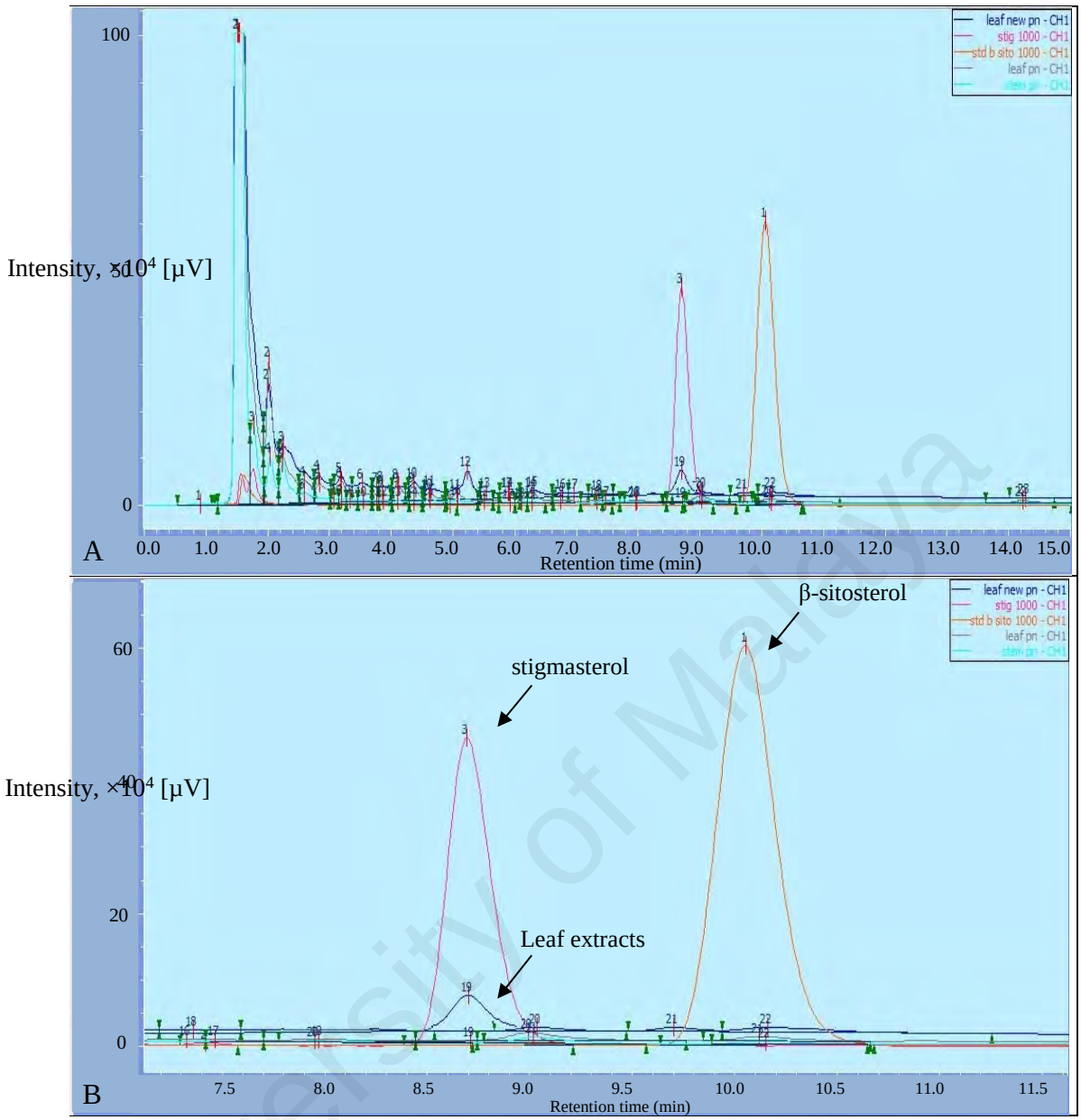


Figure 4.27: HPLC analysis on leaf and stem extracts on stigmasterol and β-sitosterol standards (A) Chromatogram of leaf and stem extracts (B) Enlargement from stigmasterol and β-sitosterol peak areas.

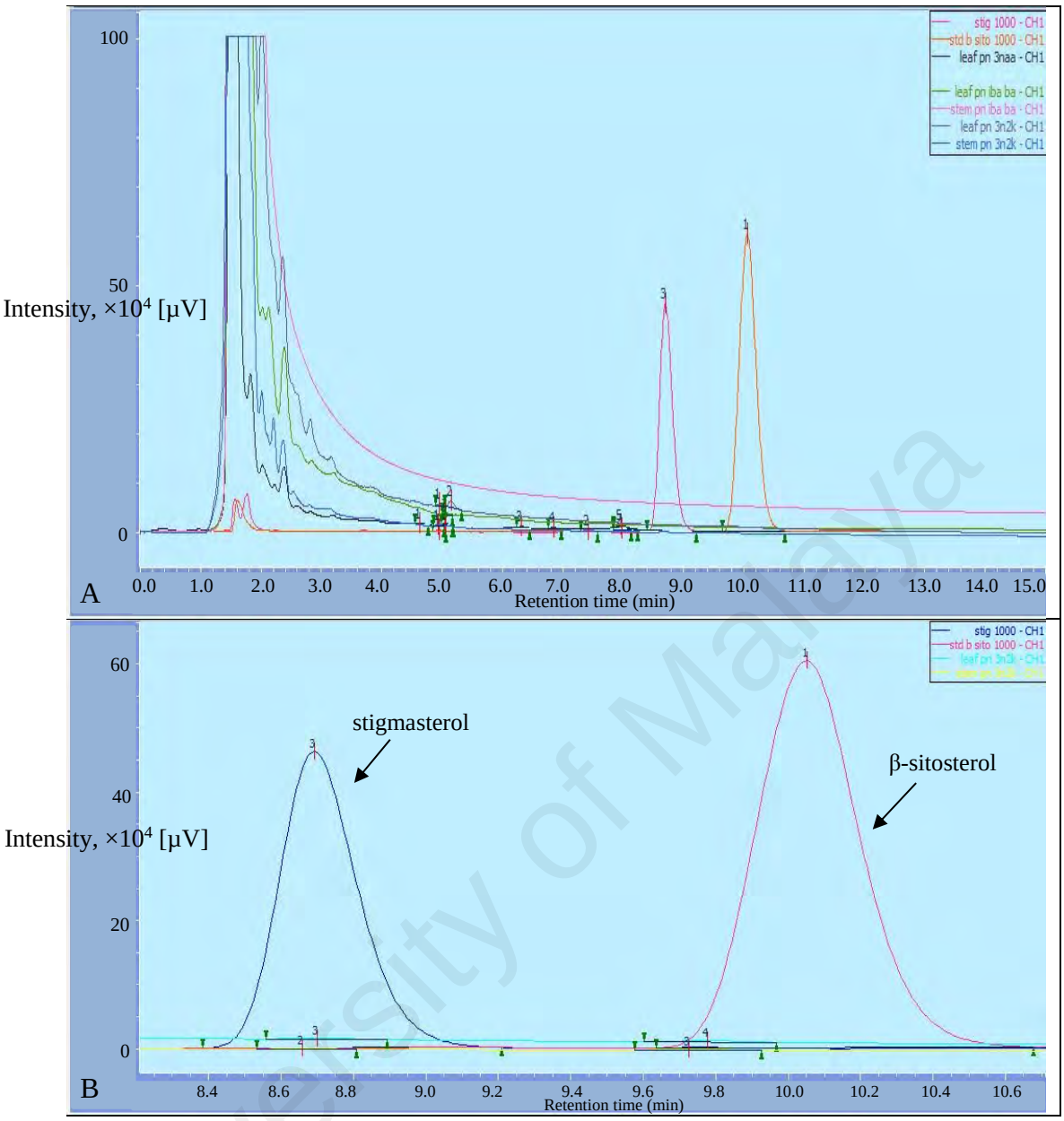


Figure 4.28: HPLC analysis callus extracts on stigmasterol and β-sitosterol standards (A) Chromatogram of six callus extracts including standards (B) Enlargement from stigmasterol and β-sitosterol peak areas.

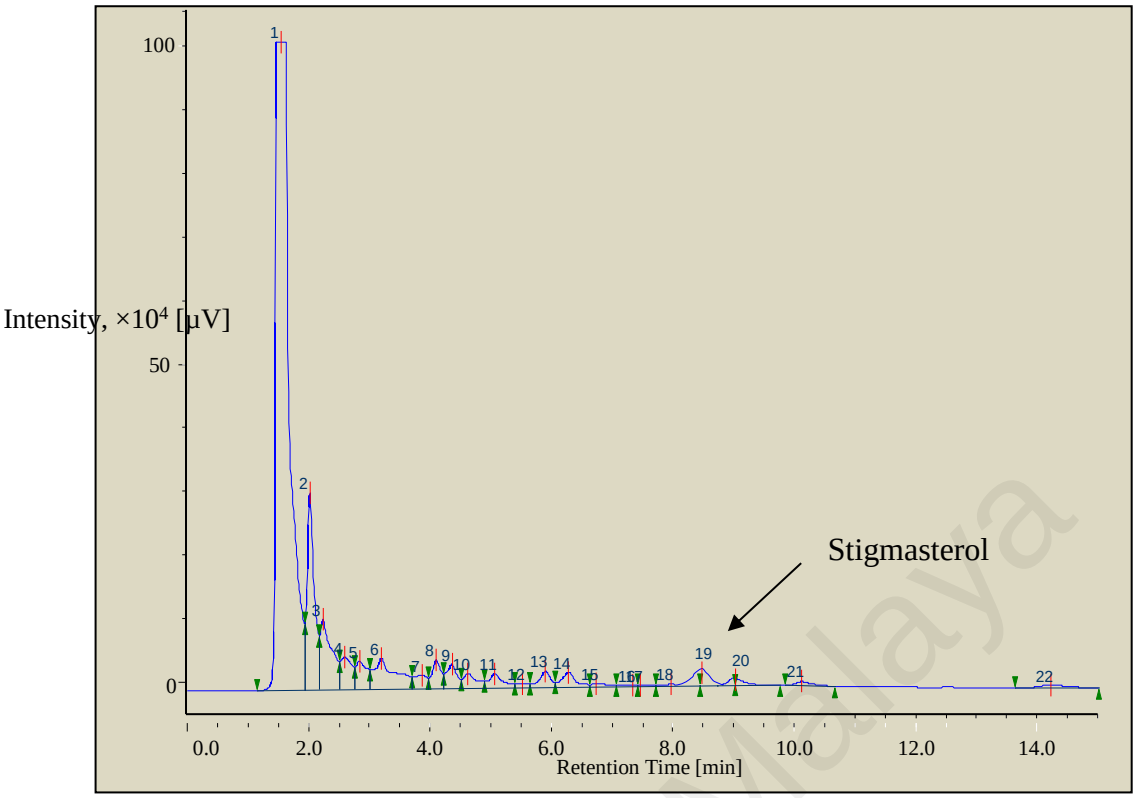


Figure 4.29: Leaf extracts chromatogram.

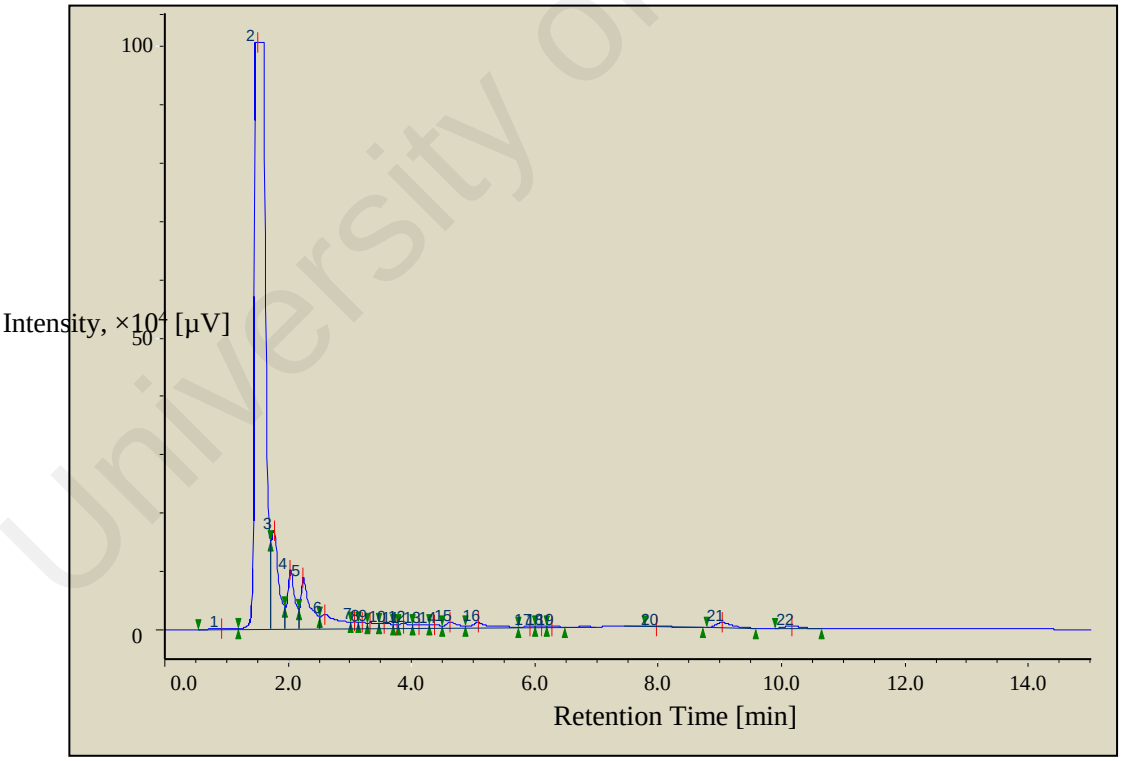


Figure 4.30: Stem extracts chromatogram.

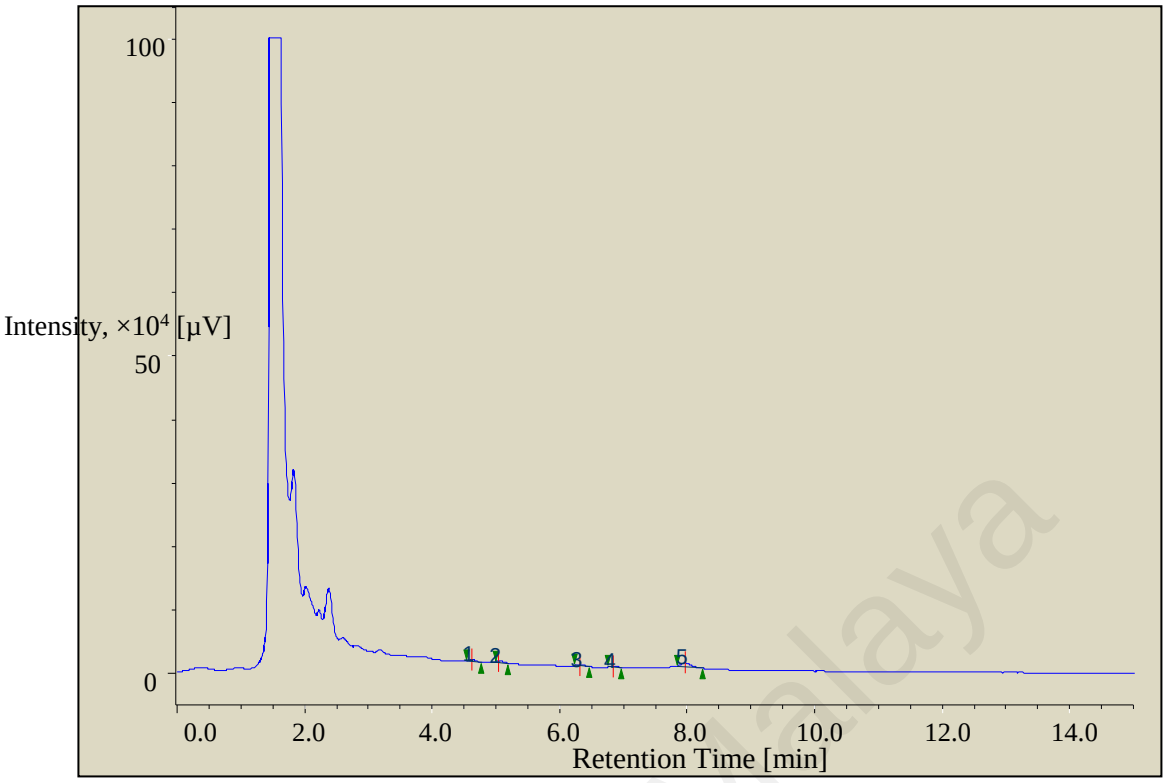


Figure 4.31: Callus extracts from callus induced on leaf explants supplemented with 3.00 mg/L NAA.

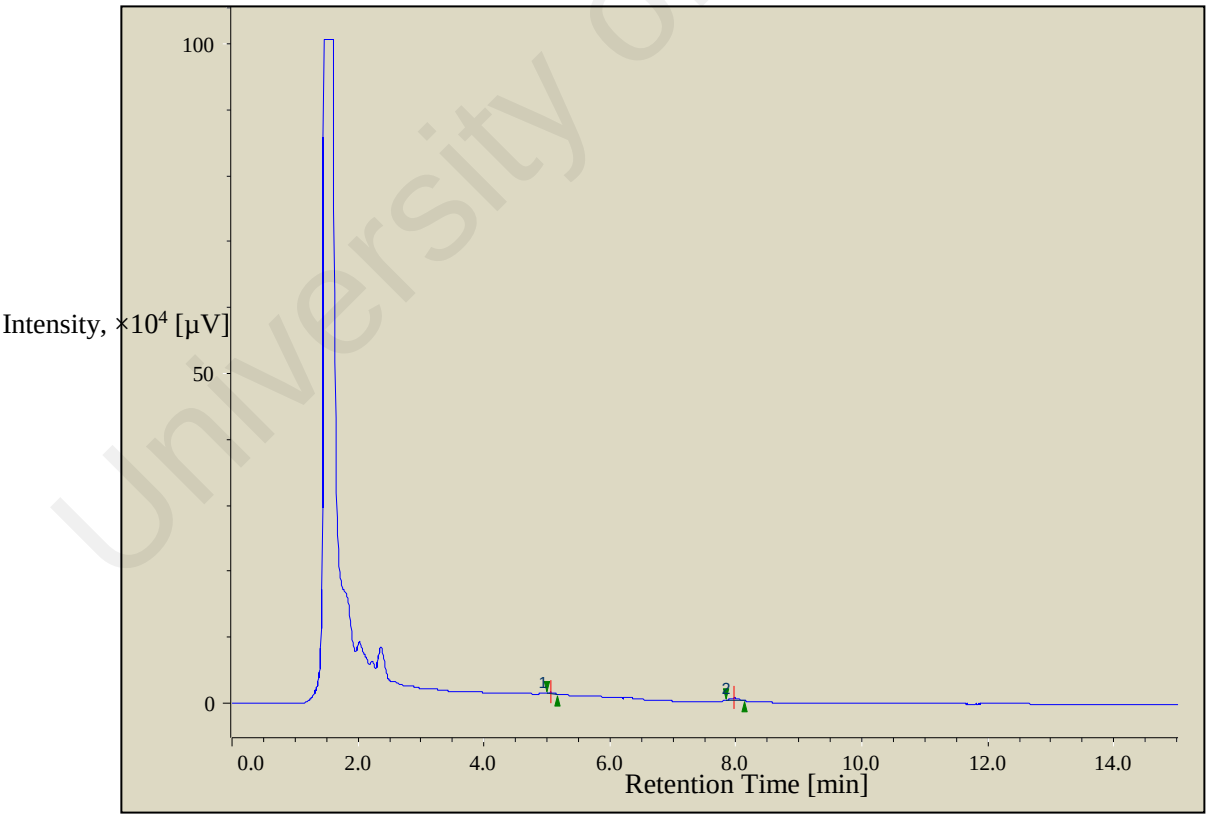


Figure 4.32: Callus extracts from callus induced on stem explants supplemented with 3.00 mg/L NAA.

Table 4.15: Concentration of stigmasterol and β-sitosterol in plant extracts for 1.50 g/mL.

Sample	Retention Time (RT, min)	Area, $\times 10^4$	Volumetric Concentration (mg/L)	Concentration (mg/g)
Stigmasterol				
Leaf	8.71	82.05	91.40	6.09×10^{-2}
Callus	8.69	1.94	4.20	2.80×10^{-3}
β-sitosterol				
Leaf	10.17	11.89	18.75	1.25×10^{-2}
Stem	10.53	70.56	11.67	7.78×10^{-3}

Fang et al. (2014) investigated the fingerprint of *L. nodiflora* methanolic extracts main significant secondary metabolites. Firstly the study had fractionated the extracts by serial purification and able to obtain eight flavonoids which are (1) 3,7,4',5'-tetrahydroxy-3'-methoxyflavone, (2) nodifloretin, (3) 4'-hydroxywogonin, (4) onopordin, (5) cirsiliol, (6) 5,7,8,4'-tetrahydroxy-3'-methoxyflavone, (7) eupafolin, (8) hispidulin, and (9) β-sitosterol. High Performance- Liquid Chromatography with ultraviolet detecttor (HPLC-UV) was used to analyse fingerprint analysis and quantitative determination of the main significant secondary metabolites which are onopordin and eupafolin, using its standard.

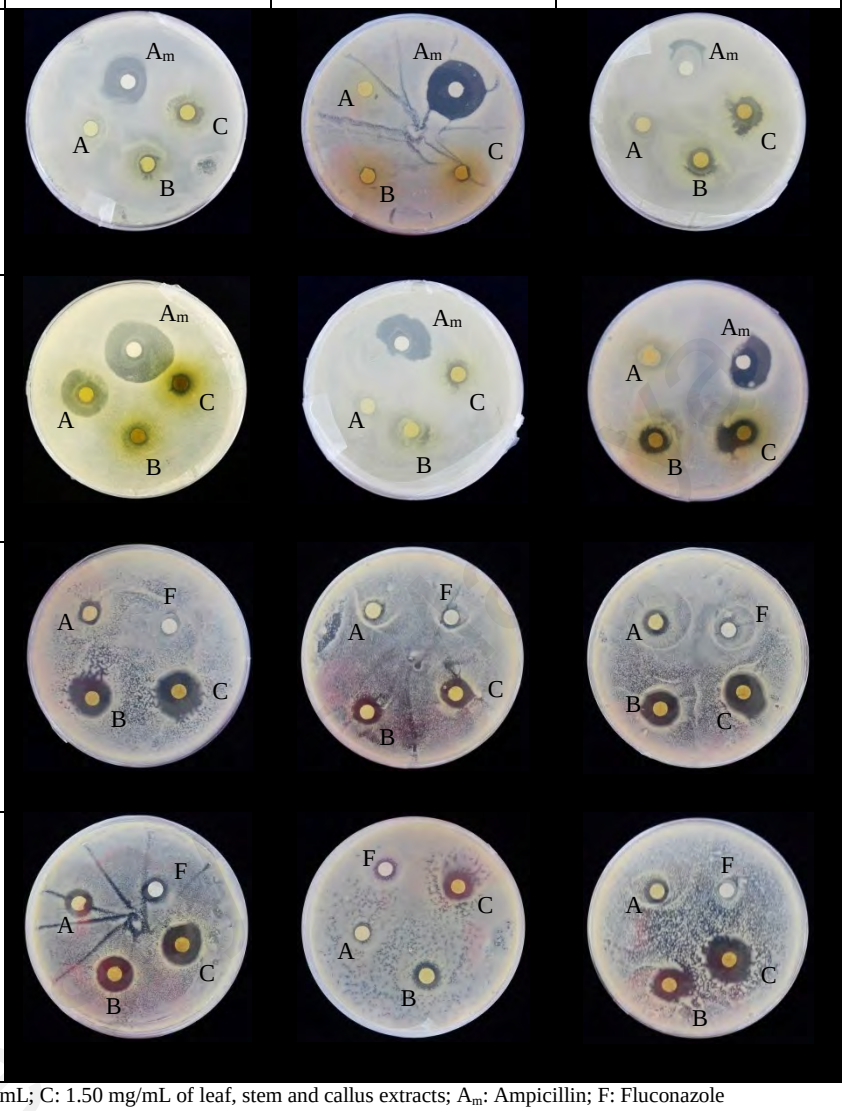
4.5.5 Antimicrobial Activity of leaf, stem and callus extracts of *Lippia nodiflora*

Two bacterial strains *Escherichia coli* and *Bacillus subtilis* with two fungal strains *Candida albicans* and *Aspergillus niger* were tested their susceptibility to *L. nodiflora* methanolic extracts. The positive control used for antibacterial and fungal compounds were ampicillin and fluconazole respectively. The extracts were tested at three different concentrations of 0.50, 1.00 and 1.50 mg/mL. Maximum zone of inhibition for all strains was observed using highest concentration 1.50 mg/mL for each leaf, stem and callus extracts as it gave the highest inhibition zone toward the microbial species either it is the nearest value to compare with the control or over it.

Firstly the bacterial strain *B. subtilis*, callus extract was more resilient than leaf and stem extracts with 9.00 mm inhibition zone however, not significant as the standard ampicillin. As for *E. coli*, leaf extracts inhibited the highest with 15.33 mm, followed by callus extracts, 11.33 mm. Leaf and stem extracts did not show potential on antibacterial property compared to callus extracts. *L. nodiflora* extracts on fungal strains gave significant inhibition zone for all leaf, stem and callus extracts. Maximum zone of inhibition for *C. albicans* was observed and callus extracts gave the highest inhibition zone with 15.00 mm while for *A. niger*, leaf extracts gave the highest with inhibition zone 11.33 mm with tiny margin compare to callus extracts with 11.00 mm.

Based on antifungal property, *L. nodiflora* extracts from leaf, stem and callus has shown greater potential than the control used which was fluconazole. In short, *L. nodiflora* plant has antimicrobial properties especially on its leaf explants compare with stem, however the callus induced from leaf explants was able to give better results than its initial form.

Table 4.16: Zone of inhibition of sample extract.

Type of microbes	Leaf	Stem	Callus
Bacillus subtilis			
Escherichia coli			
Candida albicans			
Aspergillus niger			

*A: 0.50 mg/mL; B: 1.00 mg/mL; C: 1.50 mg/mL of leaf, stem and callus extracts; A_m: Ampicillin; F: Fluconazole

Table 4.17: Mean value of zone of inhibition *L. nodiflora* extract on selected microbes.

Species of microbes	Type of extracts	Mean (mm)		
		Leaf	Stem	Callus
B. subtilis	Standard (ampicillin)	24.30±0.79^{a}	21.00±0.00^{a}	20.33±0.67^{a}
	0.50 mg/mL	6.16±0.20^{c}	5.33±0.33^{b}	5.00±0.00^{c}
	1.00 mg/mL	7.10±0.21^{bc}	5.33±0.33^{b}	7.67±0.67^{bc}
	1.50 mg/mL	8.10±0.31^{b}	6.07±0.13^{b}	9.00±0.00^{b}
E. coli	Standard (ampicillin)	23.00±0.11^{a}	24.50±0.68^{a}	24.33±0.13^{a}
	0.50 mg/mL	15.33±0.29^{b}	5.83±49^{c}	6.47±0.17^{c}
	1.00 mg/mL	8.07±0.43^{c}	7.13±0.23^{bc}	7.70±0.36^{bc}
	1.50 mg/mL	8.10±0.26^{c}	8.60±0.15^{b}	11.33±0.37^{b}
C. albicans	Standard (fluconazole)	5.33±0.33^{c}	5.23±0.12^{c}	5.70±0.21^{c}
	0.50 mg/mL	6.00±0.00^{c}	5.33 ±0.33^{c}	8.23±0.27^{c}
	1.00 mg/mL	8.56±0.53^{b}	8.27±0.14^{b}	11.33±0.33^{b}
	1.50 mg/mL	11.03±0.37^{a}	10.33±0.24^{a}	15.00±0.57^{a}
A. niger	Standard (fluconazole)	6.33±0.33^{c}	7.70±0.21^{ab}	7.70±0.10^{ab}
	0.50 mg/mL	5.00±0.00^{d}	6.67±0.44^{c}	7.33±0.73^{b}
	1.00 mg/mL	8.33±0.33^{b}	7.50±0.29^{ab}	9.00±0.00^{ab}
	1.50 mg/mL	11.30±0.33^{a}	8.67±0.33^{a}	11.00±0.00^{a}

Values are expressed as Mean±SE of triplicate measurements. A value of $p < 0.05$ was considered statistically significant (By one way ANOVA followed by Tukey's Honest Significant Difference test at a confidence level of 95%)

Malathi et al. (2011) reported that *L. nodiflora* extracts extracted from hexane, chloroform and alcohol respectively inhibited *B. subtilis.* A different extraction method was used by Zare et al. (2012) and it shows inhibition of gram positive bacteria were more susceptible than negative gram bacteria however, similarly *L. nodiflora* extracts shown better results for fungi. Even though smaller concentrations were used the inhibition percentage is higher may be due to different methods where they have used percolation method. *Aloysia triphylla* (Verbenaceae) was investigated by Parodi et al. (2013) and they found that the extracts acquired from three different temperatures and pressures produced different compounds and indicated that the present of monoterpenoids were important for its antimicrobial activity.

Overall, standard compounds for antibacterial shows better inhibition compared to other concentration of all extracts while fluconazole mostly had lower inhibiton than other concentration of all extracts. The figures showed that the higher the extract's concentration, the higher inhibition zone observed.

4.5.6 Antioxidant Activity on *Lippia nodiflora* Leaf and Stem Explants

Free radical scavenging activity was done by using DPPH assay to obtain information on the mechanisms of the antioxidative effects of *L. nodiflora* extracts. Their radical scavenging effects was examined by measuring changes of DPPH radicals at 517 nm in absorbance using spectrophotometer. The absorption maximum of a stable DPPH radical in methanol was at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidative activity of antioxidants (Chang et al., 2002).

Figure 4.33 and Table 4.18 showed the results obtained from DPPH assay in IC_{50} (μg/mL), ascorbic acid was used as a standard for this experiment and IC_{50} value for it is 205 μg/mL. The results revealed that the stem explants exhibited the radical scavenging activity with concentration of 5932 μg/mL, while leaf was 14535 μg/mL and callus was 30291 μg/mL. Figure 4.32 shows that as the concentration of sample increased, the percentage of scavenging activity also increasing. This result can be applied for all three sample extracts. However, stem explants possessed higher percentage of scavenging activity compared to leaf explants and callus with 92.20% compared to 36.40% and 16.40% for the highest concentration (10, 000 μg/mL) of extracts respectively.

Sudha and Srinivasan (2014) reported that methanolic extracts on aerial part of *L. nodiflora* was found to had significant scavenging effects with increasing concentrations with 79.35% inhibition compare to the control used 86.42% 2-deoxy-D-ribose, butylated hydroxyl toluene (BHT). Aerial part of *L. nodiflora* consists of exposed leaf and stem from an intact plant. This study on the other hand, extract leaf and stem separately to

identify which part of plant possess higher antioxidant activity. The average of both explants POI resulted with 64.3%, showing lower than Sudha & Srinivasan (2014).

Teoh et al. (2013) investigated *L. nodiflora* extracts with methanol and ethyl acetate however, the results showed that other than methanol extraction gave better result than ethyl acetate extraction, the leaf extracts gave also better result than stem extracts with 93.26%, 90.1% and 89.82%, 72.7% respectively, opposite with the current study. Initially, Teoh freeze dried her sample while the current study used raw materials to do soxhlet extraction. Also, it was found that the duration of the soxhlet apparatus was running during extraction was differed which Teoh run the extraction with 13 hours while the current study was 24 hours. Other than that, the extracts was kept at -80ºC while the current study mentioned that it was kept at -20ºC. So, these differences might affected the results from both studies and Teoh methods gave better results in antioxidant activity.

Thamaraiselvi et al. (2013) also investigated antioxidant activity on the leaf explants of *Lippia nodiflora* using Soxhlet apparatus and fractionated using methanol, chloroform, petroleum ether and hexane solvents. The result showed that the leaf extracts gave IC_{50} value of 79.37 µg/mL on ascorbic acid as control with 30.09 µg/mL.

To conclude, it shows that stem extracts has significant antioxidant property however, leaf and callus extracts obtained from the study was not as significant. As discussed earlier on callus growth curve of callus induction section, the extraction was done during the highest biomass collection which is around third to fourth week of callus induction. However, the callus might had not prepared or fully equipped with secondary metabolites comparable to *in vivo* plant similarly reported by Ng et al. (2016) mentioning primary metabolites has high concentration in callus and secondary metabolites has higher concentration in shoot, compared to callus in *Boesenbergia rotunda*. Ashokkumar et al. (2008) reported that phenolic compounds are associated with antioxidant activity.

Furthermore, the process of methanolic extraction usually extracted phenolic compounds, thus assuming this phenolic group contributed to the antioxidant activity of *L. nodiflora*. It has been suggested by Tanaka et al. (1998) that up to 1 g of polyphenolics compound ingested daily has inhibitory effects on mutagenesis and carcinogenesis in humans (Ashokkumar et al., 2008). Callus extracts on the other hand, gave good result on antimicrobial activity compare to leaf and stem explants when leaf and callus extracts possess antioxidant activity but it was not as significant as stem explants of *L. nodiflora.* Handling experiments need extra caution where trivial matters such as collection of young leaf as sample, overheating solvent on soxhlet apparatus, degradation of compounds during drying of extracts and the condition of extracts storage may lead to wrong data collection and interpretation.

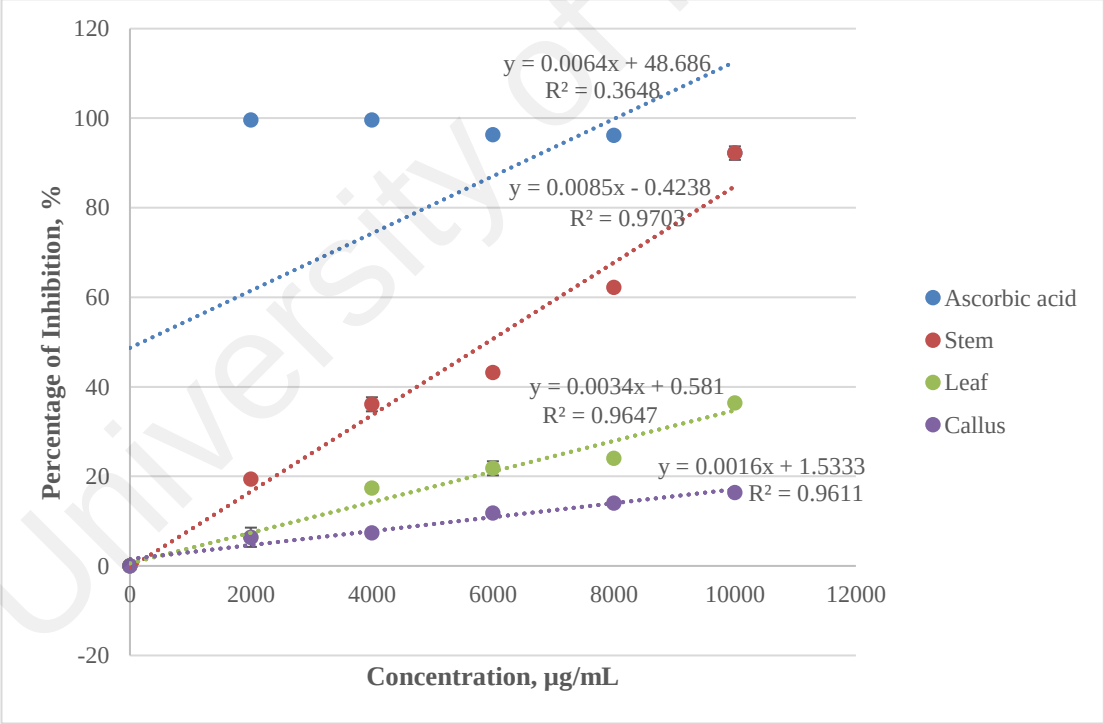


Figure 4.33: Scavenging activity against final concentration of sample.

Table 4.18: IC_{50} value antioxidant activity of for *L. nodiflora* extracts.

Sample Extracts	IC_{50} (µg/mL)
Ascorbic acid	205
Stem	5932
Leaf	14535
Callus	30291

4.6 Anticancer Study on Cell Line HCT 116

The anticancer study has been investigated on HCT116 human colon cancer cells with *Lippia nodiflora* leaf and stem extracts. The study has shown the effect of the extracts on the cancer cells with the percentage of inhibition (POI).

Figures 4.34 to 4.37 showed all four extracts percentage of inhibition (%) on HCT116 cancer cells against concentration (µg/mL) of *L. nodiflora* extracts. Table 4.19 shows half maximal inhibitory concentration of MTT assay for fresh and dried leaf and stem extracts. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide) assay is commonly used to identify the proliferation of cells by the determination of mitochondrial reductive function and hence it gave a good indicator of cell death or inhibition of growth (Pope, 2010). It was done to evaluate the cytotoxity of *L. nodiflora* extracts on HCT116 based on the percentage of inhibition of cells after 72 hours of treatment. According from percentage of inhibition, dried stem extracts gave the highest number with 95% followed by dried leaf extracts, 91%, then, fresh leaf extracts and stem extracts with 86 and 83% respectively. Reduction of purple color of the MTT had occurred from bottom to top (G to A well) at the 96 well plate was due to cell's death. The lighter color indicates higher cell's death and the dark color shows that higher cell counts.

IC_{50} value was calculated by the linear equation of every graphs shown on Figure 4.34 to 4.37. Where x is IC_{50} value and y is the percentage of inhibition. The IC_{50} values produced here were an indication where it was the first known reporting of their activity against cancer cell lines. Based on the result of IC_{50} value, which was not similar to percentage of inhibition, the dried leaf gave the lowest IC_{50} value followed by dried stem, fresh leaf and fresh stem extracts (19.15 µg/mL, 40.23 µg/mL, 47.80 µg/mL and 60.98 µg/mL respectively). The lower the IC_{50} value indicates the higher susceptibility of the cells towards the treatment. The cytotoxicity level of the extracts can be divided into

strong (<100 µg/mL), moderate (101- 200 µg/mL), and weak (>200 µg/mL) (Garbi et al., 2015). Therefore, all of the four samples had a strong level of inhibition and were capable of inhibiting HCT116 colon cancer cell lines.

Punitha et al. (2012) reported *Gmelina arborea* (Verbenaceae) was able to inhibit another type of colon cancer (COLO 201) along with Gastric cancer cells (HT- 29) and Human oesophagel cancer cells (TE- 2) cell by. The maximum cell proliferation percentage of inhibition was found to be 62% in Colon cancer cell (COLO 201), 80% in Gastric cancer cell (HT- 29) and 70% in Human Oesophagal cancer cells (TE- 2).

Other than colon cancer, Teoh et al. (2013) had found that *L. nodiflora* extracts was able to inhibit MCF7 breast cancer cell growth via apoptosis induction with leaf extracts which was extracted using ethyl acetate and methanol (EC_{50} value 0.4271 mg/mL and 0.6177 mg/mL respectively). Based on HPLC analysis, stigmasterol compound had been identified to be present in *L. nodiflora.* Stigmasterol had several records on successful anticancer study by elongating tested subject survival duration against Ehrlich Ascites Carcinoma (EAC) and also other study found reduction of tumor size in skin cancer (Ashokkumar et al., 2009; Ghosh et al., 2011; Ali et al., 2015).

Considering that *L. nodiflora* has a strong level of cytotoxicity based on its IC_{50} value, it has potential on anticancer activity for pharmaceutical use. It also proven to inhibit other cancer cells as discussed earlier and Verbenaceae family had many research on anticancer properties.

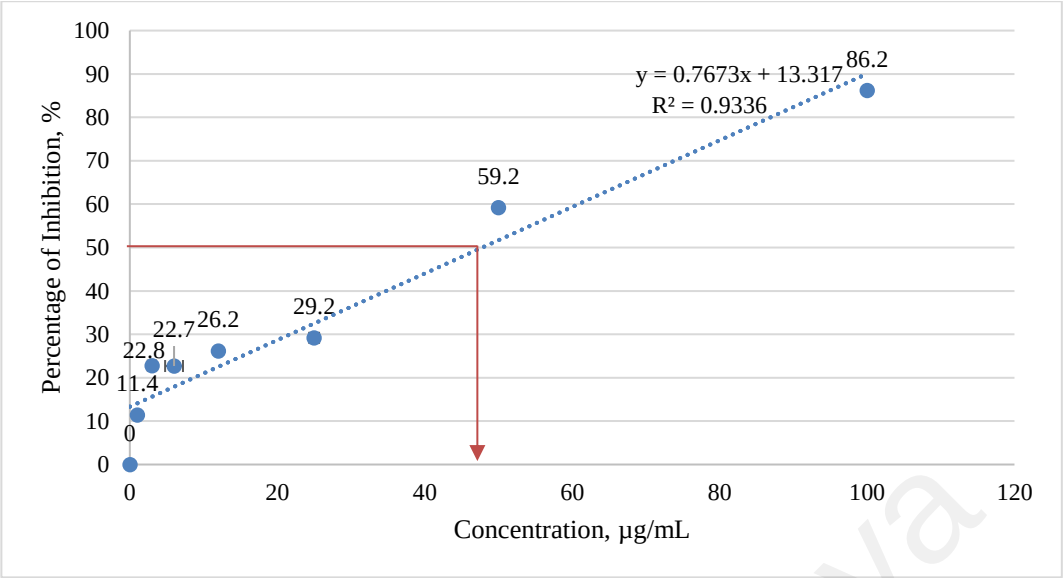


Figure 4.34: Percentage of inhibition, % versus concentration of *L. nodiflora* fresh leaf extracts.

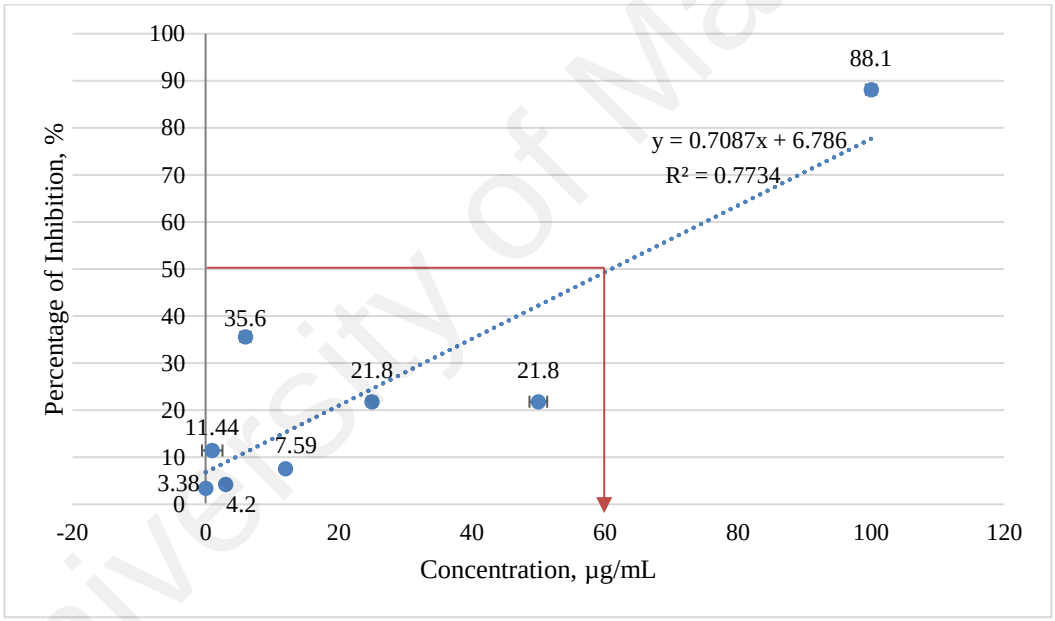


Figure 4.35: Percentage of inhibition, % versus concentration of *L. nodiflora* fresh stem extracts.

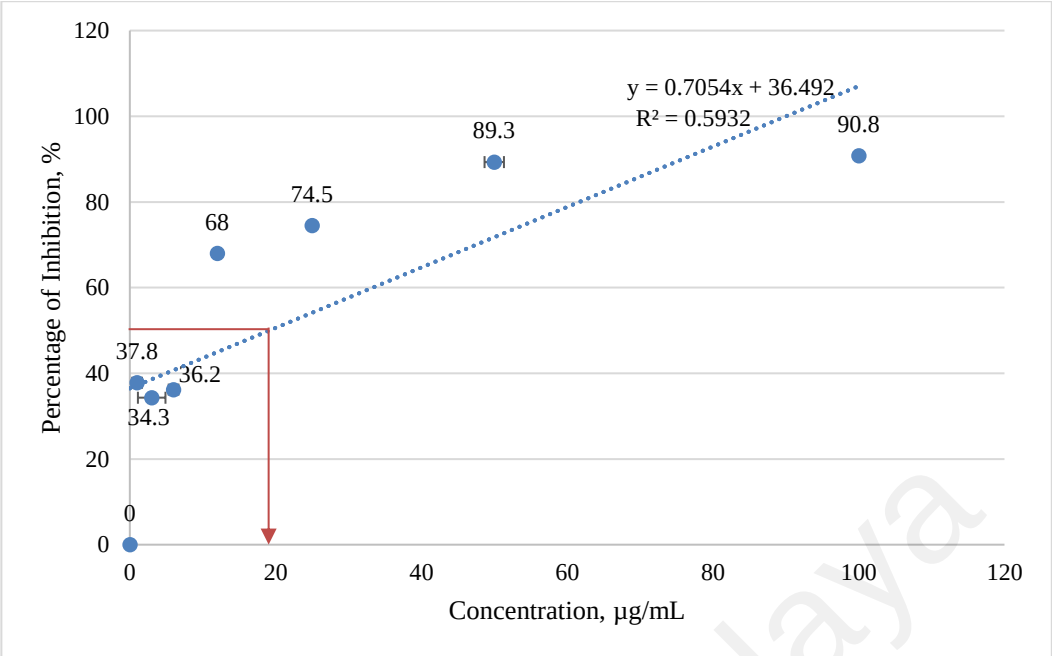


Figure 4.36: Percentage of inhibition, % versus concentration of *L. nodiflora* dried leaf extracts.

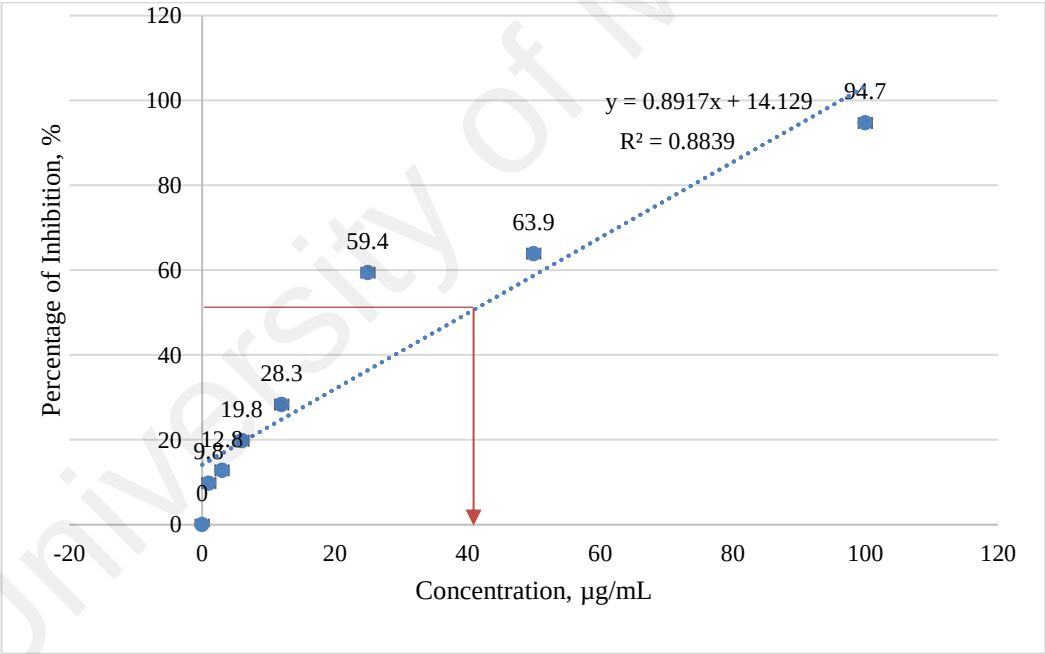


Figure 4.37: Percentage of inhibition, % versus concentration of *L. nodiflora* dried stem extracts.

Table 4.19: IC_{50} value of MTT proliferation assay for *L. nodiflora* extracts.

Sample Extracts	IC_{50} (µg/mL)
Fresh Leaf	47.80
Fresh Stem	60.98
Dried Leaf	19.15
Dried Stem	40.23

4.7 Correlation Analysis

Table 4.20 shows β- sitosterol (BS) was negatively correlated to antioxidant activity (AO) while stigmasterol (S) was shown a significant correlation towards anticancer activities (AC), as proven to be found in *L. nodiflora* based on HPTLC and HPLC analysis, which indicate that this substance is directly included in such activities. Hinkle et al. (2003) interpreted the size of correlation explaining that the range of 0.70 to 0.90 (-0.70 to 0.90) has high positive (negative) correlation between two variables while .00 to .30 (.00 to -.30) has negligible correlation. As correlation coefficient are used to assess the strength and direction of linear relationship, correlation coefficient of zero indicates no linear relationship while -1 or +1 indicates a perfect linear relationship and any correlation coefficient showing stronger correlation as it gets closer to ±1. Positively correlation coefficient described the variables are directly related while negative coefficient is inversely related (Mukaka, 2012).

Table 4.20: Pearson's correlation coefficients between the variables.

Correlation				
	BS	**S**	**AO IC50**	**AC IC50**
BS	1			
S	.829**	1		
AO IC50	-.745*	-.244	1	
AC IC50	.246	.747*	.463	1

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed)

CHAPTER 5: CONCLUSION

Lippia nodiflora was known for its ornamental value and also as a medicinal plant. The micromorphological study on the leaf of *Lippia nodiflora* also had shown the presence of oil gland along with the arrangement of the diacytic stomata and a distinct simple unicellular trichomes on the abaxial and adaxial leaf surfaces with stomata index 19.52% and 19.86% respectively. The trichomes exhibit an uncommon simple unicellular with two ends, connected to the epidermal tissue at the center of the trichome.

The induction of callus was successful on most MS media supplemented with plant growth regulators especially auxin and combinations of auxin and cytokinin. Callus induction was not obtained on the control condition which was MS media without plant growth regulators. Light green friable callus was obtained from callus induction with MS media supplemented with NAA and 2,4-D while mostly green compact callus was obtained on callus induction with MS media supplemented with auxins IAA, IAA, cytokinin BA, KN and combinations auxins and cytokinin.

The highest biomass and best nature obtained for single PGRs were 2.50 mg/L NAA (118.60 mg/L) with light green friable callus for auxin and 1.00 mg/L BA (42.60 mg/L) with green compact callus for cytokinin. The highest biomass and best nature for combination PGRs were 3.00 mg/L NAA + 2.00 mg/L KN (165.00 mg/L) with green compact and friable callus and 2.50 mg/L IBA + 1.00 mg/L BA (139.60 mg/L) with green compact callus. The *in vitro* growth curve of callus had shown that the optimum biomass was obtained on 3rd week for single PGRs and 3rd to 4th week for combination PGRs. Mass propagation of callus can be done through the exponential phase of the callus before the highest biomass reached as delays will resulted in stationary phase and cause slowing down of callus proliferation.

Overall, *in vitro* growth curve showed that stem explants has shown better callus induction response and biomass to compare with leaf explants. Compact callus also had given longer duration of callus proliferation than in friable callus from the exposure of browning effect that leads to dead callus.

Plant regeneration of *Lippia nodiflora* was successful in both control (MS media without plant growth regulators) and MS media supplemented with plant growth regulators. However, high response of plant regeneration was found in control (90%) in comparison with the tested treatment but the control was only able to produce one shoot at a time compare with other treatment. The control condition also gave the highest length (mean 6.40 ± 0.45 cm) with maximum over 8 cm long (8.50 cm longest). The highest number of shoots had found on combination PGRs IAA + BA (2.70 ± 0.14 shoots) maximum number of four shoots. The successfully regenerated plant was able to generate roots by inoculation in MS media supplemented with IAA and IBA PGRs. All concentrations of IAA and IBA was able to produce roots and 3.00 mg/L IAA had the highest amount five roots per plantlet.

Mass propagation of *Lippia nodiflora* plant *in vivo* and *in vitro* can be done using the control as the acclimatization took place, the plant was able to propagate healthily in a few weeks duration.

Lippia nodiflora has potential anticancer compounds including antimicrobial and antioxidant properties. The extraction process of *Lippia nodiflora* plant which was through methanolic soxhlet extractions was able to provide for antimicrobial, antioxidant and anticancer activities experiments. The Thin Layer Chromatography (TLC) experiment has showed the presence of steroids compound and High Performance Thin layer Chromatography (HPTLC) verify the presence of steroids compounds. High Performance Liquid Chromatography (HPLC) was tested with both steroids standard

compound stigmasterol and β-sitosterol which resulted that stigmasterol was present in *Lippia nodiflora.* Both stigmasterol and β-sitosterol had almost similar molecular formula ($C_{29}H_{50}O$ and $C_{29}H_{48}O$ respectively) as they were categorised under phytosterols that only present in plant, having similar chemical structure as cholesterols. Experiments that has been done was difficult to differentiate both compounds except for HPLC as their retention time (RT, minute) was different in acetonitrile: methanol (80:20) as mobile phase with RT 8.69 and 10.05 minutes respectively. Multiple experiments were done on TLC and HPTLC but the R_f values were almost similar with each other, hence stigmasterol and β-sitosterol were not able to be differentiate from the plant extracts.

Lippia nodiflora extracts has antimicrobial property which inhibit bacteria and fungi. However, its potential as antifungal activity is greater compared to antibacterial activity that was able to give better result than the control used (15 mm inhibition zone over 5 mm on *Candida albicans* respectively). Callus extracts gave the highest inhibition zone compared with leaf and stem extracts. Gas Chromatography Mass Spectrometry (GC-MS) chromatogram had identified 14 compounds from the methanolic extracts of *Lippia nodiflora* and one of the compound, tetradecanoic acid has the property of antimicrobial and antioxidant activities. The compound might be the source of antimicrobial activity as well as antioxidant activity. The antioxidant property present in *Lippia nodiflora* extracts showed potential from stem explants compared with leaf and callus extracts with IC_{50} 5932 µg/mL (stem extracts) over 205 µg/mL (ascorbic acid as control). Overall, stem extracts has significant antioxidant property that can assist in the pharmaceutical industry.

This study has been aimed on colon cancer HCT116 cells, where preliminary assay (MTT assay) was done to investigate the inhibition of cancer cells production. *Lippia nodiflora* extracts was able to inhibit the cancer cells where dried leaf extracts gave the highest inhibition (19.15 µg/mL) however, the IC_{50} value of all tested fresh and dried leaf

and stem extracts had a near value which all treatments gave IC_{50} value of strong level of cytotoxity against cancer cells with IC_{50} value <100 µg/mL.

Stigmasterol is a natural product that has anticancer property with successful records against several types of cancer cells. As the phytochemical screening proven that stigmasterol was present in *Lippia nodiflora*, this compound can be suggested as the source of the anticancer property that assist in the anticancer activity. Thus, *Lippia nodiflora* has high potential to assist in the medical and pharmaceutical industry especially on anticancer study to aid successful treatment for cancer.

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