IN VITRO CULTURES OF *CURCUMA MANGGA* VAL. FOR THE PRODUCTION OF (*E*)-LABDA-8(17), 12-DIENE-15, 16-DIAL

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ABSTRACT

Curcuma mangga Val., commonly known as the mango ginger has been used traditionally as a seasoning for food and as a treatment for stomach aches, fever, and cancer. Due to its medicinal importance, a systematic approach was taken to establish a rapidly growing suspension culture of *C. mangga*. In initiating callus, various responses were obtained from shoot bud explants cultured on MS basal medium supplemented with different concentrations of 2,4-D, NAA and IAA either alone or in combinations. Different concentrations of sucrose were also tested. Rapid growing friable callusobtained from shoot explants cultured on MS basal medium supplemented with 1 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose and 2 g l^{-1} gelrite were selected for the initiation of suspension cultures based on histological morphology. From various medium screened, rapid growing suspension cultures were established by using MS liquid medium supplemented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 30 g l^{-1} sucrose, 0.1 g l^{-1} malt extract, 0.5 mg l^{-1} d-Biotin, 100 mg l^{-1} glutamine, 5 mg l^{-1} ascorbic acid and citric acid respectively. Phenolic compounds production was most effectively controlled by the incorporation of ascorbic and citric acid as antioxidants.

(*E*)-labda-8(17),12-dien-15,16 dial is one of the bioactive compounds isolated from rhizomes of *C. mangga*. Recently, the cytotoxicity of this compound against cancer cells has been reported. (*E*)-Labda-8(17),12-dien-15,16 dial was extracted from cells, callus, rhizomes and shoots using solvent extraction method. Gas Chromatography (GC) and Gas Chromatography Flame Ionization Detector (GCFID) was used to compare the quantity of (*E*)-labda-8(17),12-dien-15,16 dial production in suspension cells and callus induced through various treatment and at different growth period.The presence of this compound in field grown rhizomes and *in vivo* shoot buds samples were also tested. The amount of (*E*)-labda-8(17),12-dien-15,16 dial in *in vivo*

sources was higher than the *in vitro* sources, but the presence can be enhanced through various method in future studies.

ABSTRAK

Curcuma mangga Val. atau lebih dikenali sebagai temu pauh, secara tradisinya digunakan sebagai perasa dalam makanan dan juga untuk merawat demam, sakit perut dan kanser. Disebabkan kepentingan perubatan spesis ini, langkah yang sistematik telah disusun untuk menghasilkan kultur sel ampaian yang aktif membiak dan membahagi. Dalam uji kaji menghasilkan kalus, pelbagai keputusan didapati daripada eksplan tunas pucuk yang dikultur di atas media pepejal MS yang dirawat dengan beberapa kepekatan dan kombinasi hormon 2,4-D, NAA dan IAA yang berbeza. Kesan kepekatan sukrosa yang berbeza turut diuji. Kalus rapuh (friable) yang giat membahagi diperolehi daripada media pepejal MS yang dirawat dengan 1 mg l^{-1}2,4-D, 30 g l^{-1}sukrosa dan 2 g l^{-1} fitagel. Kalus ini dipilih untuk memulakan kultur sel ampaian berdasarkan histologi kalus ini. Berbanding dengan pelbagai rawatan berbeza yang dikaji, sel ampaian yang aktif membahagi diperolehi daripada media cecair MS yang dirawat dengan 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 30 g l^{-1} sukrosa, 0.1 g l^{-1} ekstrak malt, 0.5 mg l^{-1} d-Biotin, 100 mg l^{-1} glutamin, 5 mg l^{-1}asid askorbik dan 5 mg l^{-1}asid sitrik. Penghasilan sebatian fenolik berjaya dikawal melalui penggunaan asid askorbik dan asid sitrik sebagai antioksidan.

(*E*)-labda-8(17),12-dien-15,16 dial adalah salah satu sebatian bioaktif yang diekstrak daripada rizom *C. mangga*. Sitotoksiti sebatian ini terhadap sel-sel kanser telah dilaporkan baru baru ini. Dalam kajian ini, (*E*)-Labda-8(17),12-dien-15,16 dial telah diekstrak daripada sel ampaian, kalus, rizom dan tunas pucuk dengan menggunakan teknik “cold soak”atau pengekstrakan pelarut. Gas Kromatografi (GC) dan “Gas Chromatography Flame Ionization Detector” (GC-FID) digunakan untuk memeriksa dan membandingkan kuantiti (*E*)-labda-8(17),12-dien-15,16 dial yang dihasilkanoleh sel-sel suspensi dan kalus yang diperolehi daripada pelbagai rawatan media dan pada tempoh pertumbuhan yang berbeza dengan rizom biasa dan tunas pucuk. Keputusan menunjukkan kehadiran (*E*)-labda-8 (17),12-dien-15,16 dial dalam

sampel yang diuji. Kuantiti (*E*)- labda-8 (17),12-dien-15,16 dial dalam sumber *in vivo*di dapati lebih tinggi daripada sumber *in vitro*. Walaubagaimanapun, manipulasi faktor kimia dan fizikal dipercayai dapat mempertingkatkan kuantiti (*E*)- labda-8 (17),12-dien-15,16 dial dalam kajian pada masa depan.

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I FEEL TRULY BLESSED

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Description
%	Percent
ºC	degree celsius
±	more less
:	is to
&	and
^{13}C NMR	carbon-13 nuclear magnetic resonance
2,4-D	2,4-dichlorophenoxyacetic acid
<	less than
>	more than
µl	microlitre
amp	ampere
cm	centimeter
C	carbon
$C_{20}H_{30}O_2$	(*E*)-labda-8(17),12-diene-15,16-dial
DCM	dichloromethane
DNA	deoxyribonucleic acid
e.g.	*exempli gratia* (for example)
et al.	*et alia* (and others)
Fig	Figure
g	gramme
g l^{-1}	gramme per litre
GC	gas chromatography
GC-FID	gas chromatography- flame ionisation detector

GC-MS	gas chromtography-mass spectrometry
H	hydrogen
HCl	hydrogen chloride
$HgCl_2$	mercury chloride
HOCl	hypochlorous acid
IAA	indole-3-acetic acid
kPa	kilopascal
LC	liquid chromatography
min	minute
mg	milligramme
mg l^{-1}	milligramme per litre
ml	millilitre
mm	millimetre
m/z	mass-to-charge ratio
M	Molar
MeOH	methanol
MS	Murashige and Skoog
MS	mass spectrometry
nm	nanometre
NAA	napthaleneacetic acid
NaOCl	sodium hypochlorite
NaOH	sodium hydroxide
NMR	nuclear magnetic resonance
O	oxygen
pH	potential hydrogen

ppm	part per million
pTLC	preparative thin layer chromatography
PGR	plant growth regulators
rpm	range per unit
R_f	retention factor
RNA	ribonucleic acid
sec	second
SCV	settle cell volume
S.D	standard deviation
S.E	standard error
TLC	thin layer chromatography
UV	ultra violet light
v/v	volume per volume
V	volt
w/v	weight per volume

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CHAPTER 1: INTRODUCTION

Curcuma mangga Valeton & C. van Zijp belongs to Zingiberaceae family, and it is among 80 species of rhizomatous herb plants of *Curcuma* genus. This species of *Curcuma*manifests most distinct identity of raw mango flavour combined with that of ginger in the rhizome due to the presence of car-3-ene, cis- and trans- dihydroocimene, ocimene and mycrene (Achut & Bandyopadhyaya, 1984; Singh *et al*., 2003; Sasikumar, 2005). The natural pigment from this species has been reported to show antioxidant, anti-inflammatory, antitumour, anti-tubercular, antifungal, antibacterial and antiprotozoal activities (Richomme *et al*., 1991; Habibi *et al*., 2000, Kirana *et al*., 2003, Chan *et al*., 2008; Tewtrakul &Subhadhirasakul*et al*., 2007, Liu & Nair, 2011; Malek *et al*., 2011).

Various secondary metabolites have been isolated from *C. mangga*(Liu & Nair, 2011; Malek *et al*., 2011, Abas *et al.*, 2005; Singh *et al.* 2002; Gupta *et al.* 1999; Ibrahim *et al*, 1999; Wong *et al.* 1999), however there is little information on the *in vitro* production of secondary metabolites. Although these secondary metabolites can be obtained from field grown rhizomes, enhanced production of secondary metabolites from *in vitro* sources could ensure continuous supply of consistently high quality compounds under controlled environment.

To date only two reports are available on *in vitro* direct regeneration of *C. mangga* (Raihanna *et al*., 2011; Abraham *et al*., 2011). However, no reports are available on *in vitro* production of secondary metabolites or establishment of suspension culture of this species. A stable callus and suspension culture established, with a systematic protocol is critical to enable the manipulation of cultures for enhanced production of secondary metabolites. The success of *C. mangga* cultures and the production of its secondary metabolites are greatly dependent on the culture medium used and particularly the exogenous application of plant growth regulators such as 2,4-

D, IAA and NAA. Growth period of *in vitro* cultures also plays a crucial role in production of secondary metabolites.

(*E*)-Labda-8(17),12-diene-15,16-dial is the compound of interest reported in this study. Its inhibition activity against lung, colon, gastric and breast tumour cell lines have been reported earlier (Malek *et al*., 2011; Liu & Nair, 2011). Other than these, (*E*)-Labda-8(17),12-diene-15,16-dial also shows antibacterial, antiprotozoal, antifungal, antitubercular, antioxidant and anti-inflammatory activity (Chan *et al*., 2008; Yunbao & Muraleedharan, 2010; Tewtrakul & Subhadhirasakul *et al*., 2007; Kirana *et al*., 2003; Habibi, *et al*., 2000; Richomme *et al*., 1991). In this study, (*E*)-Labda-8(17), 12-diene-15, 16-dial production was quantitatively compared between selected *in vivo* and *in vitro C. mangga*sources.

Cold soak extraction method were utilized for extraction of compounds. Combination of gas chromatography (GC) and flame ionization detector (FID) were used to quantitate and detect the amount and presence of (*E*)-Labda-8(17),12-diene-15,16-dial in various *in vivo* and *in vitro* sources of *C. mangga.*

OBJECTIVES

The objectives of this study:

1) To develop suitable callus culture from shoot bud of*Curcuma mangga* Val. for esthablishment of suspension culture
2) To establish protocol for cell suspension culture of*C. mangga*
3) To compare production of (*E*)-Labda-8(17), 12-diene-15, 16-dial from different sources of *C. mangga* namely *in vivo* rhizomes, *in vivo* shoot buds, calli of different morphology initiated through different treatments, friable calli and suspension cultures at different growth period

CHAPTER 2: LITERATURE REVIEW

2.1 General description

The genus *Curcuma* belongs to the family Zingiberaceae comprising more than 80 species of rhizomatous herbs (Hussain *et al.*, 2008; Lemmo & Schulman, 2004). This family comprises of about 52 genera and 1300 species. Besides *Curcuma*, which morphologically resembles ginger and turmeric plants, this family also includes cardamon, large cardamon, melegueta pepper and grain of paradise. They originated from the warm and humid environment of the Indo-Malayan region and are distributed widely by migrants in the tropics from Asia, Africa and Australia (Sasikumar *et al.*, 2005). Mango ginger (*Curcuma mangga* Val.) or locally known as temupauh or temumangga, which has mango flavour that has been attributed to volatile compounds such as car-3-ene and *cis*-ocimene (Achut *et al*., 1984). *Curcuma mangga*, as all the other plant species in zingiberae tribe has both modern and traditional medicinal values besides culinary value that are extensively used in the preparation of pickles, preservatives, candy, sauce and salad. The classification of *C. mangga* is described in Table 2.1.

Table 2.1: Classification of *C. mangga.* Source (Bisby *et al*, 2007)

Kingdom : Plantae
Division : Magnoliophyta
Class : Liliopsida
Order : Zingiberales
Family : Zingiberaceae
Tribe : Zingiberae
Genus : *Curcuma*
Species : *Curcuma Mangga*

2.2 Common names

Curcuma mangga Valeton & Zijp has many common names as listed in Table 2.2.

Table 2.2: Common names of *C. mangga*Val.

Language	**Name**
English	Mango ginger
French	Gingembremangue
Hindi	Am Haldi, Amada Haldi
Malay and Indonesia	Temupauh, Temumangga
Tamil and Malayalam	Mangainchi, Mangayincchi
German	Mangoingwer
Japanese	Mango Taamerikku
Nepalese	KapuurHaledo
Portuguese	Acafrao
Thailand	Khamin-kao
Arabic	Daruhaldi
Persian	Darchula
Bengali	Amada

Source: CAB International, 2007 ; Velayudhan *et al.*, 1999

2.3 Plant description

This herbaceous perennial plant can grow up to 90 to 130 cm tall. The leaves are narrowly ovate or elliptic with a purple costa or entirely green. Young inflorescence is available during the beginning of rainy season. Spike-like and cylindrical inflorescences are usually produced outside the leaves. The sterile flowers are pure white with yellow midlobe on lip. The stamen with filament is 3 mm by 3 mm with anther affixed near its base, pollen sacs are 4 to 5 mm long, spurs are narrow parallel and slightly curved (figure 2.1). The flower can last for 3 months (Newman *et al*., 2004; Holttum, 1950).

Botanically mango ginger is not related to neither mango nor ginger but to turmeric (*C. longa*). The inner part of the rhizomes are less yellow compared to the outer part, with a sweet smell of unripe mango when crushed (figure 2.2). The young rhizomes are usually white in colour and are produced 4 to 5 months after planting (refer figure 2.2). Eventhough this perennial herb has rhizome that is morphologically similar to ginger (Newman *et al*., 2004), it has a shorter life cycle of six months and it differ in biochemical composition including starch granules. Structure and size of the mango ginger starch granules varies significantly from turmeric and gingers by the absence of fissures on the surface and also its X-ray diffraction pattern (Policegoudra & Aradhya, 2007). The fresh rhizomes compose of 86% moisture, 0.8% ash, 0.8% total sugars, traces of reducing sugars, 1.4% fibre, 0.1% essential oil and 6.9% starch whereas dried rhizomes consistof 5.7% ash, 5.8% total sugar, traces of reducing sugars, 10.6% crude fibre, 0.9% essential oil and 45.6% starch. The mango ginger starch contains of 43% amylase (Policegoudra & Aradhya, 2007).

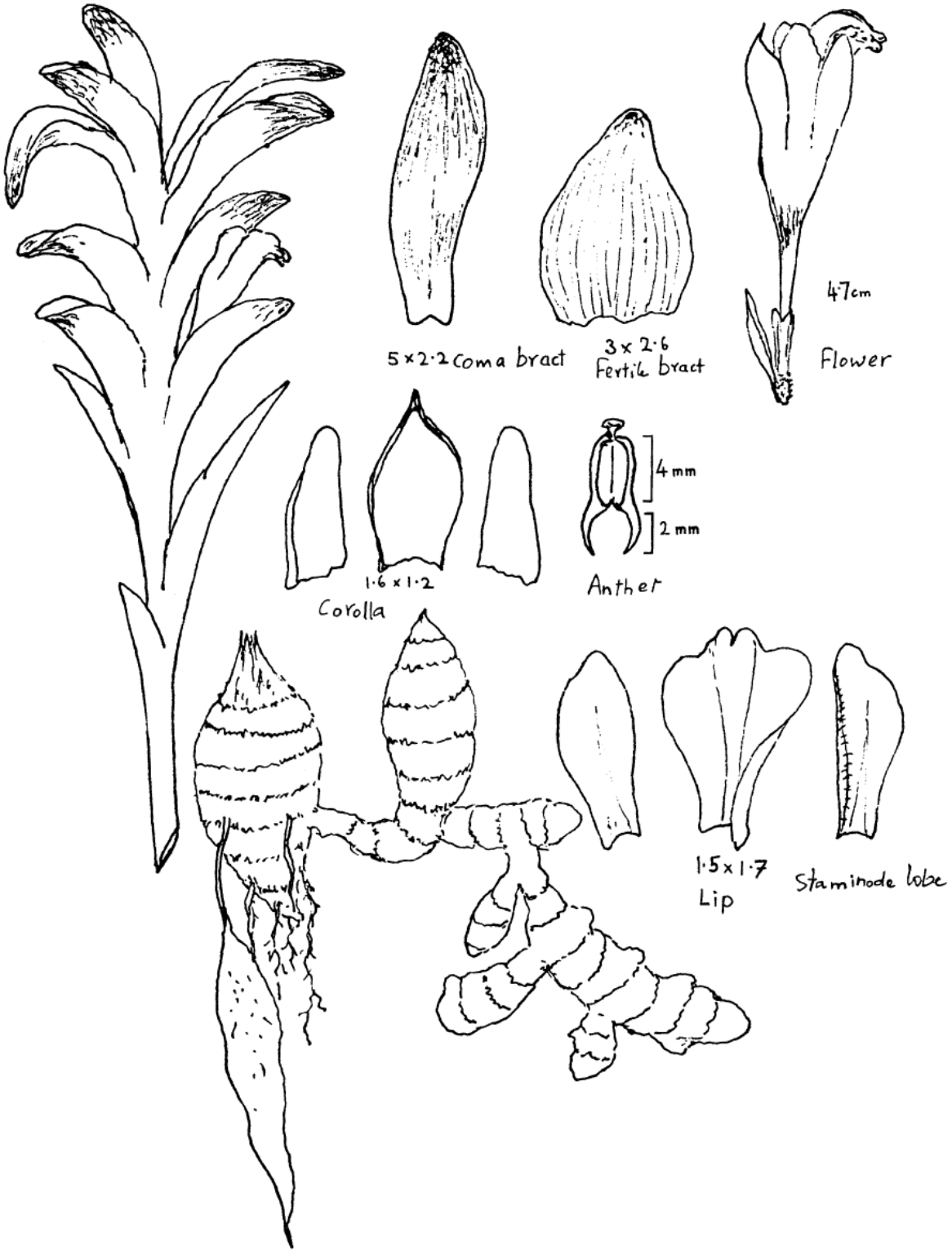

Figure 2.1: Morphology of *C. mangga* Val. (Adapted from Velayudhan *et al.*, 1999)

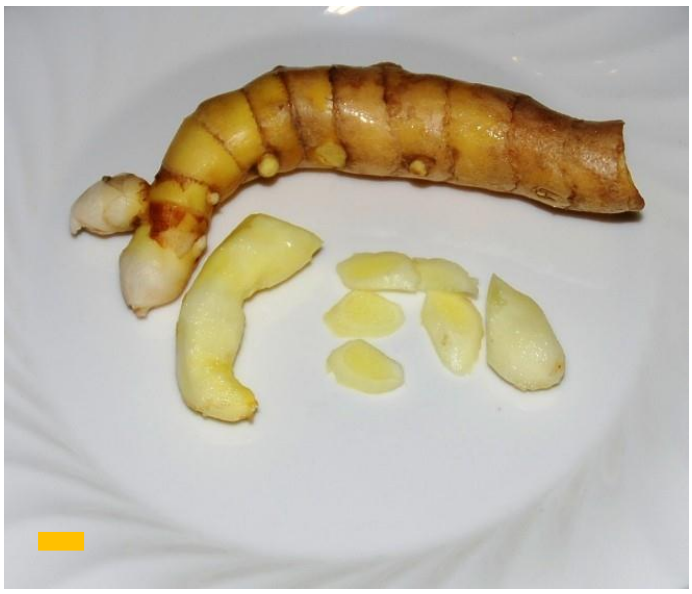

Figure 2.2: Rhizome of *C. mangga*(Bar represent = 1 cm)

2.4 Common and medicinal use

C. mangga has many traditional uses. The rhizomes of mango ginger are used to treat stomach and chest pains, fever and debility (Abas *et al*., 2005; Park & Kim, 2002; Hong, *et al*., 2001; Syu *et al*., 1998). As in ayurveda, the oldest system of Indian medicine, importance has been given to mango ginger rhizomes as an appetizer, aphrodisiac, and laxative. It is also used against biliousness, itching, skin diseases, bronchitis, asthma, hiccups, and inflammation due to injuries. Moreover, according to the Unani system of medicine, it is a diuretic, maturant, emollient, expectorant, antipyretic and appetizer. This species of ginger is also used against inflammation in mouth, ear and gleets; ulcers in male sex organs, scabies, lumbago and stomatitis (Abas *et al*.,2005; Warrier *et al*., 1994; Kirtikar& Basu*et al*., 1984; Burkill, 1966). *C. mangga* is also widely used in pharmaceutical and cosmetic industry mostly as cream in skincare therapy. Recent studies have shown that *C. mangga* has diverse bioactive molecules demonstrating antibacterial, antifungal, insecticidal, aphrodisiac, antipyretic, anti-

inflamatory, antihypercholesterolemic, anti-tumour and antioxidant properties (Liu & Nair, 2011; Philip *et al*.,2009; Tewtrakul & Subhadhirasakul, 2007; Abas *et al*.,2005; Kirana *et al*.,2003). Due to its exotic flavour and medicinal property, they are also used in the preparation and as natural preservatives of foods and beverages. To date it is still used in postpartum care, specifically to aid healing of wounds (Abas *et al*., 2005; Hong *et al*., 2001; Park & Kim, 2002; Syu *et al*., 1998).

2.5 Secondary metabolites and its importance

There are two types of metabolites produced by plants; primary and secondary metabolites. Carbohydrates, lipids, amino acids and protein are primary metabolites. Primary metabolites are very essential for plant growth and metabolism hence they are synthesised in large quantity. Secondary metabolites are compunds biosynthetically derived from primary metabolites through modifications, such as methylation, hydroxylation and glycosylation. They are synthesised due to biotic or abiotic stresses, limitation of nutrients or attack by micro-organisms (Yazaki *et al*., 2008; Yeoman & Yeoman, 1996). Secondary metabolites are not involved in the basic metabolic processes of living cells.

Naturally, secondary metabolites are more complex than primary metabolites. They are classified on the basis of chemical stuctures (e.g., aromatic rings, sugar), composition (presence of nitrogen) and their solubility in various solvents or pathway by which they are synthesized. Secondary metabolites are categorised into terpenes (e.g. monoterpenes, labdanes, sesquiterpenes, diterpenes and sterols), phenolics (e.g. coumarins, flavonoids, and lignans) and nitrogen and/or sulphur containing compounds (e.g. alkoloids, glucosinolates). Terpenes are composed entirely of carbon and hydrogen, meanwhile phenolics are composed of simple sugars, benzene rings,

hydrogen and oxygen (Chinou, 2008; Ramawat, 2007; Sarin, 2005; Verpoorte *et al*., 2002). Terpenoid is the largest group of secondary metabolites and is about one third of all known compounds (Verpoorte *et al.,* 2000).

Plants produce secondary metabolites which are often specific to an individual species or genus. Usually, secondary metabolites are present in smaller quantities but are very much essential on the plants interaction with its environment. Plant secondary metabolites play major roles on plant survival by producing constitutive chemical and phytoalexins to defend against predators, pathogens, insects, microbial infection and disease (Verpoorte & Memelink, 2002; Harbrone, 2001; Dixon,2001). As in reproduction system, plant secondary metabolites help in attracting pollinators.

In our everyday life, plant secondary metabolites are used as medicines, flavours, fragrance, dyes and insecticides. Food quality, colour and smell of ornamental plants and flowers can also be determined through secondary metabolites (Verpoorte & Memelink, 2002; Verpoorte *et al,* 2000). Recently, many secondary metabolites from plants have been studied for their medicinal values. Among them are extract of *Alpinia officinarum* and *Ocimum Basilicum* with anti-microbial and anti-inflammatory properties (Subramanian *et al*., 2008; Selvakkumar *et al*., 2007) and extract of *Costus pictus* with anti- diabetic property (Shilpa *et al.,* 2009). Taxol, vincristine, vinblastine, metformin, morphine and aspirin are also very important bioactive molecules discovered in plants (Raskin & Ripoll, 2004).

2.5.1 Secondary metabolites of *Curcuma* sp.

Secondary metabolites in most *Curcuma* sp. are used in our everyday life as fragrance, medicine and dyes. Most of its usage involves the component of secondary metabolites in its rhizome such as linalool, saprole, sesquiterpenoids, sesquiterpense, pinene, terpenoids, zingiberol and zingiberene (Aggarwal *et al*., 2007)

The main pigment in *Curcuma* genus is curcuminoids which comprised of curcumin, demethoxycurcumin and bisdemethoxycurcumin. Curcuminoids are found in *Curcuma* species such as in *C. domestica, C. xanthorrhiza, C. mangga, C. zedoria* and *C. viridiflora* (Jang *et al.,* 2004; Park & Kim, 2002; DNP 2001; Matsuda *et al.,* 2001; Gupta *et al*., 1999; Nakayama *et al*.,1993). Curcuminoids has been shown to exhibit antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, anticancer activities and thus has potential against diseases such as diabetes, allergies, arthritis and Alzheimer's disease(Aggarwal *et al*., 2007). There are various other essential bioactive compounds in this genus that exihit important biological activities. Table 2.3 shows a summary of chemical compounds and Table 2.4 shows a summary of biological activities of some *Curcuma* species.

Table 2.3: Overview of chemical compounds in some *Curcuma* species

Species	**Compounds**	**References**
C. aeruginosa	Dehydrocurdione	Jirovetz *et al*., 2000
	Curcumenol	DNP, 2001
	β- Elemene	DNP, 2001
	Curzerenone	DNP, 2001
C. parviflora	4R-acetoxycadina-2,9-diene-1,8-dione	Sadhu *et al.*, 2009
	1R,3R,4-trihydroxy-9-cadinen-8-one	Sadhu *et al.*, 2009
	Cadalenequinone	Toume *et al*., 2004
	8- Hydroxycadalene	Toume *et al*., 2004
	Parviflorene A, B, C, D, E, F	Toume *et al*., 2004
C. heyneana	Epoxide	Sukari *et al.*, 2010
	Isocurcumenol	Sukari *et al*., 2007
	Curcumanolide A, B	DNP, 2001
C. longa	curculonone A, B, C, D	Jih *et al.*, 2010
	6α-hydroxycurcumanolide A	Jih *et al*., 2010
	β-Curcumene	Sacchetti *et al*., 2005
	Terpinolene	Sacchetti *et al*., 2005,
	Curcuminoids	Jitoe *et al.*, 1992
C. xanthorrhiza	Xanthorrhizol	Wibowo *et al*., 2012
		Devaraj *et* al., 2010
	Zingiberene	Hwang *et al*., 2000
C. zedoaria	Germacrone	Makabe H *et al.*, 2006
	Dihydrocurdione	Navarro *et al*, 2006.
	Curcumenone	Christiane *et al*., 2006

Table 2.4: Overview of biological activities of some *Curcuma* species

Species	**Bioactivity**	**References**
C. aeruginosa	Antioxidant Antitumor	Jitoe *et al*.,1992 Murakami *et al*., 1998
C. aromatica	Xanthorrhizol 1H-3a, 7–methanoazulene Curcumene NO-scavenging Anticancer	Choochote *et al*, 2005 Choochote *et al*, 2005 Choochote *et al*, 2005 Baliga *et al*., 2003 Baliga *et al*., 2003
C. heyneana	Cytotoxity Antimicrobial Antioxidant	Sukari *et al*., 2010 Sukari *et al*., 2010 Jitoe *et al*., 1992
C. longa	Anti-inflammatory Antidepressant Anticancer	Jih *et al*., 2010 Yu *et al*., 2002 Mahady *et al*.,2002
C. xanthorrhiza	Antimicrobial Antitumor	Wibowo *et al*., 2012; Hwang *et al*., 2000 Vimala *et al*., 1999
C.zedoaria	Antifungal Anti-inflammatory Analgesic	Ficker *et al*., 2003 Hong *et al*., 2002 Navarro *et al*., 2002

In *C. mangga*, there are only few reports on isolation, chemical constituents and bioactivity of compounds (Malek *et al*., 2011; Liu & Nair, 2011; Tewtrakul & Subhadhirasakul, 2007; Abas *et al*., 2005; Kirana *et al*., 2003; Jitoe *et al*., 1992). Cytotoxic activity against six human cancer cell lines, namely the hormone-dependent

breast cell line (MCF-7), nasopharyngeal epidermoid cell line (KB), lung cell line (A549), cervical cell line (Ca Ski),colon cell line (HCT 116), colon cell line (HT-29) and one non-cancer human fibroblast cell line(MRC-5) have been reported in seven compounds of *C. mangga*, namely (*E*)-labda-8(17),12-dien-15,16-dial, (*E*)-15,16-bisnorlabda-8(17),11-dien-13-one, zerumin A, β-sitosterol, curcumin, demethoxycurcumin and bisdemethoxycurcumin (Malek *et al*., 2011). Kirana *et al.*, (2003) also investigated *C. mangga*for its cytotoxicity effect on two cancer lines, breast cell line (MCF-7) and colon cell line (HT-29). Another recent paper reported antioxidant, anti-inflammatory and anti-tumour activities of *C. mangga* rhizomes extract (Liu & Nair, 2011). Purification of these active extracts yielded (*E*)-labda-8(17),12-dien-15,16-dial, calcaratarin A, communic acid, copallic acid, zerumin B, 14,15,16-trinor-labdan-8,12-diol and two decalins. Anti- allergic activities were observed in aqueous ethanol extracts and volatile oils from rhizome of *C.mangga* (Tewtrakul & Subhadhirasakul 2007). Abas *et al.*, 2005 reported the presence of curcumin, demethoxycurcumin, bisdemethoxycurcumin, curcummanggoside, scopoletin, calcaratarin A, (*E*)-labda-8(17),12-dien-15,16-dial, *p*-hydroxycinnamic acid and Zerumin-B. Scopoletin showed antioxidant activity meanwhile another five compounds exhibited cytotoxic activity against five human cancer cell line: HL-60 (human leukemia), HepG2 (liver cancer), MCF-7 (breast cancer), DU-145(prostate cancer) and NCI-H460 (lung cancer). The essential oil of the rhizomes were found to be a good source of β- myrcene and β- Pinene comprising of about 78- 80% of the total oil (Ibrahim *et al.*, 1999; Gupta *et al.*, 1999; Wong *et al.*,1999). Jitoe *et al.*, (1992) reported the antioxidant activity of crude extract of rhizomes of *C. mangga.*

2.5.1.1 Labdane-type Diterpenes

The occurrence of diterpenes in *Curcuma* species is less common. Labdanes are the only type of diterpene known to present in the Zingiberaceous plant, and a number of related compounds have been isolated from the genera *Alpinia* (Kong *et al.*, 2000) and *Hedychium* (Nakatani *et al.*, 1994). Breitmaier, (2006) reported more than 500 labdanes were isolated from higher terrestrial plants. Labdane-type compounds resulted from two ring closure of C-6 with C-11 and C-10 with C-15 of phytane (Breitmaier, 2006).

(*E*)-Labda-8(17),12-diene-15,16-dial (figure 2.3) is one of the labdane isolated from Zingiberaceous plant. (*E*)-Labda-8(17).12-diene--15,16-dial is a thick, light viscous oil. The molecular formula is determined as $C_{20}H_{30}O_2$. The ^{13}C NMR spectrum indicated the presence of three methyl carbons (CH_3), five methine carbons (CH) and eight methylene carbons (CH_2). Four quartenary carbons were also observed (Lee, 2011).

In the past, (*E*)-Labda-8(17),12-diene-15,16-dial has been isolated from the extract of the *Alpinia formosana* (Itokawa *et al.* 1988), *A. chinensis* (Lai *et al*., 1997), *C. longa* (Roth *et al*., 1998) and *C. heyneana* (Firman *et al*., 1988). Malek *et al*., (2011); Liu & Nair, (2011) and Abas *et al*., (2005) reported the presence of (*E*)-Labda-8(17),12-diene-15,16-dial in *C. mangga*. This compund shows cytotoxic activity, against MCF-7 (breast cancer), HCT 116 (colon cancer) and HT-29 (colon cancer) cell lines (Malek *et al*., 2011). Antioxidant, anti-inflammatory and anti-tumour activity of this compound was also reported by Liu & Nair (2011). Other than these, (*E*)-Labda-8(17),12-diene-15,16-dial also shows antibacterial, antiprotozoal, antifungal and antitubercular activity (Chan *et al*., 2008; Tewtrakul & Subhadhirasakul *et al*., 2007; Abas *et al*., 2005; Kirana *et al.*, 2003; Habibi *et al.*, 2000;Roth *et al*., 1998; Richomme *et al*., 1991)

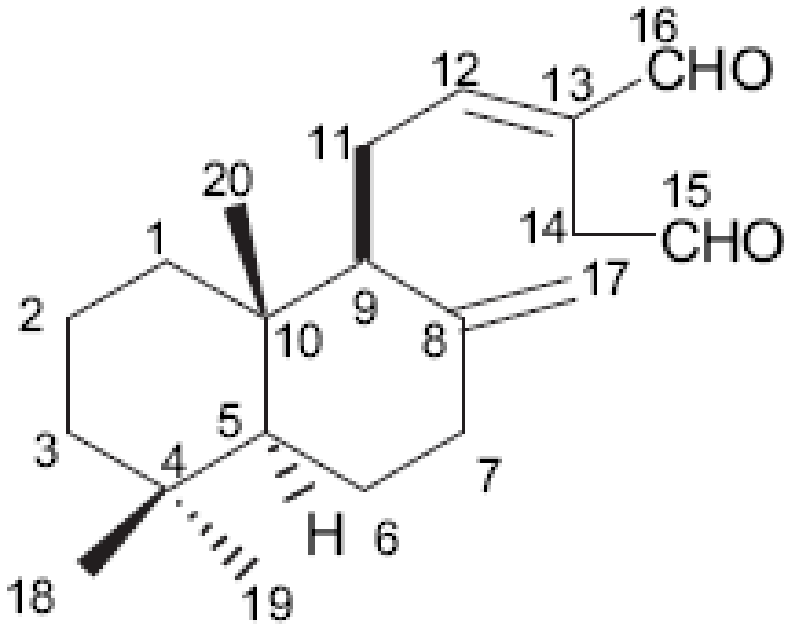

Figure 2.3: Structure of (*E*)-labda-8(17), 12-diene-15,16-dial (Adapted from Malek *et al*., 2011)

2.6 *In vitro* plant culture andproduction of secondary metabolites

Many useful compounds are left undiscovered due to environmental and geopolitical instabilities which destroy the plants natural habitat. The growth of plants and production of their secondary metabolites are very much dependent of environment and natural climate changes (Ramawat & Mathur, 2006). Besides, a loss in activity is also observed when these compounds are extracted from whole plant source. Moreover, highly complex structures of these compounds make them difficult to be synthesized chemically (Kolewe *et al*., 2008). Plant callus and cell culture cultivated *in vitro* offers an alternative to natural biomass for production of useful secondary metabolites. The basic principle of plant callus and cell culture is that each and every plant cells have full

set of genes that is used for all its function. This principle is known as totipotency or plasticity (Veerporte *et al*., 1999). The process of initiating callus and cell suspension cultures includes selecting mother plant and explants, sterilization, media preparation and culture of explants on the media.

Under aseptic and controlled condition (controlled nutrients and pH and free from microorganisms and predators), plant callus and cell cultures could grow and produce secondary metabolites *in vitro* independent of natural climate changes (Karuppusamy, 2009; Ramawat & Mathur, 2006). *In vitro* cultivation undergoes rapid multiplication and less time consuming in comparison to plants grown in the field. Upscaling of the cell and callus culture in shake flasks and bioreactor could enhance the biomass production and synthesis of secondary metabolites. There have been successful reports on production of many desirable metabolites from callus and cell cultures as shown in Table 2.5.

Table 2.5 Overview of production of desirable compounds from callus and cell suspension cultures

Species	**Compounds**	**References**
Commiphora wightii	Guggulsterone	Tanwar*et al*.,2007
Catharanthus roseus	Ajmalicine	Almagro *et al*., 2011
Silybum marianum	Flavonolignan	Hasanloo *et al*., 2009
Eurycoma longifolia	Alkaloids	Chan *et al*., 2010
Orthosiphon stamineus	Rosmarinic acid	Lim *et al*., 2006
Hypercium perforatum	Hypericin	Hohtola *et al*., 2005
Corydalis ambigua	Corydaline	Hiraoka *et al*., 2004

Table 2.5 Continued

Stevia rebaudiana	Flavonoids	Tadhani *et al*., 2007
Salvia officinalis	Rosmarinic acid	Grzegorczyk *et al*., 2007

In most plant cell culture, the main obstacle faced is either low production or absence of secondary metabolites. Therefore several strategies have been extensively studied to simultaneously optimize the secondary metabolites production and the growth of the cultures as follows

1) Standardization of culture environment (Ramachandra-Rao & Ravishankar, 2002)

2) Manipulation of plant culture (Zhao *et al*., 2005)

2.6.1 Standardization of culture environment

2.6.1.1 Medium

In order to increase the secondary metabolite production and optimization of callus and cell growth**,** medium standardization has been an important parameter. Murashige & Skoog (1962) medium is one of the most commonly used medium for growth of callus and cell suspension of many plants. However this medium, with different combination of exogenous plant growth regulators, different culture condition and alternation in its medium combination have influenced the secondary metabolite accumulation (Ramawat & Mathur, 2006; Seabrook 1980).

Nitrogen and phosphate source of the medium were found to affect the production of the *in vitro* secondary metabolites. Addition of nitrate to the medium before nitrate exhaustion (days 6–8) reduced the final product yield (by 70–80 %) while addition of nitrate in the later stationary growth phase (days 14–16) had no significant effect on the production of shikonin in *Lithospermum erythrorhizon* cell cultures (Srinivasan & Ryu, 1992). Nitrogen deficiency led to increased levels of phenolics and

increased activity of phenylalanine ammonia lyase (Kovacik *et al*., 2007). In tobacco, nitrogen deficiency led to a shift from nicotine production to production of more carbon rich metabolites (Fritz *et al*., 2006). In *Catharanthus roseus* culture, adding phosphate in culture medium prolonged the cell growth (Mac-Carthy *et al*., 1980). High level of phosphate also produced digitoxin in *Digitalis purpurea* cell culture (Hagimori *et al.*, 1992). Sucrose and glucose as carbon source also influenced cell growth and secondary metabolites production (Dornenburg & Seydel, 2008). Ramachandra-Rao & Ravishankar, (2002) reported 2 to 8% carbon source in culture media was appropriate for optimal secondary metabolite production in plant cell culture. Increasing sucrose level of *Salvia officinalis* cell suspension culture medium to 5%, improved the yield of rosmarinic acid 5 fold when compared to using 3% sucrose (Whitaker *et al*., 1984)

It was also reported that there are usually two stages of medium for maintaining plant cell cultures and production of secondary metabolites (Lee & Chan, 2004; Sierra *et al.*, 1992). The first stage involved growing and maintaining the cells at a high density on standard maintenance medium and subsequently transferring to production medium. Usually in production medium, the effect was directly observed after transferring the plant cells to the production medium, whereas in growth medium, the effect of a change in compound composition was only observed after a number of subcultures. In general, productivity improvement of about 10-20 folds were obtained by combining these approaches (Verpoorte *et al.*, 1999).

2.6.1.2 Selection of high producing cell lines

Prior to standardizing medium, high producing cell lines must be screened and selected. It was shown for certain plant species that extensive screening programmes can lead to stable high producing cell lines, such as in the case of berberine production in *Coptis japonica* cell cultures ((Verpoorte *et al.*, 1999; Verpoorte *et al*, 1992)

2.6.1.3 Plant growth regulators

Auxin and cytokinin concentration individually or in combination effects the growth, division and elongation of cells and also secondary metabolites accumulation in cell cultures. In suspension culture, auxin was found to promote cell dispersion (Gamborg, 2002). Auxins are involved in the establishment and maintenance of polarity in organised tissues, meanwhile in whole plant, auxins affect the maintenance of apical dominance and mediation of tropisms (Friml, 2003). 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA) and indole-3- acetic acid (IAA) are some of the most commonly used auxins. In plant tissue culture, auxin is generally used for undifferentiated callus and suspension culture induction and growth (George *et al*., 2008). It had been considered for a long time that such a callus or cell suspensions initiated by using auxins, were not able to produce secondary metabolites, unlike differentiated cells and specialized organs (Gadzovskaa *et al*., 2005). However many reports have proven this wrong. In the presence of 2,4-D, callus culture of *Daucus carota* (Rajendran *et al*., 1992), *Oxalis linearis* (Meyer & Staden, 1995), *Panax ginseng* (Bonfill *et al*., 2002) and *Curcuma zedoaria* (Nguyen *et al.*, 2008)were found to produce carotenoid, solasodine, anthocyanin. ginsenoside and zedoary respectively. In *Tabernaemontana divaricata* cell cultures replacing 2,4-D with NAA in the medium resulted in increase production of alkaloids (Sierra *et al*., 1992).

In nature, cytokinins are involved in cell division, shoot proliferation, regulate differentiation, inhibit root formation, activate RNA synthesis, and stimulate protein and enzyme activity. There are various types of natural and synthetic cytokinins such as kinetin, zeatin , 6-benzylaminopurine, 6- benzylaminopurine, diphenylurea and thidiazuron (Gamborg, 2002). These plant growth regulators are added in plant tissue culture medium for cell division, differentiation of callus and organs and also for shoot proliferation. Cytokinins have different effects on cultures depending on plant species

and metabolites. Kinetin has been found to stimulate the production of anthocyanin in *Haplopappus gracillus* but inhibited the formation of anthocyanin in *Populas* cell culture (Seitz & Hinderer, 1988).

2.6.1.4 pH, light, temperature and subculture period

pH has also been reported as one of the factor effecting cell growth and metabolite production. Usually, optimum growth of plant cell culture is obtained between pH 5.5 and 6.0 (Husemann *et al*., 1992). Increasing intensities of light, was also found to improve the production of secondary metabolites in callus culture of *Oligodon affinis* (Dornenburg & Seydel, 2008). UV irradiation increase the production of flavanoids in *Bosenbergia rotunda* callus culture (Tan *et al*., 2005). Temperature between 17 to 25^{o}C are normally used for callus induction and growth of cultured cells. Toivonen *et al*., (1992) however found that fatty acid content per cell of dried weight increased when temperature was lowered.

Period of subculture is also very important in determining the success of the suspension culture. The time to subculture is usually between 7-14 days depending on the stage of culture and usually at the end of log phase (Mohamed, 2010). After 2 weeks medium containing phenolic exudation, enlarge and vacuolated non-embryogenic cells and other debris would accumulate. This medium must be removed and substituted with fresh medium to avoid the culture media turning brown leading to cell death.

2.6.2 Manipulation of plant cell cultures

2.6.2.1 Organ culture

Although many studies have reported the presence of secondary metabolites in de-differentiated callus and cells, there are certain compounds that are organ specific which is produced in differentiated cells at specific developmental stage. *In vitro* shoots of *Centaurium pulchellum* were found to contain secoiridoids and xanthones (Krstic, 2003). The undifferentiated cells of *Nicotiana tobaccum* only produces trace amount of nicotine, lysine and anabasine when compared to its root culture (Karuppusamy, 2009).

2.6.2.2 Hairy root culture

Soil bacteria *Agrobacterium rhizogenes* are capable of infecting plant cells through insertion of Ri plasmid into wounded tissue causing proliferating growth of hairy roots. Hairy root culture is a promising culture method in high secondary metabolites production, pathway engineering, induction of metabolite excretion and elicitation (Guillon *et al*., 2006). Hairy root cultures of *Papaver somniferum* produced larger quantities of morphine, sanguinarine and codeine than wild plants, (Flem-Bonhomme *et al*., 2004). Herein, Sudha *et al*., (2003) also reported the secretion of ajmalicine and ajmaline from *Rauvolfia micrantha* hairy root culture.

2.6.2.3 Elicitation and precursor feeding

In nature, plant secondary metabolites are produced to protect plants against environmental stresses. Elicitors are environmental stresses (microbial, physical or chemical factors) that lead to the increase in secondary metabolites production. Elicitation in cell cultures is induced through the addition of trace amount of elicitors (Radman *et al.*, 2006). Examples of abiotic elicitors are heavy metals, fungicide and

antibiotics meanwhile polysaccharides, polyamines and galacturonide are biotic elicitors (Shilpa *et al*., 2010). Copper chloride was used as abiotic elicitors for production of herniarin and umbelliferone in *Metricaria chamomilla* culture (Eliasova *et al*., 2004). Production of beta-amyrin was observed when *Medicago truncatula* cellculture was elicitated using yeast (Broeckling *et al.*, 2005)

Precursor feeding to medium stimulates secondary metabolites production. Precursor feeding serves as an intermediate at the beginning of a biosynthetic route which stimulates secondary metabolites production (Mulabagal & Tsay, 2004). Phenylalanine, tyrosine, and tryptophan are few examples of precursors. Phenylalanine is a precursor for most phenolic compounds in plants and has been successfully used to induce metabolite production *in vitro* in many different plant systems (Fett-Neto *et al*., 1993). For example, *Salvia officinalis* cell suspension culture and *Taxus* cultures stimulate rosmarinic acid and taxol production respectively upon addition of phenylalanine (Fett-Neto *et al*., 1993).

2.7 Problems in plant cell and callus culture

2.7.1 Establishment of aseptic cultures

Explants that are collected from field-grown plants are usually contaminated by various microorganisms. This microorganisms could either be endogenous or exogenous in nature. Endogenous contaminants are latent and could surface even after years in culture. A standard eradication method has not been achieved so far for internal contaminants. The commonly used surface sterilants are bleach (containing sodium or calcium hypochlorite as active ingrediants), ethyl alcohol and mercuric chloride. Type of sterilants used, concentration and exposure time depends on explant type and species.

In the process of surface sterilization, explants only bacteria and fungal should be eradicated without the explant losing their biological activity.

Prakash *et al*., (2004) developed disinfection protocol for rhizomes of *C. amada* by washing under running tap water and immersing in Teepol detergent immediately after excision followed by surface-disinfestion in ethanol, and mercuric chloride solution. In establishing *C. manggain vitro* cultures, its apical buds were soaked in commercial detergent prior to surface sterilizing with Clorox (commercial bleach) and Tween-20 (Raihana *et al*., 2011).The best surface sterilization of *Zingiber montanum* rhizome budswas obtained by using 20% Clorox which resulted in axenic explant (Hamirah *et al*., 2010). Incorporation of antibiotic such as tetracycline and biocide PPM into culture medium also helped to reducebacterial contamination (Hamirah *et al*., 2010; Jiminez *et al*., 2006;Salvi *et al*., 2002).

2.7.2 Tissue browning

In initiation of plant cultures, explant browning and subsequent death is a major problem due to oxidation of phenolic compounds (Thomas & Ravindra, 1997). Major metabolic and developmental changes are found as the cause of oxidation and tissue culture manipulations (Benson, 2000). These changes may predispose *in vitro* cultures to increase free radical formation, resulting in cellular dysfunction and recalcitrant cultures. Oxygen free radicals are generated by wounding and result in increase in the activity of peroxidase and catalase enzymes (Salin & Bridges, 1981), which act to overcome the effect of oxidising radicals. Reducing contact with oxygen reduces the rate of oxidation of phenols at the wounded site (Elmore *et al.*,1990) leading to the use of submersion of explants in water as a means to reduce tissue oxidation. Browning is often associated with the use of 2,4-D, especially if high concentration are added to the

medium. This problem was found in cultures of *Aconitum heterophyllum* (Giri *et al.*, 1993) and *Dioscorea composite* (Viana & Mantell, 1989).

Several antioxidants such as ascorbic acid and citric acid have been evaluated with varying success in the management of phenolic damage to excised tissues. Antioxidant is defined as an electron donor which inhibit the oxidation of labile substrates with a high stoichiometric efficiency. Combination of ascorbic acid and citric acid have been used as a pretreatment rinsing agent to overcome browning process in few plant species such as *Conostephium pendulum* (Panaia *et al*., 2000) *Aechmea fasciata* (Jones & Murashige, 1974), *Musa textilis* (Mante & Tepper, 1983), *Phoenix dactylifera* (Tisserat ,1979) and *Rhododendron* (Anderson, 1975). Antioxidants have also been incorparated into tissue culture media. Ascorbic acid combined with citric acid and cysteine prevented browning in *M. textilis* (Mante & Tepper, 1983) while in *Vigna radiata,* 100 mg l^{-1} each of ascorbic acid and citiric acid added to the medium prevented browning.

Polyvinylpolypyrrolidone (PVP) is also an antioxidant that has been incorporated into media to overcome browning in *Eucalyptus tereticornis* (Subbaiah and Minocha, 1990) and *Castanea sativa* (Vestri *et al.,* 1991). Ohkatsu *et al.* in 2001 reported that high antioxidant activities are also found in tocopherols. In olive cultures, gluthathione have been found to reduce blackening (Rugini, 1986). Other antioxidant include thiothreitol in liquid medium of *Strelitzia reginae* culture (Ziv & Havley, 1983) and rosmanol that suppresed browning of *Hypericum canariensis* (Mederos, 1991). Thomas (2006) reported the usage of activated charcoal to prevent browning in many plant species.

2.8 Compound Extraction

Compound extraction is a method of separating the constituents of a mixture utilizing preferential solubility of one or more components in a second phase. Commonly this added second phase is a liquid, while the mixture to be separated may be either solid or liquid. If the starting mixture is liquid, then the added solvent must be immiscible or partially miscible with the original and of such a nature that the components to be separated have different relative solubility in the two liquid phases. There are various techniques for extractions such as solid/liquid extraction, liquid/gas extraction and acid base extraction. In our studies solid/liquid extraction will be studied and utilized.

Solid/ liquid extraction is the most common technique in isolating compounds from natural products since it allows soluble components to be removed from solids using solvent. In solid/liquid extraction, a solvent is first added to a solid. Insoluble material is then separated from the solution by gravity or vacuum filtration, while soluble material is extracted into the solvent. In natural product extraction, often the raw materials such as leaves and bark of plants are soaked with an appropriate solvent for several days before filtering (Azarkan *et al.*, 2003).

2.9 Chromatography

Chromatography is a broad range of physical methods used to separate and analyze complex mixtures. Usually, components to be separated are distributed between two phases: a stationery and a mobile phase which percolates through the stationery bead.

Technically, in chromatography process, different components are flushed through the system at different rates when a mixture of components pass through any

chromatography system. The differential rates of migration when the mixture moves over adsorptive materials displaythe separation. Molecule with weaker affinity for the stationery phase takes shorter time to pass through the column and will be separated first. Thin layer chromatography (TLC), gas chromatography (GC) and liquid chromatography (LC) are few types of chromatography routinely used in chemical extraction and separation.

2.9.1 Thin layer chromatography

Thin Layer chromatography (TLC) is one of the easiest chromatography technique. Extraction of components and separation of mixtures can be done in a very quick and inexpensive way using TLC method. It is possible to identify the numbers of component in a mixture and usually used to support the identity of a compound in a mixture when the retention factor (R_f) of a compound is compared with the R_f of a known compound on a same TLC plate. TLC plate is either a sheet of glass, aluminum or plastic foil which is coated with usually silica, aluminum oxide or cellulose as a thin layer of solid adsorbent. This adsorbent layer is known as stationery phase. Usually a minute amount of mixture that is being analyzed is spotted one or half centimeter from the bottom of the plate. When the TLC plate is placed in solvent (mobile phase), it slowly rises up the TLC plate by capillary action.

Different analytes ascend the TLC plate at different rates, so separation is achieved due to their differences in their attraction to the stationery phase and the difference in their solubility in the solvent. Separation of components can be adjusted by changing the solvent, or perhaps using a mixture of solvent. Compounds on the TLC plate can be visualized directly if coloured or under UV lamp if otherwise. R_f value is the measurement of the distance each compound travels up the TLC plate. Based on the

R_f value of the standard compound, identification of the unknown compound in the mixture will be known (Harwood *et al*., 1999; Vogel *et al.*, 1989)

2.9.2 Gas chromatography

Gas-Liquid chromatography or better known as gas chromatography (GC) was first developed by Fritz Prior in 1947 and was later upgraded by Archer John Porter Martin and Erika Cremer. The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator"). GC differs from other type of column chromatography by its component of mobile phase and stationery phase. In GC, the mobile phase (moving phase) is usually a carrier gas such as helium or nitrogen, meanwhile a column consisting of polymer or microscopic layer of liquid on inert solid support in a glass or metal tubing forms the stationery phase.

The chemicals are detected and identified electronically when they exit at the end of the column by comparing the retention time. The gaseous compounds being analyzed will interact with the walls of the column, which is coated with different stationery phases which causes each compound to elute at a different time (retention time of the compound). In GC, the temperature of the gas can be controlled, where the column of the gas phase passes is located in an oven. Moreover, vapour pressure of the gas is correlated with the concentration of compounds in the gas phase. GC separate the components of a mixture primarily based on boiling point (or vapor pressure) differences. In this aspect, GC is similar to fractional distillation. However fractional distillation is typically used to separate components of a mixture on a large scale, meanwhile GC can be used on a much smaller scale.

Hydrogen and helium are the most common carrier gas (mobile phase) used in GC. Hydrogen provides the best separation. However, helium has a larger range of flow rates compared to hydrogen. Moreover, helium is not flammable, and works with a greater number of detectors. Therefore, helium is the most common carrier gas used (Pavia *et al.*, 2006; Gary *et al.*, 1998)

2.9.3 Flame ionisation detector

Flame ionisation detector (FID) was introduced in 1958. FID as a universal, high-performance device in modern instrumental analysis has been one of the choice for GC analysis of biological samples (Crockford *et al.*, 2006). FID responds practically to all organic compounds, it is resistant to small fluctuations of the gas flow, insensitive to gas impurities and the FID response is very predictable, obeying the rule of equal carbon response (Holm, 1999). The FID works by passing the gas phase output from the column into a hydrogen flame. 100 to 200 V voltage is applied between the flame. An electrode is located away from the flame. Increasing current due to electrons emitted by burning carbon particles is finally measured. The signal current (the ionization efficiency is only 0.0015%) and the noise level (<10-13 amp) is very small. FID is able to detect all carbon containing compounds (except for a very few organic compunds, e.g. carbon monoxide). In conclusion, the FID is a useful general detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise. It is also robust and easy to use, but unfortunately, it destroys the sample.

CHAPTER 3: METHADOLOGY

3.1 Plant materials

The fresh yellow rhizomes of *Curcuma mangga* Val. were purchased from a local market at Jalan Chow Kit, Kuala Lumpur. The rhizomes were harvested from a farm at Temerloh, Pahang. Rhizomes were thoroughly washed under running tap water until all the sand particles were removed. The cleaned rhizomes were drain dried and placed in paper boxes to allow shoots to sprout to at least 3 to 4 cm in length. Since the shoots would be used as explants, the rhizomes were placed in few different conditions to obtain optimal sprouting (Table 3.1). In each paper boxes, 10 rhizomes were placed.

Table 3.1: Different treatment on rhizome for sprouting

Treatments	Photoperiod	Temperature
1	16 hours light and 8 hours dark	20 ^{o}C at air-condition
2	16 hours light and 8 hours dark	30 ^{o}C at room temperature
3	24 hours dark	20 ^{o}C at air-condition
4	24 hours dark	30 ^{o}C at room temperature

3.2 Preparation of culture medium

3.2.1 Callus induction and propagation medium

Callus was initiated on medium consisting of Murashige and Skoog (1962)(MS) basal salts (Appendix A) augmented with 2 g l^{-1} gelrite and different concentrations of sucrose and PGRs combinations. In terms of PGR, the medium were divided into 2 groups; the first group supplemented by different concentrations of 2,4-D (Table 3.2) and the second group supplemented with different concentration of IAA and NAA combinations (Table 3.3). In these two series, concentration of sucrose was kept

constant at 3 % (w/v). Meanwhile in third series the medium were supplemented with 1 mg l^{-1} 2,4-D with two different concentration of sucrose (Table 3.4). For control experiment, medium without any PGR were prepared. The pHs of all medium were adjusted to 5.7 with 0.1 M NaOH and 0.1 M HCL before adding gel rite. The medium were autoclaved at 121^{o}C, 103.4 kPa for 20 minutes to ensure sterility. IAA was filter sterilized prior to addition to cooled autoclaved media because of its heat labile nature. Medium was left for 1 week prior to culturing to ensure aseptic condition.

Table 3.2: Different concentration of 2,4-D in MS basal medium for callus induction

[2,4-D] mg l^{-1}
0.0
0.1
1.0
2.0
4.0
8.0

Table 3.3: Different concentration of IAA and NAA combinations in MS basal medium for callus induction

[IAA] mg l^{-1}	**[NAA] mg l^{-1}**
1	1
3	1
5	1
1	3
1	5
5	5
3	3
5	3
3	5

Table 3.4: Different concentration of sucrose in MS basal medium for callus induction

Sucrose (%)
3
8

3.2.2 Liquid multiplication (suspension culture) medium

Suspension cultures were established and multiplied in liquid multiplication medium, consisting of MS supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. After 3rd subculture, suspension cultures were transferred to MS medium consisting 0.1 g l^{-1} malt extract, 100 mg l^{-1} glutamine and other supplements as in table 3.5. pH of the medium was adjusted to 5.8 with 0.1 NaOH and 0.1 HCL prior to autoclaving.

Table 3.5: Different combination and concentrations of supplements in liquid MS medium for suspension culture proliferation after 3rd subculture

2,4-D (mg l^{-1})	**Sucrose (g l^{-1})**
0.05	30
0.1	30
0.3	30
0.5	30
0.05	15
0.1	15
0.3	15
0.5	15

3.2.3 Liquid multiplication medium with antioxidant

After 6th subculture, suspension cultures were transferred to MS medium supplemented with 30 g l^{-1} sucrose, 100 mg l^{-1} glutamine, 0.1 g l^{-1} malt extract, 0.1 mg l^{-1} NAA, 0.1 mg l^{-1} d-Biotin and other supplements and antioxidants as in Table 3.6. pH of the media was adjusted to 5.8 with 0.1 NaOH and 0.1 HCL prior to autoclaving.

Table 3.6: Different combination and concentrations of supplements in liquid MS medium for suspension culture proliferation after 6th subculture

2,4-D (mg l^{-1})	**Ascorbic acid (mg l^{-1})**	**Citric acid (mg l^{-1})**
0.1	0	0
0.3	0	0
0.5	0	0
0.1	5	0
0.3	5	0
0.5	5	0
0.1	0	5
0.3	0	5
0.5	0	5
0.1	5	5
0.3	5	5
0.5	5	5

3.3 Sterilization procedure

All apparatus used in tissue culture procedures were sterilized to eradicate microorganisms present. Glassware, metal tools (i.e. forceps and scalpels) and autoclavable containers to be used were soaked in detergent (Teepol ^{TM}), washed with tap water, rinsed with distilled water and dried in drying oven at 50 ^{o}C**.** Uncovered materials and instruments are covered with 2 layers of aluminium foils. Subsequently, autoclaving was carried out at 121 ^{o}C,103 kPa for 20 minutes to ensure sterility.

All tissue culture procedures were done aseptically in laminar flow chamber. Ultraviolet lamp (UV) lamp (model UVP) was switched on 15 minutes before using the laminar flow chamber to disinfect the chamber from microorganisms. Forceps and scalpels that have been autoclaved were placed in hot beads sterilizer at 250 ^{o}C prior to use. 70 % (v/v) ethanol was sprayed and wiped on the surface of laminar flow chamber. Eventually, all materials that had to be used in laminar flow were wiped with 70 % (v/v) ethanol. Sterile gloves were used, to hold and handle sterilized materials. Sterile distilled water was used to cool hot scalpels and forceps before being used for culturing.

3.4 Explant culture

3.4.1 Preparation of explant

Suitable explants (2 to 3 cm) were chosen and rinsed under running tap water for 10 minutes. The summary of the general sterilization method is shown in the Figure 3.1 and different treatments used are shown in Table 3.7. Tween-20 was added to reduce surface tension. The surface sterilized shoots had their external leaves removed and were finally dried on filter papers under laminar air flow prior to inoculation on media. The contamination, necrotic and survival percentage of cultures were calculated using the following formula:

$$\frac{\text{No. of cultures contaminated/necrotic/ survived}}{\text{Total number of cultures inoculated}} \times 100$$

Figure 3.1: Surface sterilization methods

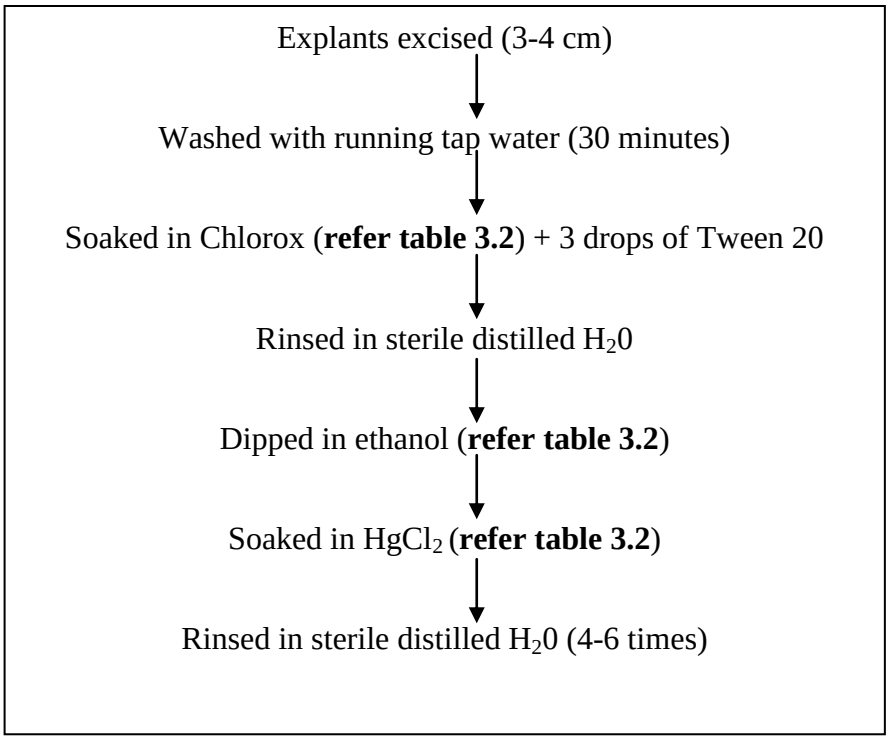

Table 3.7: Surface sterilization treatments

Disinfectant	**Treatment 1 (%)**	**Treatment 2 (%)**	**Treatment 3**
Chlorox (v/v)	50 (15 minutes)	50 (15 minutes)	50 (15 minutes)
Ethanol (v/v)	70 (5 minutes)	70 (5 minutes)	70 (5 minutes)
$HgCl_2$ (w/v)	0.1 (30 seconds)	0.5 (10 minutes)	0.3 (5 minutes)

3.4.2 Callus induction and propagation

Outer leaves were removed from the sprout and trimmed to a meristematic shoot tips and aseptically sliced into cross sections of about 1 mm pieces of explants. These pieces were placed onto callus induction medium as in chapter 3.2.1. The cultures were kept at 25 $\pm$ 1^{o}C under dark condition and observation was made fortnightly. For every treatment 15 replicates (15 plates of same medium) were used with 10 explants cultured on each plate. Observations on the morphology of the callus induced were recorded after two weeks. The callus induction percentage was calculated using the following formula:

$$\frac{\text{No. of explants initiating callus}}{\text{Total number of explants inoculated}} \times 100$$

When the callus is about 1 cm in diameter, they were subcultured onto media of the same formulation in separate aggregate of about 3 mm size after discarding the original explant and any brown tissue. Subculturing was done forthnightly over a period of 2 months. Subculture was discontinued for callus that turned brown or remained unchanged in size. Percentage of explants forming callus and growth rate of callus were recorded.

3.4.3 Establishment of suspension culture

Suspension culture was initiated by using friable callus. Prior to inoculation, the callus was gently crushed using a spatula to break up the callus. About 1 g callus was inoculated into 10 ml of liquid suspension culture medium in 100 ml Erlenmeyer flasks under aseptic conditions. The flasks were then sealed and placed on an orbital shaker at 90 rpm in growth room at the temperature of 24 $\pm$ 2^{o}C and under a photoperiod of 16 hours light: 8 hours dark regime culture condition. Cells were suspended in MS medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. Due to cell browning, medium as

listed in table 3.5 were used (subsection 3.2.2). Cultures were maintained by subculturing in liquid medium of same formulation every 10 days. The standard procedure of subculturing involve decanting off 10 ml of the liquid medium, followed by the inoculation of 10 ml of the suspension culture to 40 ml of the fresh medium in a 250 ml Erlenmeyer flask with the ratio of 2 : 5 (cell : liquid medium). Due to cell browning, after the 6th subculture, medium as listed in table 3.6 (subsection 3.2.3) were used. Standard subculture method is as described earlier

At every subculture, small aliquots of cells from suspension cultures were randomly withdrawn and observed under inverted microscope for cell morphology, proliferation and growth. Occasionally crushing was done on a need basis to fragment the clumps which were larger than 0.5 cm in diameter before inoculation at every subculture.

3.5 Observation and growth measurement

3.5.1 Growth curve of callus culture

The growth of friable callus was assessed by measuring the fresh weight forthnightly for 4 months. MS supplemented with 1 mg l^{-1}2,4-D were used as culture media. Healthy callus (about 1 cm diameter), were subcultured onto MS medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose,separated into 10 aggregates of callus weighing about 0.03 g each, in a petri dish. Graph of biomass of callus against time were plotted. All procedures were done aseptically in laminar flow chamber as stated in subsection 3.3. Each treatment had four replicates and was repeated twice.

3.5.2 Growth curve of suspension culture

The growth of suspension cultures were determined using method adopted from Philips *et al*.,(1995). MS medium supplemented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 mg l^{-1} malt extract, 5 mg l^{-1} ascorbic acid and 5 mg l^{-1} citric acid were used as culture media. Stable suspension cultures were used to determine the growth curve. Suspension cultures were considered stable once single clumps of callus were formed. To determine settle cell volume (SCV), a known volume of uniformly dispersed suspension was transferred to a 50 ml polypropylene tube under aseptic condition. The cells were left to settle in polypropylene tube for 10 minutes before reading was taken. SCV is volume of settled cell expressed as ml per litre culture or a percentage. The cells were resuspended and transferred in 250ml flask on a shaker. These steps were repeated every 3 days over a period of a month. The means of SCV were calculated and growth curves were plotted. Each treatment had four replicates and was repeated twice.

3.6 Histology

The histological sectioning of callus was done in Malaysian Palm Oil Board, MPOB, according to the following techniques. Histology techniques using resin developed by the ORSTOM – CIRAD team LRGAPT France (Maril *et al*., 1995) were used to fix, dehydrate, infiltrate, embed, cut and stain the sections. The samples were initially sliced to the size of the available mould and then fixed for 24 to 48 hours at room temperature in Glutaraldehyde-Paraformaldehyde-caffeine (GPC) fixative (50 ml 0.2M phosphate buffer, pH 7.2 + 20 ml paraformaldehyde +4 ml 25% glutaraldehyde + 1 g caffeine + topped up to 100ml with distilled water). The samples were then dehydrated in ascending ethanol percentage in the order of: 30% (30 min); 50% (45

min); 70% (45 min); 80% (60 min); 90 % (60 min); 95 % (60 min) and twice in absolute ethanol for 60 minutes each. The tissues were then prepared for filtration with basic resin (Leica Historesin Embedding Kit) for 24 to 48 hours at 4^{o}C under slight vacuum. The sinking of slightly translucent specimen to the bottom signaled the completion of infiltration. The specimens were embedded into moulds and the resin was allowed to be fully polymerized after which holders were attached and 3 micrometer sections were sliced using a microtome. Good stains are then stained with 1% periodic acid for 5 minutes, rinsed four times with distilled water at pH 4.5 and the submerged in Schiff's reagent (1g basic fuchsin, 2g disodium metasulfite in 1M HCl, and 0.5 g neutralized activated charcoal) for 20 minutes in dark. The four times rinsing in distilled water pH 4.5 was repeated. Finally for counter staining, Naphthol Blue Black (1g Naphthol Blue Black in 100 ml 7% acetic acid) was used at 60^{o}C for 5 minutes. After further rinsing under running water, the sections were dried before viewing under light microscope.

3.7 Plant materials and sample extraction

Cold soak extraction method was used for compound extraction from all samples.

3.7.1 Rhizomes (*In vivo*)

In vivo rhizomes were thinly sliced and oven dried until constant mass. The dried slices were grounded using a blender. For extraction, batches of powdered dried rhizomes were used. The dried rhizomes (200 g) were soaked in one litre methanol for three days. Then, the mixture was filtered and remaining solids were re-soaked in 500 mL methanol for 24 hours in two consecutive cycles. Solvent was evaporated using a rotary evaporator (Buchi Rotavapor R114) leaving behind a sticky extract.

3.7.2 Shoot buds

Shoot buds were sliced and oven dried until constant mass. The dried slices were pulverised using mortar and pestle. For extraction, cold soak method was used. Five grams of powdered dried shoot buds were soaked in 200 ml methanol. Subsequent treatment was as outlined in subsection 3.7.1.

3.7.3 Callus

Calli of different morphologies in different medium treatments were oven dried until constant mass. Friable calli at different growth phases were also tested. The dried calli were pulverised using mortar and pestle. One gram of powdered dried calli were soaked in 50 ml methanol for three days. The mixture was filtered and the remaining solids were re-soaked twice overnight, each time with 20 ml methanol. Solvent was evaporated using a rotary evaporator (Buchi RotaVapor R114) leaving behind a sticky extract.

3.7.4 Suspension culture cells

Cell clumps from suspension cultures were harvested every three days for a period of 30 days. They were dried until constant mass. The dried cell clumps were pulverised and methanol soaked before the solvent was evaporated as described in subsection 3.7.1.

3.8 Standard calibration of (*E*)-labda-8(17),12-diene-15,16-dial using gas chromatography (GC)

Pure labda-8(17), 12-diene-15, 16 dial stock (crystal form) was obtained from Biochemistry Division, University of Malaya, Kuala Lumpur. Pure (*E*)-labda-8(17),12-diene-15,16-dial was used in the standard calibration. A known amount of pure stock was weighed and dissolved in chloroform.

The standard stock solution was then diluted into 1, 2, 3, 4 and 5 mg/ml concentrations. Each concentration was injected into the GC in triplicate. (*E*)-labda-8(17),12-diene-15,16-dial was detected at 20.8 minutes. The average peak area for each concentration was determined and plotted against the concentrations to obtain a standard calibration.

3.9 Characterization of labda-8(17), 12-diene-15,16 dial

3.9.1 Thin layer chromatography (TLC)

TLC was used to detect the presence of a compound in a mixture. The retention factor, R_f of a compound is compared with the R_f of a known standard compound. R_f is determined as follows:

$$R_f : \frac{\text{Distance travelled by the compound spot } (x \text{ cm})}{\text{Distance travelled by solvent front } (y \text{ cm})}$$

Pre-coated silica gel 60 F_{254} TLC plates from MERCK were used for TLC analysis. A small drop of sample was spotted about 1.0 cm from the bottom of the TLC plate by using a capillary tube. Once the sample dried, the TLC plate was placed into a developing tank filled with premixed organic solvents. The mobile phase used was a

mixture of 30% acetone with 70% hexane (Lee, 2011). The TLC plate was removed from the developing tank when the solvent front was 1 cm from the plate's top edge. It was then dried using hot air blower and was viewed under short wavelength (254 nm) and long wavelength (365 nm) ultraviolet light. The TLC plates were then placed in an iodine vapour chamber to stain the spots of separated chemicals components. Sticky crude extracts and pure (*E*)-labda-8(17),12-diene-15,16-dial (standard) stock were diluted in chloroform before spotting on TLC plates. R_f value for pure (*E*)-labda-8(17),12-diene-15,16-dial was calculated at 0.4 (Lee, 2011).

Preparative TLC (PTLC) is a technique to isolate a target spot on TLC plate. Initially, the sample was introduced onto the plates as a continuous streak to form a band sample. Once the sample band had dried, the TLC plate was placed into a developing tank and removed from the tank when it was fully developed. After it was air dried and viewed under UV light, the separated bands were marked. Each band was scrapped out using spatula and fragments of the silica gels were soaked in chloroform. After desorption, analytical TLC was performed on recovered products.

3.9.2 Gas-chromatography- Flame Ionisation Detector (GC- FID)

In this study, gas chromatography equipped with flame ionisation detector (GC-FID) from Shimadzu (Model No. GC-2010) (Japan) was used for quantitative determination of (*E*)-labda-8(17),12-diene-15,16-dial. The methanol crude extracts (0.1 g) from the different sources (section 3.5) studied, were diluted in chloroform (1 ml) and injected into the machine.

The operating parameters for GC- FID were as follow: the initial temperature was set at 150°C with 5°C ramping in a minute. For (*E*)-labda-8(17),12-diene-15,16-dial determinations, the holding time was 10 minutes and the final temperature was set at 300°C. The injector temperature was maintained at 250°C. The injection volume was

set to 1 µl for each sample, with five pre-run rinses and five post-run rinses using dichloromethane (DCM). This process took about 40 minutes to complete. A sample was mixed with chloroform in 2.0 ml vial. Then, 1.0 µl was injected into the GC-FID using an auto injector. Each treatment has three replicates and repeated thrice.

3.10 Determination of (*E*)-labda-8(17),12-diene-15,16-dial amount in 1 g of dried samples

An example of determination of (*E*)-labda-8(17),12-diene-15,16-dial amount in 0.1 g methanol crude sample is shown as follows :

Peak area = 478442.6 (obtained from GC-FID abundance peak)

Equation from standard calibration in Figure 4.2.1 (see Results and Discussion)

(*E*)-labda-8(17),12-diene-15,16-dial concentration (*x* axis) = 1/48289 x (peak area + 3149.1)

thus *x* is = 1/48289 x (478442.6+ 3149.1)

= 9.973 mg ml^{-1}

In 1 ml of chloroform, 0.1 g of methanol crude extract was diluted, hence the mass of (*E*)-labda-8(17),12-diene-15,16-dial in 1 g methanol crude

= (9.973 x 10^{-3} g / 1 ml) x (1 ml / 0.1 g)

= 9.973 x 10^{-2} g labda-8(17), 12-diene-15, 16 dial in 1 g of methanol crude extract

= **99.73 mg (*E*)-labda-8(17),12-diene-15,16-dial in 1 g of methanol crude extract**

(Note: The amount of (*E*)-labda-8(17),12-diene-15,16-dial in 1 g methanol crude in other samples are in Appendix E(i), Appendix F(i), Appendix G(i) and Appendix H(i))

As in Appendix E(ii) , 1 g of dried rhizome produce 0.276 g methanol crude extract,

So in 1 g of dried rhizome,

= (0.276 x 99.73) mg of (*E*)-labda-8(17),12-diene-15,16-dial was produced

= 27. 52 mg of (*E*)-labda-8(17),12-diene-15,16-dial was produced

(Note: The amount of methanol crude (g) obtained in 1 g dried samples of other samples are tabled in Appendix E(ii), Appendix F(ii), Appendix G(ii) and Appendix H(i))

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Explant preparation

Since the explants were taken from underground rhizomes, establishment of contamination free culture was a major task (Hosoki & Sagawa, 1997). In this study, rhizomes were sprouted in soil free conditions until shoot buds appeared (Figure 4.1 (a)). As in table 4.1, rhizomes left to sprout at room temperature under dark condition produced the highest amount of shoot buds in 2 weeks.

Table 4.1:Effect of different treatments on rhizome for sprouting

Treatments	Photoperiod	Temperature	Duration of sprouting (weeks)	Number of shoot buds (in 10 rhizomes/ 4 weeks)
1	16 hours light and 8 hours dark	20^{o}C at air-condition	4	5 to 20
2	16 hours light and 8 hours dark	30^{o}C at room temperature	4	5 to 20
3	24 hours dark	20^{o}C at air-condition	3	40 to 60
4	24 hours dark	30^{o}C at room temperature	2	40 to 60

4.2 Explant sterilization

The surfaces of explants usually carry a wide range of microbial contaminants, dead and superfluous tissues. Thus, the explants must be thoroughly surface sterilized before inoculating on culture medium to avoid infection. Usage of commercial bleach (5.25% sodium hypochlorite as active ingredient), ethanol and mercury chloride ($HgCl_2$) had been reported as very effective sterilizing agents in establishing aseptic buds of many *Zingiberaceae* species (Mrudul *et al.*, 2001; Neeta *et al.*, 2002; Chan & Tong, 2004; Yusuf *et al.*, 2007). Two to three centimetres shoot buds (Figure 4.1(b)) were excised from rhizomes, washed with running tap water and surface sterilized by

using disinfectants with different treatments, as described in Figure 3.1 and Table 3.7 (chapter 3.4.1). Shoot bud explants were first treated with Tween 20, a wetting agent that reduces the surface tension on the explant and thus allowed better access to sterilizing agent. Tween 20 also improved the disinfestation by acting as a surfactant, thereby removing surface contaminants such as soil and dust. Hypochlorite is known to be very effective against bacteria. When diluted in water, the concentration of hypochlorite salt used (NaOCl)) leads to the formation of HOCl, whose concentration is correlated with bactericidal activity (Nakagarwara *et al*., 1998). Bacteria killing by HOCl maybe due at least in part to lethal DNA damage (Dukan *et al.*, 1999; Wlodkowski &Rosenkranz, 1975). Ethanol is also a powerful sterilizing agent but explants were usually exposed to it for only a few minutes due to its extreme phytotoxicity and could cause reverse osmosis in the explants (Afolabi *et al.*, 2009). Reverse osmosis would shrink and shrive the plant explant cells and causes plasmolysis. Ethanol (70 %) washing, prior to disinfection with $HgCl_2$ enhances the contact between $HgCl_2$ and the surface of explants efficiently. Mercury ions in $HgCl_2$ solution used during surface sterilization. This ion interfered with enzymes and protein in the cell membrane and cytoplasm of contaminating pathogenic microorganisms (Smith, 2005). Singh *et al*., (2009) reported that contamination can be reduced to 5.5 % by using 0.1 % (w/v) $HgCl_2$. In contrast, our initial study showed that the contamination was high at 80 % when 0.1 % (w/v) $HgCl_2$ was used. However when the concentration of $HgCl_2$ was increased to 0.5 % (w/v), 83 % of the cultured explants were found to be dead. This is probably due to the fact that a high concentration of $HgCl_2$ is phytotoxic to plant cells. Out of three surface sterilization treatments carried out, treatment three with 0.3 % (w/v) $HgCl_2$ was the most effective with a 77 % survival rate (Figure 4.2) (Appendix B) after four weeks in MS medium. This treatment was thereafter adopted for subsequent experiments.

Figure 4.1: Shoot buds of *C. mangga*. a. Shoot buds sprouted from mature rhizomes of *C. mangga*(bar represent 1 cm) b.Sprouted shoot buds (2 cm to 3 cm) collected as explants (bar represent 0.5cm)

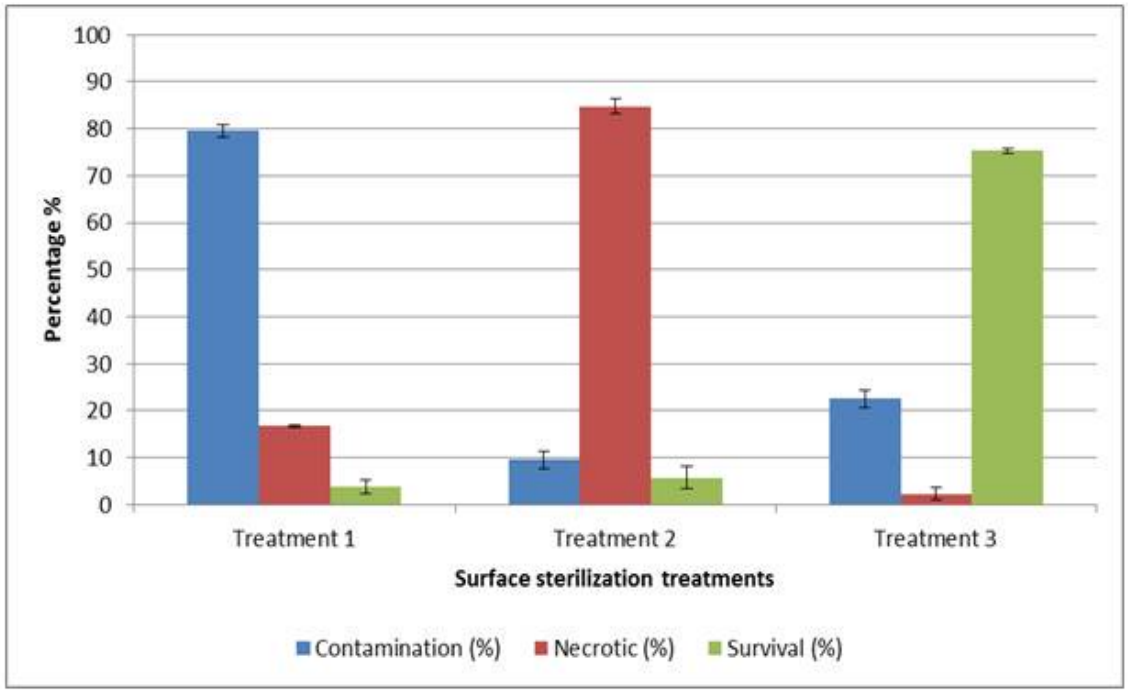

Figure 4.2: Percentage of surviving explants under different methods of surface sterilization.

Values are means ± standard deviation for 4 replicates each treatment

4.3 Initiation, proliferation and characterisation of callus

Callus initiation is the primary stage in many tissue culture processes for the establishment of cell suspension cultures (Kumar & Kanwar, 2007; Ngara *et al.*, 2008), indirect somatic embryogenesis (Kulkarni *et al*.,2002; Rahman *et al*., 2006) and other applications. Various PGRs treatments have been used for these purposes in different plant cultures (Michel *et al*., 2008; Afshari *et al*., 2011). In our present study, several different types and concentrations of auxins were tested for initiation of friable and vigorous growing callus from shoot bud explants to be used as inoculums for suspension cultures. Various auxin concentrations used in this study produced different types of callus morphology and callus induction percentages (Table 4.2 and Table 4.3). When cultured on a MS hormone-free medium, the explants produced roots (Figure 4.3). In contrast in MS medium supplemented with auxin, cultured explants were swollen after five days of inoculation, callus emerged at the cut edges within 14 to 20 days of culture and totally visible after 10 weeks. MS with 1 mg l^{-1} 2,4-D was the most effective medium for friable callus induction at 76 % (Figure 4.4(a)). 2,4-D at 1-2 mg l^{-1} was reported to be an effective PGR that produced rapid proliferating friable callus in *C. amada* (Prakash *et al*., 2004), *Gymnema sylvestris* (Roy *et al*., 2008) and *Brassica napus* (Jonoubi *et al.,* 2005) *in vitro* cultures. Substitutions with other auxins or increasing or decreasing the concentration of 2,4-D resulted in a reduced percentage of explants producing callus (Table 4.2 and Table 4.3). The callus were white, nodular/compact with roots at 0.1mg l^{-1} 2,4-D (Figure 4.4(b)) and relatively brownish with a dry friable appearance at 8 mg l^{-1} 2,4-D (Figure 4.4(c)). However, when MS in combination with higher or lower concentrations of IAA and NAA were used, the callus morphology and callus induction percentage differed but without any particular pattern (Table 4.3). In comparison to the natural auxin, IAA, synthethic auxins NAA and 2,4-D induced a better response in terms of the percentage of friable callus induction even at

low concentrations (Table 4.2 and Table 4.3). Pierik (1990) reported that this result could be attributed to the fact that synthetic PGRs are more stable in their ability to withstand physical and enzymatic degradation than naturally occurring auxins. The callus observed were whitish and globular at lower concentrations of IAA and NAA (1 mg l^{-1} IAA and 1 mg l^{-1} NAA) (Figure 4.4(d)) and were whitish with a mixed friable and globular appearance at higher concentrations of IAA and NAA (5 mg l^{-1} IAA and 5 mg l^{-1} NAA) (Figure 4.4(e)).

Auxin is believed to cause de-differentiation and elicit totipotency (Terzi & Loschiavo, 1990). In this treatment, the concentration of 2,4-D was maintained at 1 mg l^{-1} due to its response in producing favourable rapid growing friable callus. 2,4-D, a strong synthetic auxin has been used as a PGR in many callus induction media due to its ability to initiate and sustain callus growth (Ozawa & Komamine,1989; Lee *et al*.,2002). 2,4-D has a dual effects on explants, that is as a PGR (directly or through endogenous IAA metabolism) and as a stress agent.However, from this study, it could also be inferred that 2,4-D is not necessary for callus initiation and proliferation if other auxins such as exogenous IAA and NAA were used. Salvi *et al*.,(2001) used NAA to induce callus from turmeric leaf base.

In this experiment, embryogenic calli were not produced probably due to prolong exposure of explants to 2,4-D in cultures. According to Kranz, (2001) prolong exposure to high level of auxin will reduce the embryogenic nature of the callus. Feher *et al.*, (2003) reported similar finding in carrot culture where 2,4-D blocked the development of somatic embryo in pre-globular stage. In conclusion, this study showed that the formation of callus depends largely by the type and level of the PGR present in the culture medium.

Table 4.2: Effect of 2,4-D on callus induction from *C. manga* shoot bud explants cultured on MS medium with 3 % (v/v) sucrose

2,4-D (mg l^{-1})	Callus induction (%)	Morphology of callus
0.0	0.0	Explant produced roots and shoots without callus formation
0.1	64 ± 2.3	White, globular, compact/nodular with roots
1.0	76 ± 3.2	White, friable, sticky and glassy
2.0	70 ± 3.1	Yellowish white, friable, sticky and glassy
4.0	54 ± 2.9	Yellowish white, dry friable and glassy
8.0	58 ± 3.1	Brownish, dry friable and glassy

Values represent means ± standard deviation (S.D) for 15 cultures per treatment.

Table 4.3:Effect of IAA and NAA on callus induction from *C. manga* shoot bud explants cultured on MS medium with 3 % (v/v) sucrose

IAA(mg l^{-1})	NAA (mg l^{-1})	Callus induction (%)	Morphology of callus
1	1	62 ± 1.8	Whitish and globular
3	1	55 ± 2.0	Whitish and globular
5	1	58 ± 6.1	Whitish, globular and friable
1	3	42 ± 3.4	Whitish, globular and friable
1	5	57 ± 5.2	Whitish, globular and friable
5	5	70 ± 1.7	Whitish, globular and friable
3	3	68 ± 2.0	Whitish, globular and friable
5	3	67 ± 2.3	Whitish, globular and friable
3	5	66 ± 2.4	Whitish, globular and friable

Values represent means ± standard deviation (S.D) for 15 cultures per treatment.

Sucrose concentrations also affected callus morphology and callus induction percentage (Table 4.4). Mucilaginous and white callus were formed when the concentration of sucrose was increased to 8 % (Figure 4.4(f)). This morphology may be caused by the influence of sucrose on the humidity of *in vitro* culture conditions (Lee *et*

al., 2002). In plant tissue culture, sucrose is the most commonly used carbohydrate source because of the wide spread of this disaccharide as a transporter molecule, and its high solubility in water. Many *in vitro* studies have proven that sucrose supports near optimum rates of growth and also plays multiple roles in the provision of carbon and energy that promotes cell growth and division (Kranz, 2001;Swedlund & Locy, 1993).

Table 4.4: Effect of sucrose on callus induction from *C. mangga* shoot bud explants cultured on MS medium with 1mg/l 2,4-D

Sucrose (%) (v/v)	**Callus induction (%)**	**Morphology of callus**
3	76 ± 3.2	White, friable, sticky and glassy
8	65 ± 2.7	White, mucilaginous, wet and in lumps

Values represent means ± standard deviation (S.D) for 15 cultures per treatment.

Subculturing enabled amassing of a large stock of calli for other subsequent applications. In this study, callus subcultured on MS media supplemented with 1 mg l^{-1} or 2 mg l^{-1} 2.4-D grew rapidly doubling in size in less than 2 weeks producing white friable and yellowish white friable callus respectively. Whereas, callus in MS media supplemented with 4 mg l^{-1} 2,4-D was yellowish white and dry friable. It took 3 weeks to double in size when subcultured onto fresh medium of same formulation. Meanwhile in MS media supplemented with 8 mg l^{-1} 2,4-D, browning of callus was observed without any further growth. Callus subcultured on all MS media supplemented with various concentration of IAA and NAA doubled in size in 3 to 4 weeks when subcultured onto fresh medium of same formulation. Morphology observed was either white globular or white globular and friable as reported in table 4.3. The declining callus propagation with increasing concentration of 2,4-D in this study concurred well

with results reported by Kackar *et al.*, (1993). Tan, (2005) reported *Bosenbergia rotunda* callus proliferation rate appeared to diminish with increased frequency of subculture.

Figure 4.3: Root from shoot bud explant of *C. mangga* on MS media without any PGR (Scale: Bar = 1mm)

rt: root structure

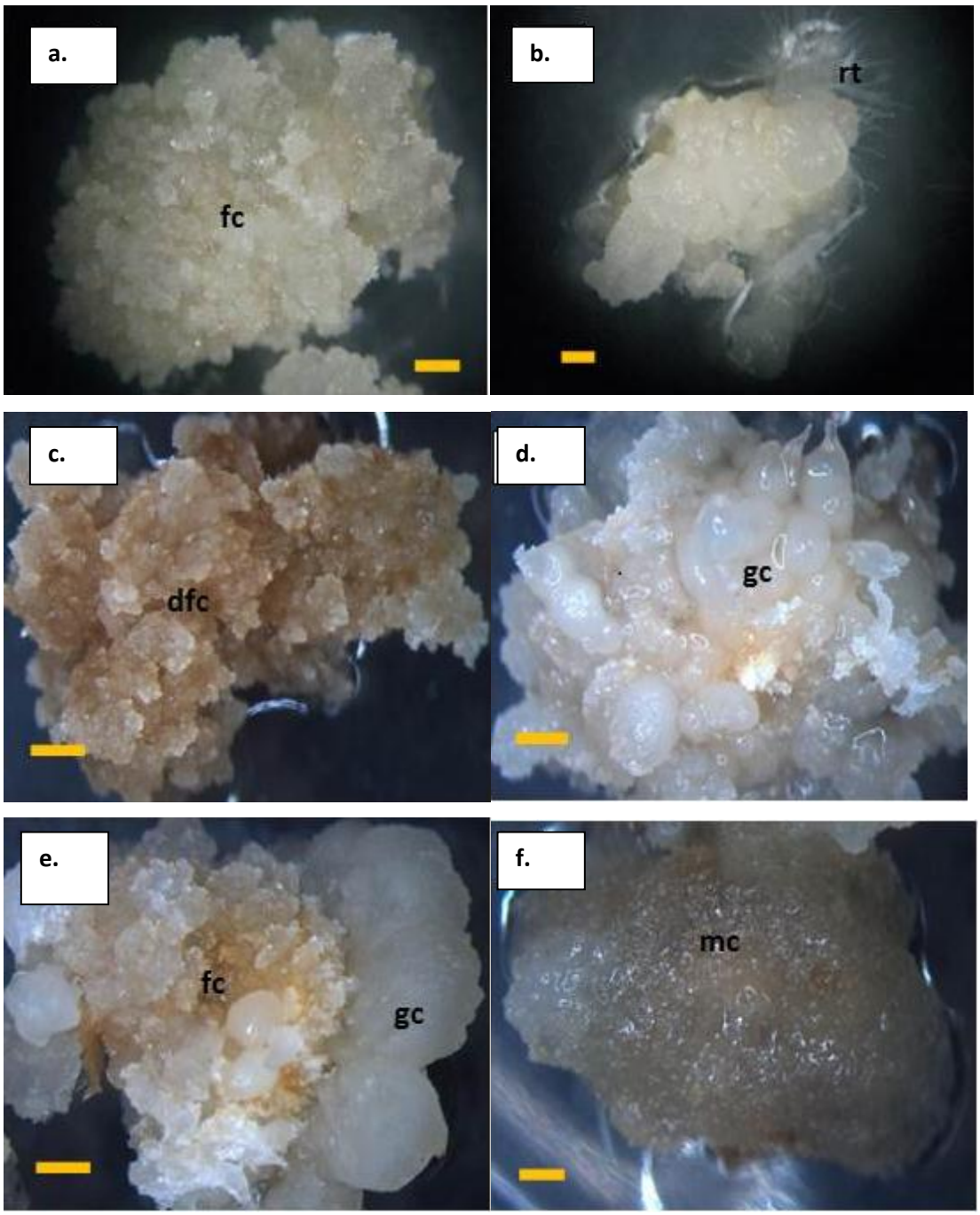

Figure 4.4: Morphology of *C. mangga* calli in MS medium. **a.** White and friable callus induced on MS with 1 mg l^{-1} 2,4- D media (Scale: Bar = 1 mm). **b.**White, nodular/compact and rooting callus on MS with 0.1 mg l^{-1} 2,4-D media (Scale: Bar = 1 mm). **c.** Brownish, dry friable callus on MS with 8 mg l^{-1} 2,4-D media (Scale: Bar = 1 mm).**d.** Whitish and globular callus on MS with 1 mg l^{-1} IAA and 1 mg l^{-1} NAA media (Scale: Bar = 1 mm). **e.** Whitish, globular and friable callus on MS with 5 mg l^{-1} NAA and 5 mg l^{-1} IAA media (Scale: Bar = 1 mm). **f.** Mucilaginous, wet and big lump callus on MS with 8% sucrose in 1 mg l^{-1} 2,4-D media (Scale: Bar = 1 mm).

dfc: dried friable callus; **fc**: friable callus; **gc**: globular callus; **mc**: mucilaginous callus; **rt:** root structure

4.4Growth of callus culture

Callus doubled in size in less than two weeks when subcultured in a fresh MS medium supplemented with 1 mg l^{-1} 2,4-D. However, callus on MS medium supplemented with lower or higher 2,4-D or other PGRs, proliferated slowly and did not produce friable callus as reported earlier. Friable callus is ideal for the initiation of cell suspension cultures in many plants (Lee & Chan, 2004). Slow growth rates were not appropriate for the establishment of suspension cultures. For this reason, the growth kinetics of callus cultured on MS media supplemented with 1 mg l^{-1} 2,4-D was further studied (Figure 4.5) (Appendix C).

The growth curve of *C. mangga* callus showed a sigmoidal-type pattern where three growth phases can be distinguished (Figure 4.5). A sigmoidal growth curve was also reported in the *in vitro* callus culture of other plant species such as *Orthosiphon stamineus* (Lee & Chan, 2004), coffee plant (Santos *et al*., 2003) and Brazil nut (Serra, 2000).

In this study, during the lag phase cells of the explants were preparing for division and accumulating energy. This phase occurred up to day 42 from inoculation which was similar to Brazil nut callus culture (Serra, 2000). This was followed by an exponential growth phase occurring between day 42 and 110. During this growth period maximum cell division and increase in cellular area (Abbade *et al.*, 2010) were observed.

Growth deceleration was observed between day 110 and 120. Lima *et al.*, (2008) reported that growth deceleration occured as a result of the usage of nutrients and the accumulation of toxic substances in the culture medium. It is appropriate to subculture the callus at the beginning of this phase (Lima *et al.*, 2008). The maximum increase of callus biomass was observed at the late stationary phase, with the initial inoculums of 0.3 g reaching 11 g.

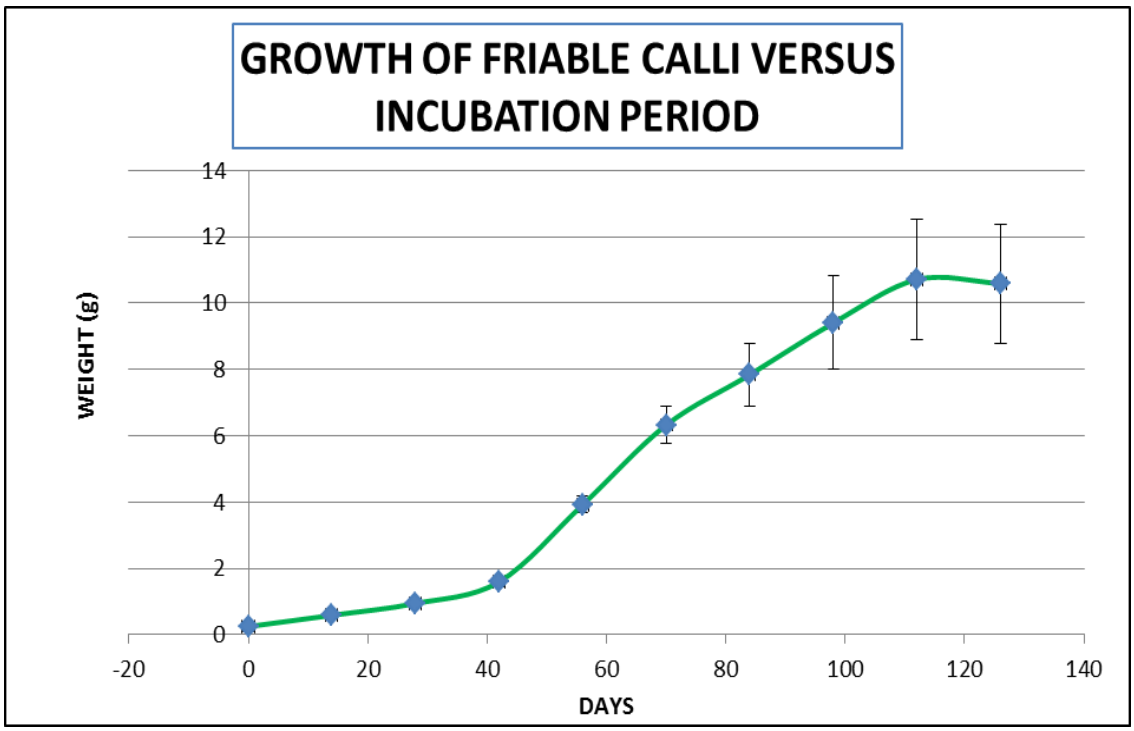

Figure 4.5: Fresh weight of *C. mangga* callus culture in MS medium supplemented with 1mg l^{-1} 2,4-D
Values represent means ± standard deviation (S.D) for 4 replicates per treatment.

4.5 Histology

Histological studies were also carried out to provide further evidence on fast growing callus of different morphology. Periodic acid Schiff (red stain), specifically stains polysaccharide (starch reserves and walls) meanwhile blue-black naphtol stains soluble or reserve protein (Fisher, 1968). Red stained cells indicate the presence of polysaccharides as the source of energy that supports proliferation of cell division (Jalil *et al.*, 2008). Histological section of friable white callus cultured on MS solidified medium supplemented with 1 mg l^{-1} and 2 mg l^{-1} 2,4-D showed large cells with large nuclei and dense cytoplasm with islets of protein compounds stained blue- black and polysaccharide stained red (Figure 4.6(a)). When supplemented with a low concentration of 2,4-D (0.1 mg l^{-1} 2,4-D), cells with dense cytoplasm and nuclei were observed (Figure 4.6(b)). However these cells (Figure 4.6(b)) grew slowly due to the absence of polysaccharide which supports cell division. Callus produced in media

supplemented with high concentrations of IAA and NAA (5 mg l^{-1} NAA and 5mgl^{-1} IAA) showed cells with less dense cytoplasm and nuclei (Figure 4.6(e)). Callus on medium supplemented with low concentrations of IAA and NAA (1 mg l^{-1} NAA and 1 mg l^{-1} IAA) media are irregular in shape with small nuclei, less cytoplasmic and the presence of vacuolated cells (Figure 4.6(d)). While, callus initiated in MS medium supplemented with 8 mg l^{-1} 2,4-D revealed that the cells are largely vacuolated (Figure 4.6(c)). The presence of vacuolated cells will affect growth of callus, thus reducing the rate of suspension culture propagation when transferred to a liquid medium and the propagation of debris will dominate the culture (Tan, 2005). Thus, the histological studies showed that the ideal callus for the initiation of cell suspension were cells with large nuclei and dense cytoplasm that were cultured on MS medium supplemented with 1 mg l^{-1} 2,4-D. This callus shows the presence of polysaccharides that support the proliferation of cell division. Histological sectioning was also performed for callus initiated on MS medium supplemented with 8 % (v/v) of sucrose. The cells also showed large nuclei with dense cytoplasm and the presence of few polysaccharides granules (Figure 4.6(f)).

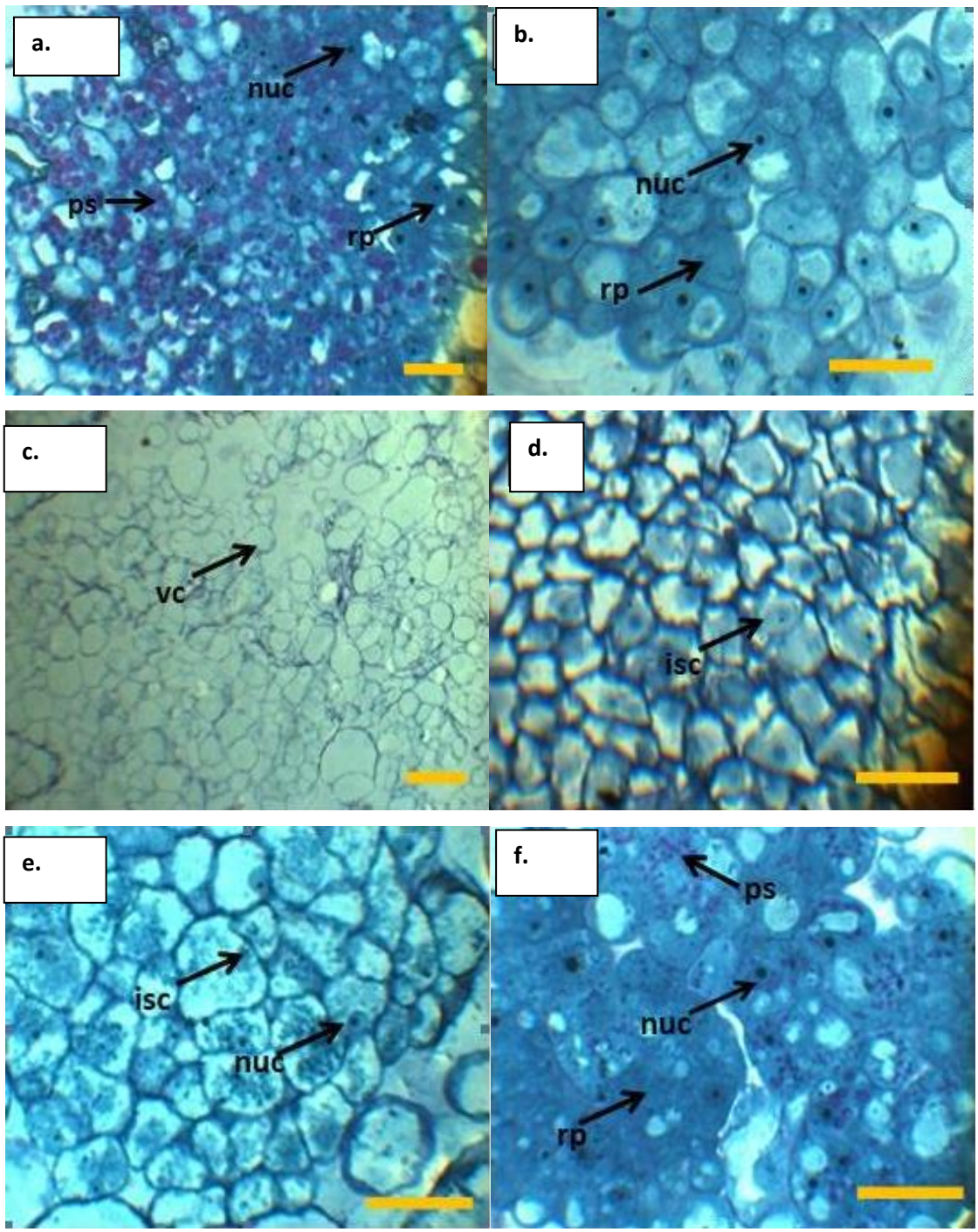

Figure 4.6: Histology of *C. mangga* calli in MS medium. **a.** Histology of dense cells of callus with large nucleus on MS with 1 mg l^{-1} 2,4- D media (Scale: Bar = 50 µm). **b.**Histology of dense cells of callus with large nucleus initiated on MS with 0.1 mg l^{-1} 2,4-D media(Scale: Bar = 50 µm). **c.** Histology of callus with large vacuolated cells on MS with 8 mg l^{-1}2,4-D media (Scale: Bar = 50 µm).**d.**Histology of callus with irregular shape cells with small nucleus and cytoplasm on MS with 1 mg l^{-1} IAA and 1 mg l^{-1} NAA media (Scale: Bar = 50 µm).**e.**Histology of callus with small nucleus and cytoplasm on MS with 5 mg l^{-1} NAA and 5 mg l^{-1} IAA media (Scale: Bar = 50 µm).**f.**Histologyof dense cells of callus withlarge nucleion MS with 8% sucrose in 1 mg l^{-1} 2,4-D media (Scale: Bar = 50 µm)

nuc: nucleus; **ps**: polysaccharide; **rp**: reserved protein; **vc**: vacuolated cells;
isc: irregular shaped cells;

4.6 Determination of optimum maintenance medium for cell suspension culture

Cell suspension culture is a viable alternative over whole plant cultivation for the production of secondary metabolites. Cell suspension culture has been proven to be a rapid propagation method for callus and has higher proliferation rate than normal plant. This makes it suitable to be a continuous source of *C. mangga* cells for this study.

Continuous shaking is essential in facilitating the transfer of oxygen and nutrients from liquid media to cells in suspension culture. Shaking will also maintain uniform distribution of the cells and cell clumps in liquid media. Size of cell clumps can be controlled through the speed of rotation and it is inversely proportional to the speed of rotation (Mohamed, 2010). In this study, the orbital shaker was set at a rotation speed of 90 rpm to disperse the cells, nutrients and oxygen uniformly without creating high shear stress that would affect the cell growth and viability in liquid media.

In this study,when cells were suspended in MS medium supplemented with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose, the proliferation rate was rapid, doubling the yield in 10 days after initiation. However, suspension cultures showed tissue browning after the third passage of subculture even though the subculture interval was shortened. Subculture was frequently done to replenish the media and remove the build-up of toxic media. Usually during the culture period, nutrients in the medium were used to support growth and increase biomass of cells. In the cell suspension culture of *Pelargonium fragrans*, accumulation of monoterpenes was reported to be toxic to the cultures (Brown *et al*., 1987). Thus,frequent subculture to fresh medium has been reported as a factor favouring culture establishment without tissue browning due to toxicity of media as reported by Tiwari *et al*.,(2002).

Different strengths of MS basal medium and different concentrations of 2,4-D (auxin) were tested to overcome browning. Decreasing the concentration of 2,4-D to 0.5 mg l^{-1} in the MS medium, earlier produced proliferating suspension cells without any sign of tissue browning. Eventhough, the use of 2,4-D and NAA in the media as common auxins have encouraged growth and maintained the viability of suspension cultures in this study and also other reports (Endress, 1994), browning was also associated with the use of 2,4-D, especially if high concentrations were added to the medium. This problem was also found in cultures of *Dioscorea* sp.(Viana & Mantell, 1989) and *Aconitum heterophyllum* (Giri*et. al.,* 1993). Evans *et al.,* (2003) reported that synthetic auxins like 2,4-D may possess herbicidal property at high concentrations, that inhibits the formation of callus and suspension cells leading to cell browning.

However, browning recurred in cells cultured in 0.5 mg l^{-1} 2,4-D supplemented media after the 6th subculture. The addition of ascorbic acid (5 mg l^{-1}) and citric acid (5 mg l^{-l}) as antioxidants and further decreasing 2,4-D concentration to 0.3 mg l^{-1} produced proliferating yellowish white and vigorous suspension cell cultures (Figure 4.7) without further tissue browning. Bushra *et al*., (2009), reported that the browning and subsequent death of explants and cultures could be attributed to the oxidation of polyphenols. Treatment with antioxidants like ascorbic acid and citric acid seemed to inhibit the polyphenoloxidase enzyme that converts the phenols to typical brown polymers. Therefore this is one of the effective strategies to control the phenolic browning of *in vitro* cultures (Pizzocaro *et al.,* 2007). The combination of 2 % ascorbic acid and 2 % citric acid reduced the percentage of browning in *F. religiosa* L. culture (Siwach *et al.,* 2011).

MS medium augmented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 g l^{-1} malt extract and antioxidant agents, 5 mg l^{-1} ascorbic acid and citric acid respectively was further used as

standardized medium for suspension cultures. Meanwhile other media used in this study did not overcome cell browning.

100 mg l^{-1} glutamine and 0.1 mg l^{-1} d-biotin were also added to the media. Glutamine is an amino acid added as a nitrogen source and also to increase embryogenic callus formation (Peterson & Smith, 1991). Ahmed *et al.* (2009) reported that glutamine at different concentrations has been used in order to reduce phenolic exudation from leaf callus. Meanwhile biotin acts as a cofactor for a small number of enzymes involved in carboxylation, decarboxylation, and transcarboxylation reactions that are concerned with fatty acid and carbohydrate metabolism (Alban *et al.*, 2000; Knowles, 1989).

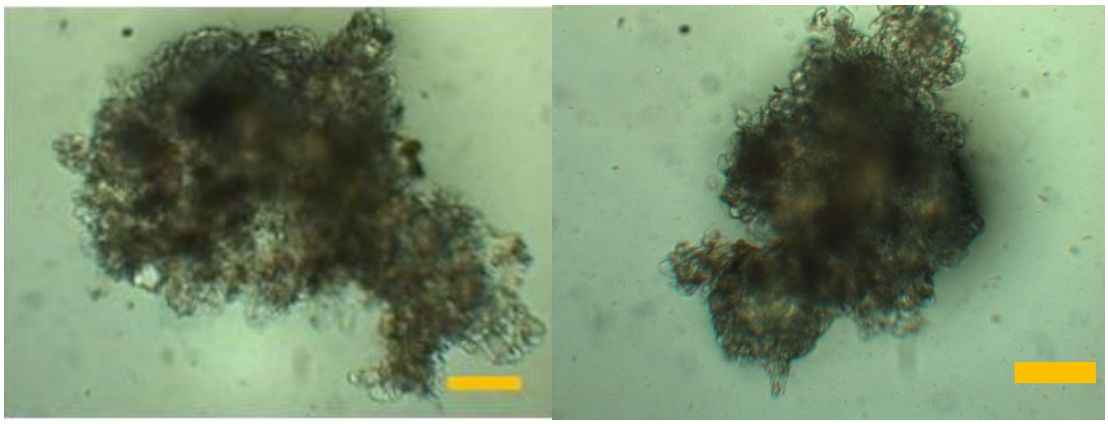

Figure 4.7**:** Rapid growing suspension cells in MS medium supplemented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 mg l^{-1} malt extract, 5 mg l^{-1} ascorbic acid and 5 mg l^{-1} citric acid (Scale: Bar = 100µm)

4.7Growth of suspension culture

Since prior results had indicated that suspension cultures of *C. mangga* could grow well in an MS medium supplemented with vitamins and augmented in 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 mg l^{-1} malt extract and added with antioxidant agent of 5 mg l^{-1} ascorbic acid and 5

mg l^{-1} citric acid, growth kinetics experiments were undertaken using this medium. The cell cultures were maintained by subculturing at 10 days intervals.

The growth of cells in the suspension culture was found to proliferate more vigorously compared to cells on the solidified culture medium (Figure 4.8)(Appendix D). This was probably due to the agitation and loosening of cells which would encourage further cell division. In this study, suspension cultures showed typical growth kinetics when 5 ml (SCV) inoculums were cultured in 50 ml of culture medium. The growth of cell suspension cultures reached a maximum growth (45ml SCV) 21 days after initiation and subsequently became stationery and started to decline after day 28 (Fig 4.8)(Appendix D). The same growth kinetics pattern was reported by Lee & Chan et al (2004) in *in vitro Orthosiphon stamineus* cell suspension growth kinetics.

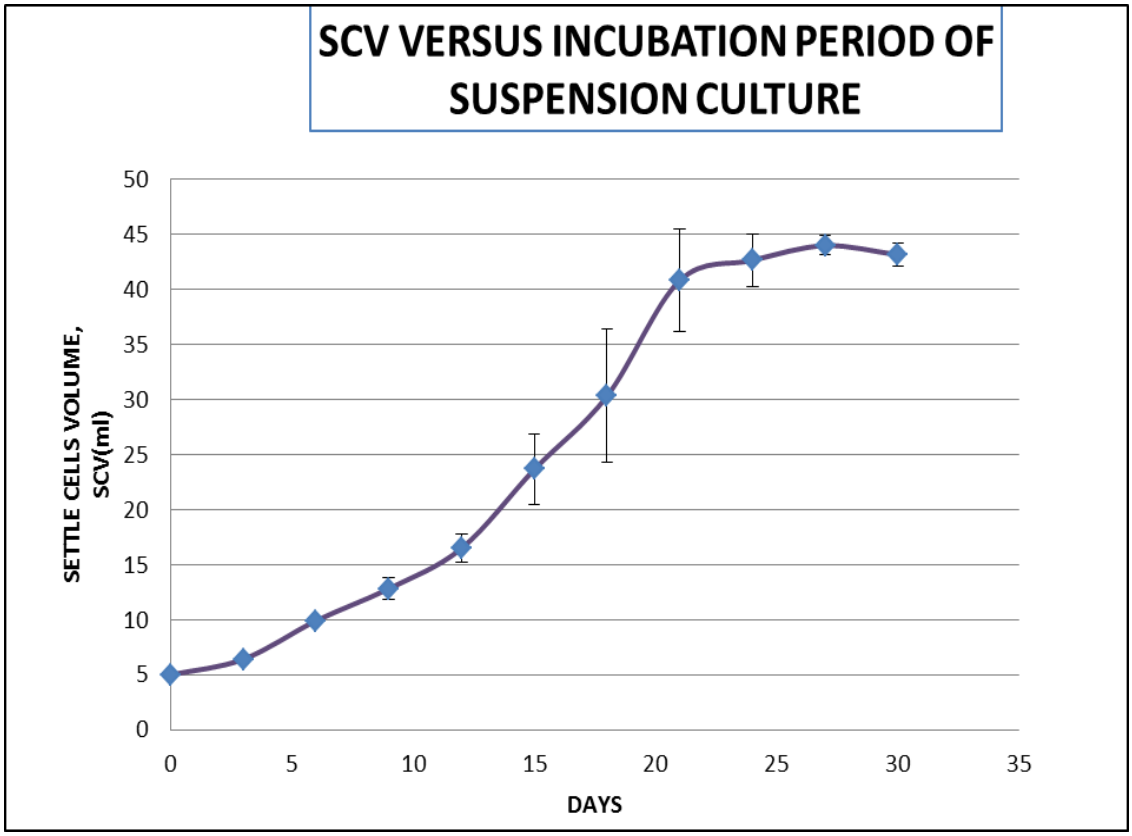

Figure 4.8: Fresh weight of *C. mangga*suspension culture in MS medium supplemented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 mg l^{-1} malt extract, 5 mg l^{-1} ascorbic acid and 5 mg l^{-1} citric acid.
Values represent means ± standard deviation (S.D) for 8 replicates per treatment.

4.8 Standard calibration of (*E*)-labda-8(17),12-diene-15,16-dial

Standard (*E*)-labda-8(17),12-diene-15,16-dial was run through GC and the retention time was determined. The retention time for (*E*)-labda-8(17),12-diene-15,16-dial was at 20.8 ± 0.05 minutes.

Figure 4.9showed the (*E*)-labda-8(17),12-diene-15,16-dial standard calibration with linear equation relating (*E*)-labda-8(17),12-diene-15,16-dial concentrations (mg ml^{-1}) to peak area. The correlation coefficient, R^2 was 0.9865.

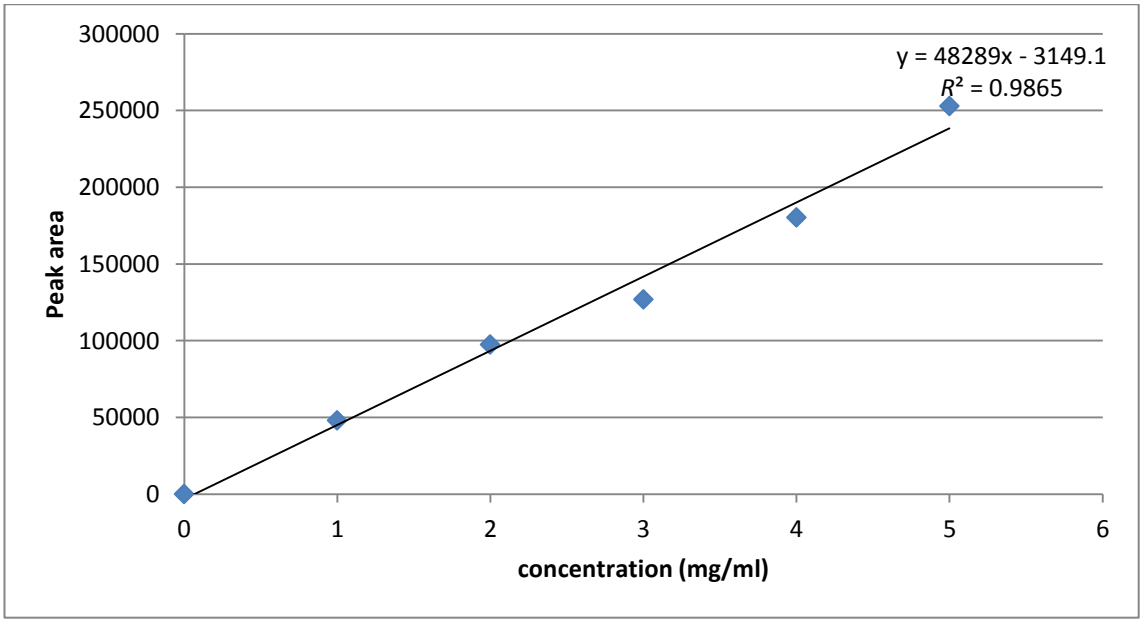

Figure 4.9:Standard calibration for (*E*)-labda-8(17),12-diene-15,16-dial

4.9 Characterization of (*E*)-labda-8(17),12-diene-15,16-dial

4.9.1 Thin layer chromatography

TLC was carried out to detect the presence of (*E*)-labda-8(17),12-diene-15,16-dial in the extract. Figure 4.10 (a) showed the TLC of crude extract from rhizome after being stained by iodine. Figure 4.10 (b) shows the purified (*E*)-labda-8(17),12-diene-15,16-dial extract from rhizomes, friable callus and suspension cells that were tested along with (*E*)-labda-8(17),12-diene-15,16-dial standard by pTLC technique. The figures clearly showed the spots which was assigned to (*E*)-labda-8(17),12-diene-15,16-dial from the extractions (blue circle). Acetone and hexane were used as mobile phase in a ratio of 3:7. Crude extract showed similar chromatographic pattern as the standard for the presence of (*E*)-labda-8(17),12-diene-15,16-dial with R_f value of 0.40 as reported by Lee, (2011).

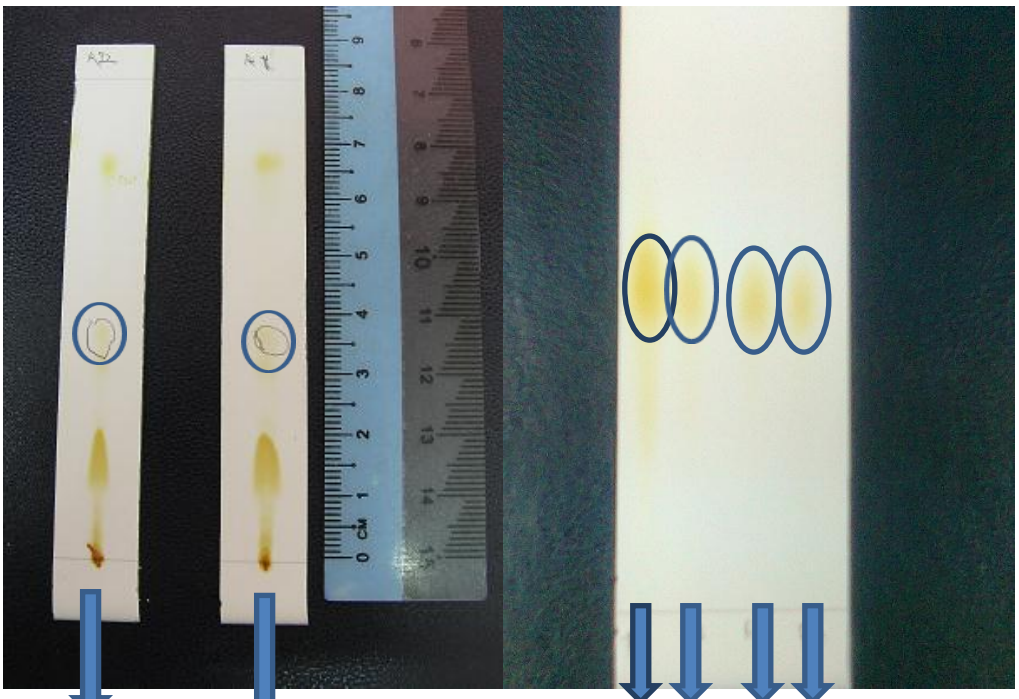

Figure 4.10 (a)

Figure 4.10 (b)

Key:

○ : (*E*)-labda-8(17),12-diene-15,16-dial
RC : rhizome crude
PR : purified rhizome methanol crude
S : standard
PC : purified callus methanol crude
PS : purified suspension cells methanol crude

Figure 4.10: (*E*)-labda-8(17),12-diene-15,16-dial detection in TLC plate

4.9.2 Gas chromatography analysis

(*E*)-labda-8(17),12-diene-15,16-dial is a light yellowish viscous oil. Lee (2011) reported that the mass spectrum showed a molecular ion peak at *m/z* 302 consistent with a molecular formula $C_{20}H_{30}O_2$, a diterpenoid. A base peak was observed at *m/z* 137suggesting a labdane-type diterpene skeleton (Itokawa and Moritha,1988). GCFID

detected the retention time for (*E*)-labda-8(17),12-diene-15, 16 dial at 20.8 ± 0.05 minutes, similar to that reported by Lee (2011) for the same compound

Table 4.5 showed the amount of (*E*)-labda-8(17), 12-diene-15, 16 dial obtained from *in vivo* rhizomes and shoots. The results showed significant difference ($p < 0.05$) between (*E*)-labda-8(17),12-diene-15,16-dial yields from these sources with the rhizome giving consistently higher amount of the compound. Figures 4.11 and 4.12 showed the peaks of (*E*)-labda-8(17),12-diene-15,16-dial () extracted from *in vivo* rhizomes and shoot buds, respectively.

Table 4.5: Quantity of (*E*)-labda-8(17),12-diene-15,16-dial obtained from 1 g dried sample of rhizomes and shoot buds

Replicates	**(*E*)-labda-8(17),12-diene-15,16-dial concentration, mg g^{-1} ± S.E**	
	***In vivo* sources**	
	Rhizomes	Shoot
1	28.7 ± 0.7	9.1 ± 0.3
2	24.3 ± 0.9	10.7 ± 0.3
3	29.3 ± 0.6	14.4 ± 0.4

Calli cultures were extracted at different growth periods to determine which time period gives the highest yield of (*E*)-labda-8(17),12-diene-15,16-dial. Friable calli of 2, 3 and 4 months after initiation on MS medium with 1 mg l^{-1}2,4-D were analysed for the presence of (*E*)-labda-8(17),12-diene-15,16-dial. The result showed significant difference ($p < 0.05$) between (*E*)-labda-8(17),12-diene-15,16-dial yields from different cultivation time (Table 4.6). Figures 4.13 and 4.14 showed the peaks of (*E*)-labda-8(17),12-diene-15,16-dial () extracted from *in vitro* callus at different time periods.

The concentrations of the (*E*)-labda-8(17),12-diene-15,16-dial varied with the age of the callus culture (Table 4.6). The concentration was the highest at three months cultivation (late exponential phase), and the level declined at the fourth month (stationery phase). The compound was not detected in two months culture. Thus, all the subsequent extractions were carried out on a three month old callus.

Table 4.6: Quantity of (*E*)-labda-8(17),12-diene-15,16-dial obtained at different growth period of callus

	(*E*)-labda-8(17),12-diene-15,16-dial concentration in 1g of dried callus , mg g^{-1} ± S.E		
Replicates	**Age of callus cultures**		
	2 months	**3 months**	**4 months**
1	—	2.3 ± 0.0	1.1 ± 0.0
2	—	1.6 ± 0.1	1.0 ± 0.0
3	—	2.4 ± 0.3	1.3 ± 0.0

Calli of different morphologies initiated on MS medium supplemented with various PGRs were also tested for the presence of (*E*)-labda-8(17),12-diene-15,16-dial. Production of (*E*)-labda-8(17),12-diene-15,16-dial in friable callus (MS + 1mg/l 2,4-D + 3% sucrose), mucilaginous callus (MS + 1 mg l^{-1} 2,4-D + 8 % sucrose) and nodular compact callus (MS + 0.1 mg l^{-1} 2,4-D + 3% sucrose) were compared. The amount of (*E*)-labda-8(17),12-diene-15, 16 dial in friable callus was found to be significantly higher ($p < 0.05$) as compared to mucilaginous and compact callus from all the three replicates tested (Table 4.7). Figures 4.15, figure 4.16 and figure 4.17 showed the peaks of (*E*)-labda-8(17),12-diene-15,16-dial () extracted from *in vitro* callus with different morphologies.

Table 4.7: Quantity of (*E*)-labda-8(17),12-diene-15,16-dial obtained from callus of different morphologies

Replicates	**(*E*)-labda-8(17),12-diene-15, 16 dial concentration, mg g^{-1} ± S.E**		
	Morphology of callus		
	Friable	**Mucilaginous**	**Compact**
1	2.3 ± 0.0	1.1 ± 0.1	1.2 ± 0.0
2	1.6 ± 0.1	1.1 ± 0.1	1.2 ± 0.0
3	2.4 ± 0.3	1.1 ± 0.1	1.6 ± 0.0

Suspension cultures were also extracted for the compound every three days for thirty days to determine the time period that yields the highest amount of (*E*)-labda-8(17),12-diene-15,16-dial. The results obtained showed significant difference ($p < 0.05$) between (*E*)-labda-8(17),12-diene-15,16-dial yields for different cultivation periods (Table 4.8). The compound was detected only after the late exponential and stationary phases which were on 18th, 21st, 24th and 27th days. Figures 4.18, 4.19, 4.20 and 4.21 showed the peaks of (*E*)-labda-8(17),12-diene-15,16-dial () extracted from cell suspension at different growth periods on day 18, 21, 24 and 27 respectively.

Table 4.8: Quantity of (*E*)-labda-8(17),12-diene-15,16-dial obtained at different growth periods of a suspension culture

Replicates	**(*E*)-labda-8(17),12-diene-15,16-dial concentration, mg g^{-1} ± S.E**									
	Age of suspension culture (days)									
	3	6	9	12	15	18	21	24	27	30
1	-	-	-	-	-	0.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	-
2	-	-	-	-	-	0.5 ± 0.0	0.4 ± 0.0	1.2 ± 0.1	0.4 ± 0.0	-
3	-	-	-	-	-	0.6 ± 0.0	0.8 ± 0.0	2.0 ± 0.0	0.6 ± 0.0	-

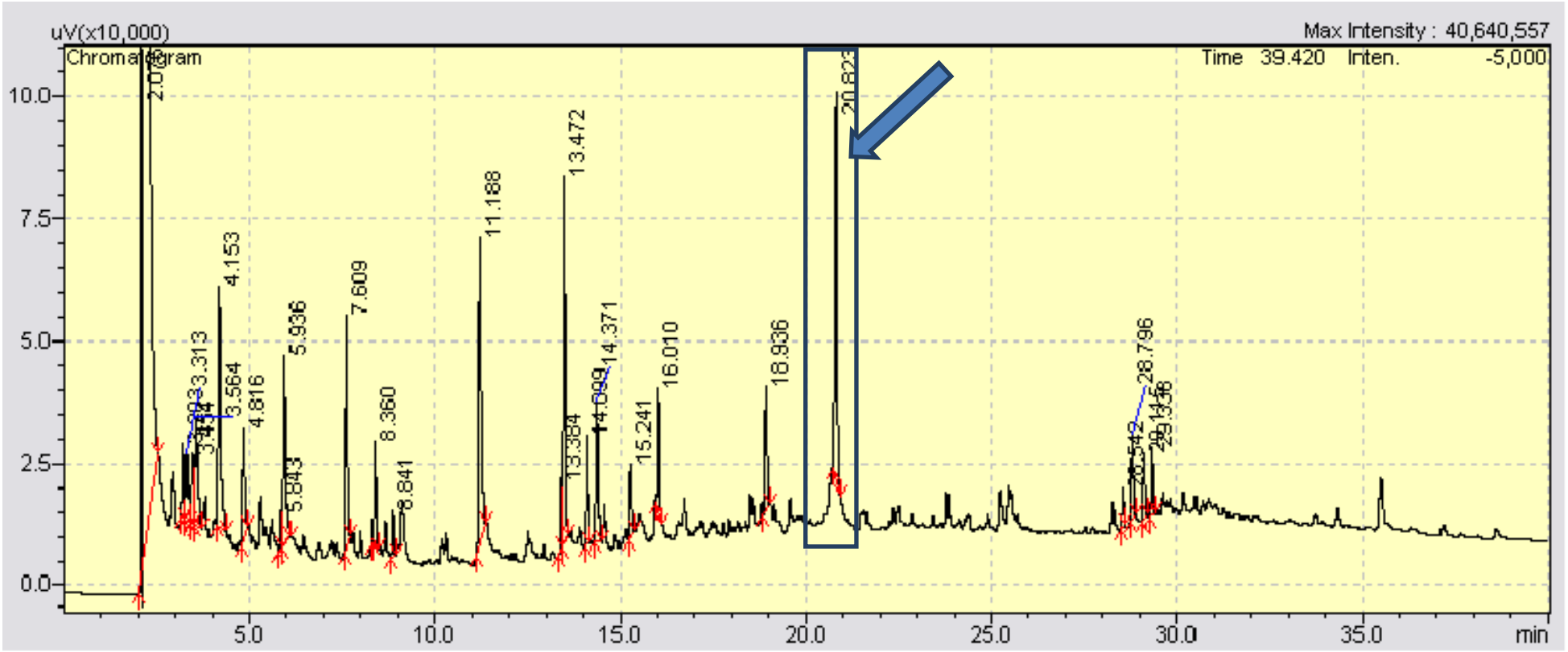

Figure 4.11: GC-FID chromatogram of methanol crude extract from *in vivo* rhizome

: (*E*)- Labda-8(17),12-diene-15, 16 dial

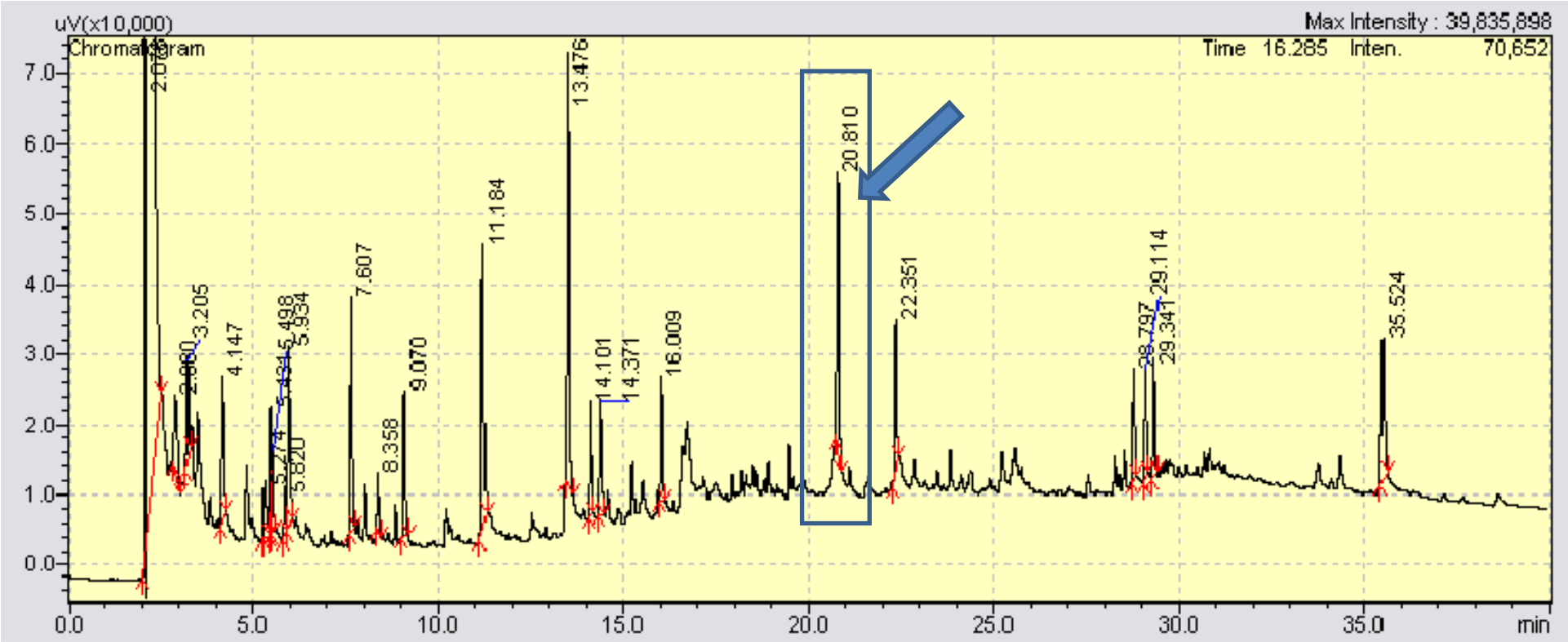

Figure 4.12: GC-FID chromatogram of methanol crude extract from *in vivo* shoot bud

: (*E*)- Labda-8(17),12-diene-15, 16 dial

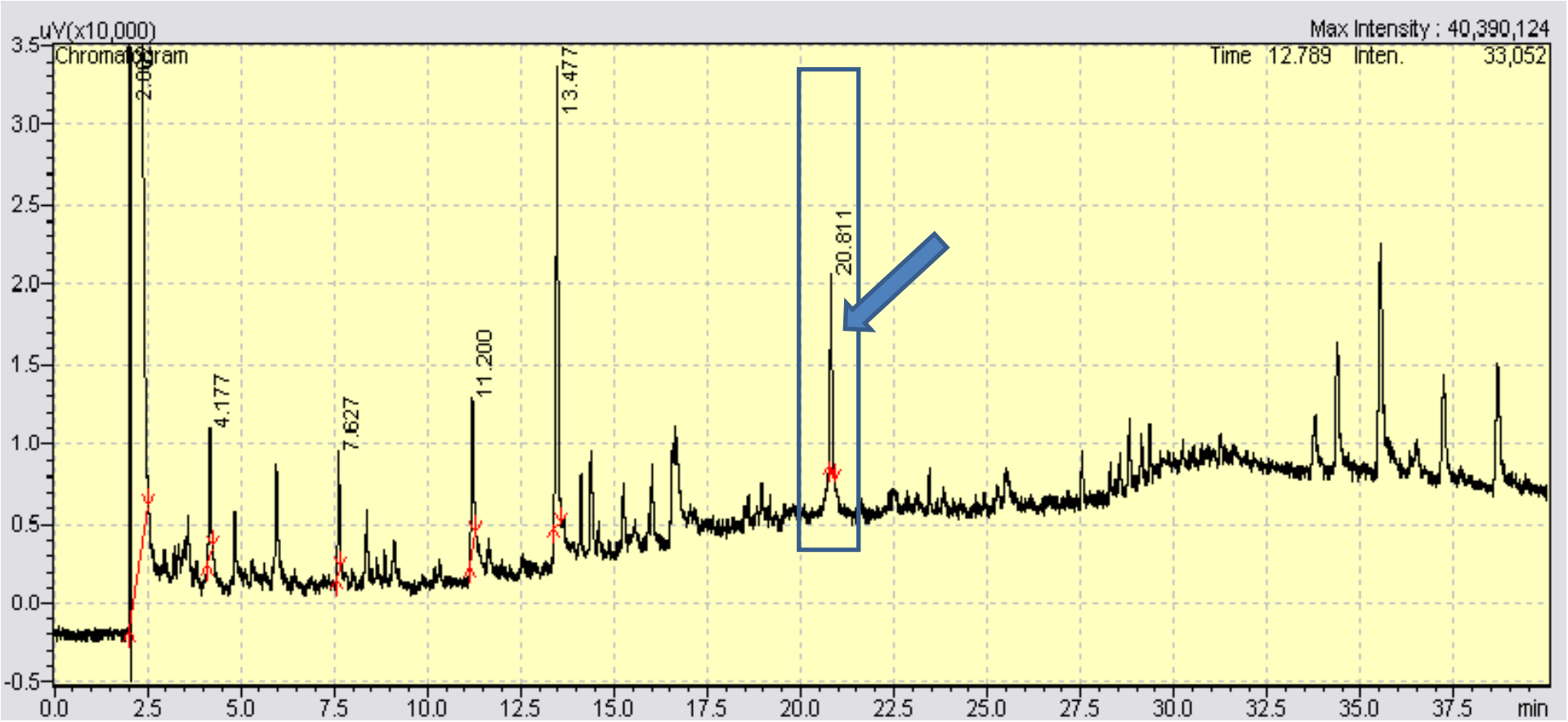

Figure 4.13: GC-FID chromatogram of methanol crude extract from friable callus (3 months)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

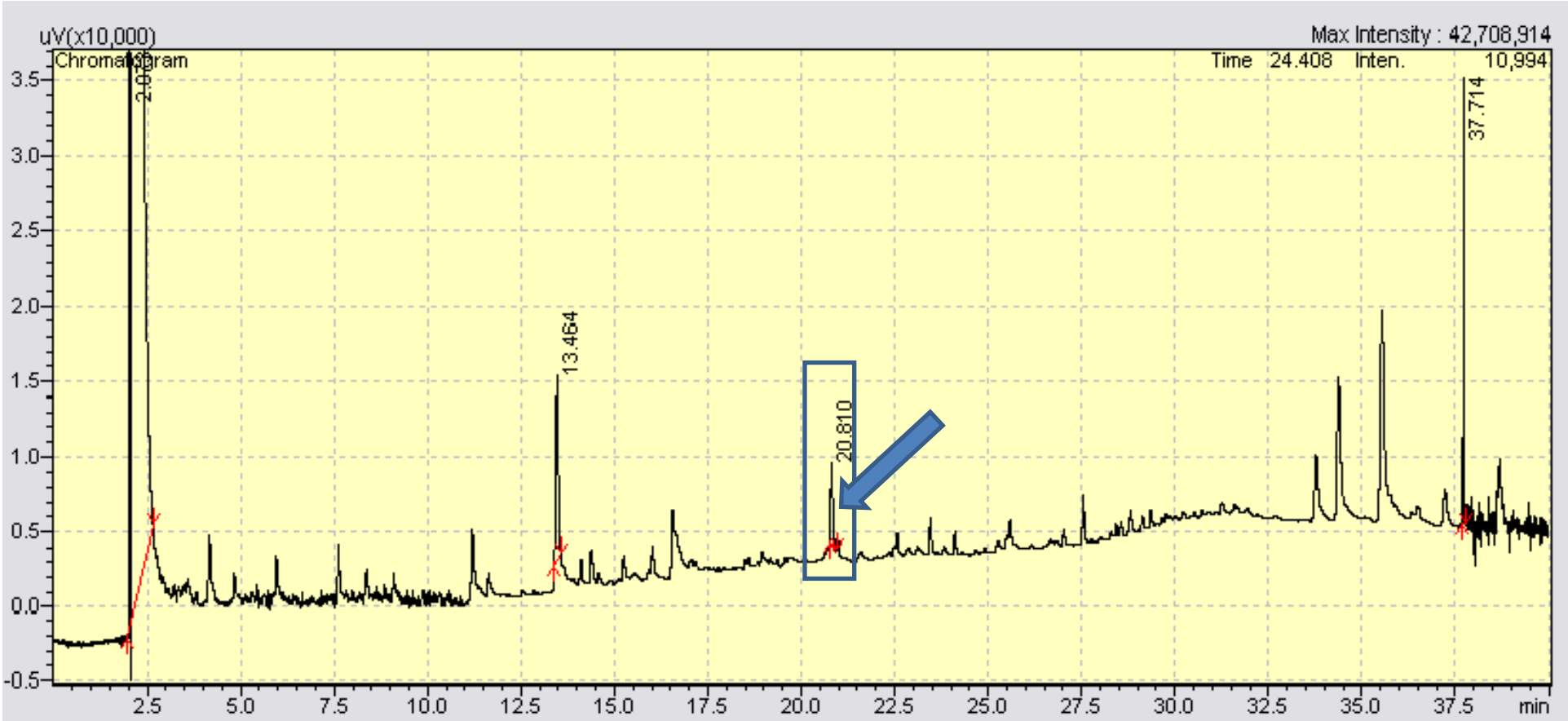

Figure 4.14: GC-FID chromatogram of methanol crude extract from friable callus (4 months)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

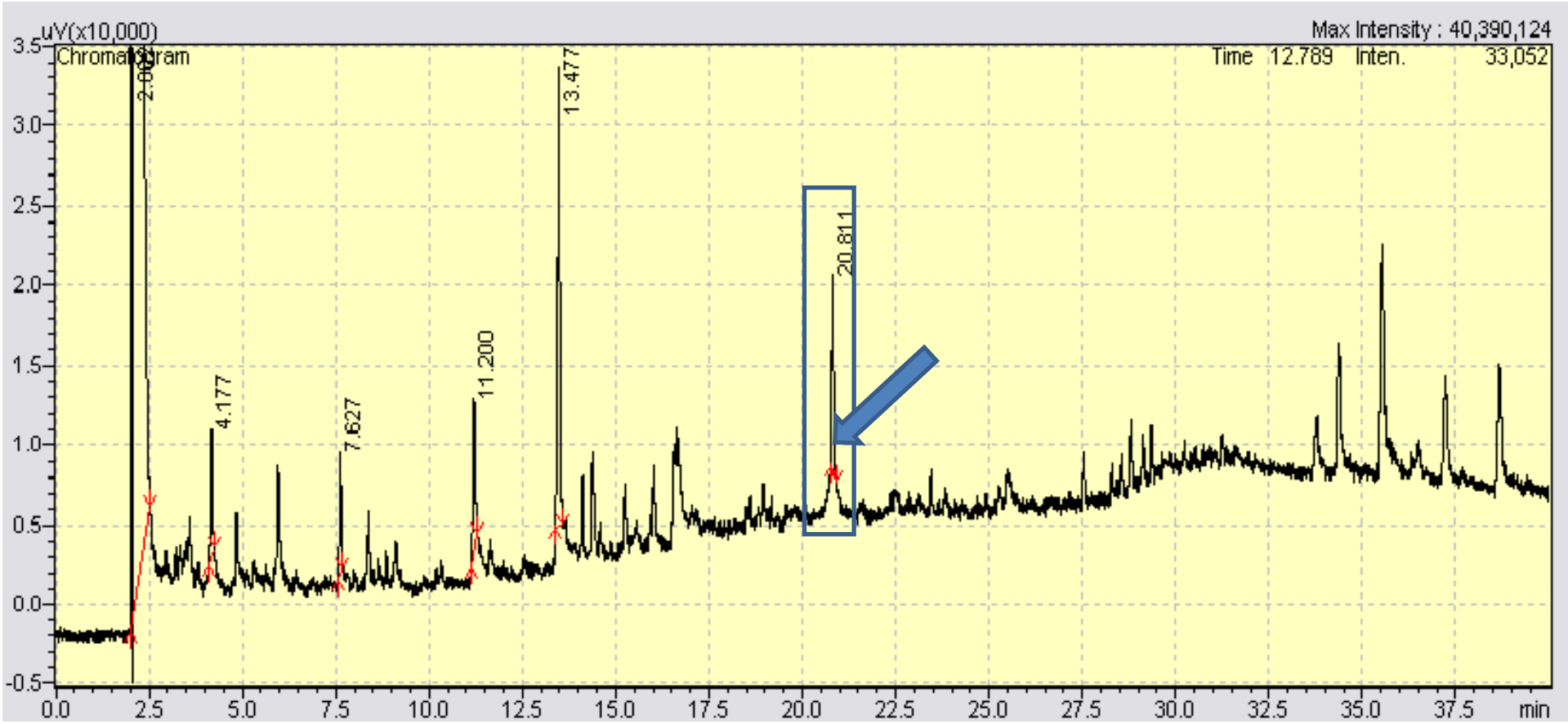

Figure 4.15: GC-FID chromatogram of methanol crude extract from friable callus (MS + 1 mg l^{-1} 2,4-D with 3% sucrose)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

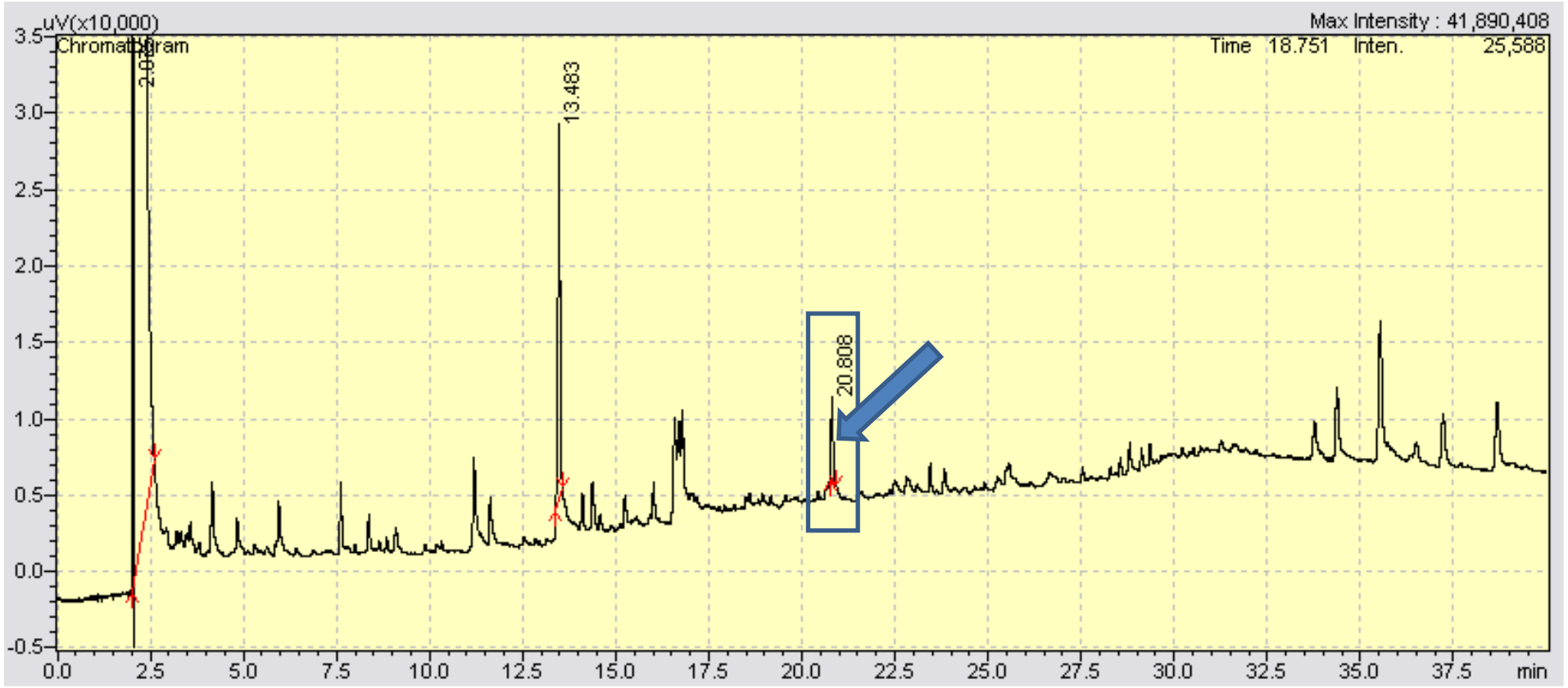

Figure 4.16: GC-FID chromatogram of methanol crude extract from mucilaginous callus (MS + 1mg l^{-1} 2,4-D with 8% sucrose)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

Figure 4.17: GC-FID chromatogram of methanol crude extract from compact and nodular callus (MS + 0.1 mg l^{-1} 2,4-D with 3% sucrose)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

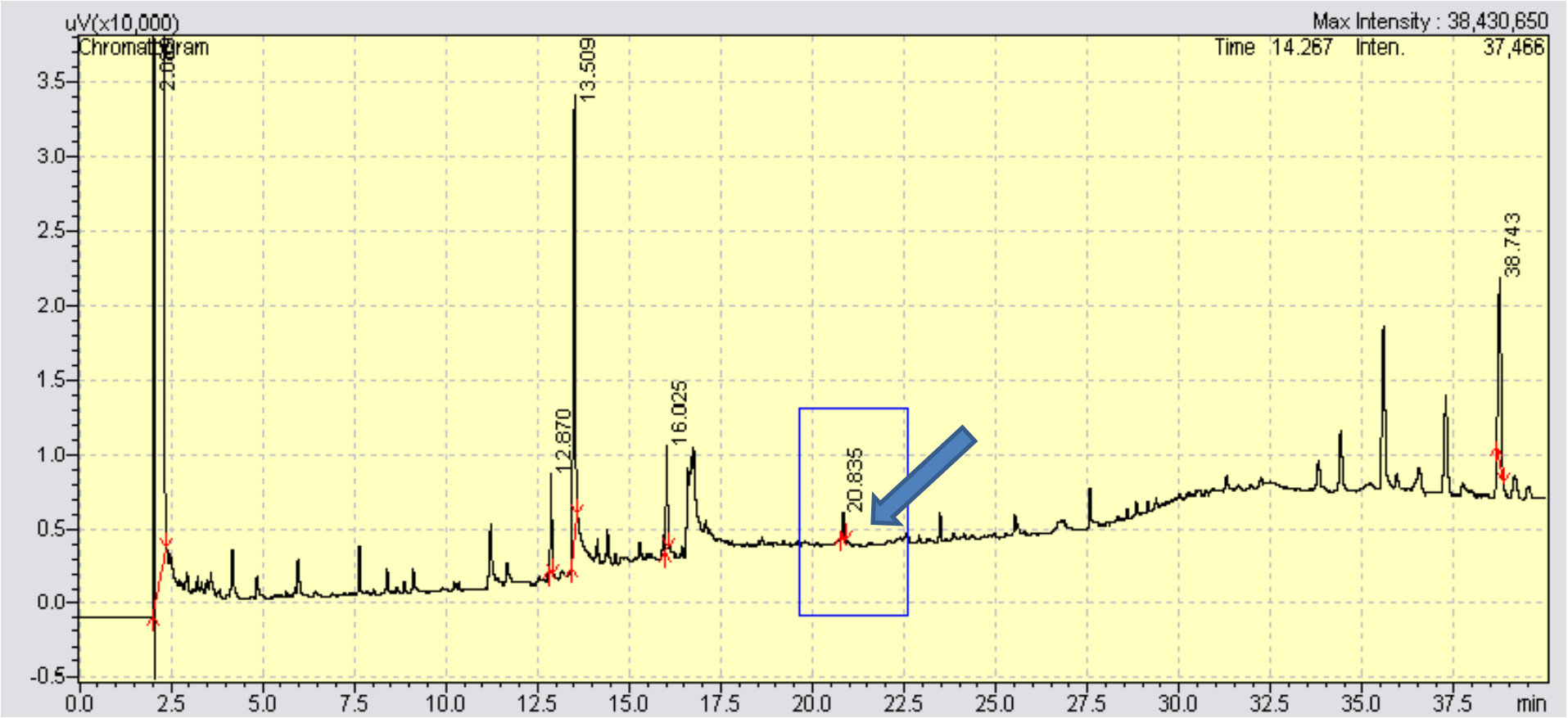

Figure 4.18: GC-FID chromatogram of methanol crude extract from suspension culture cells (18 days)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

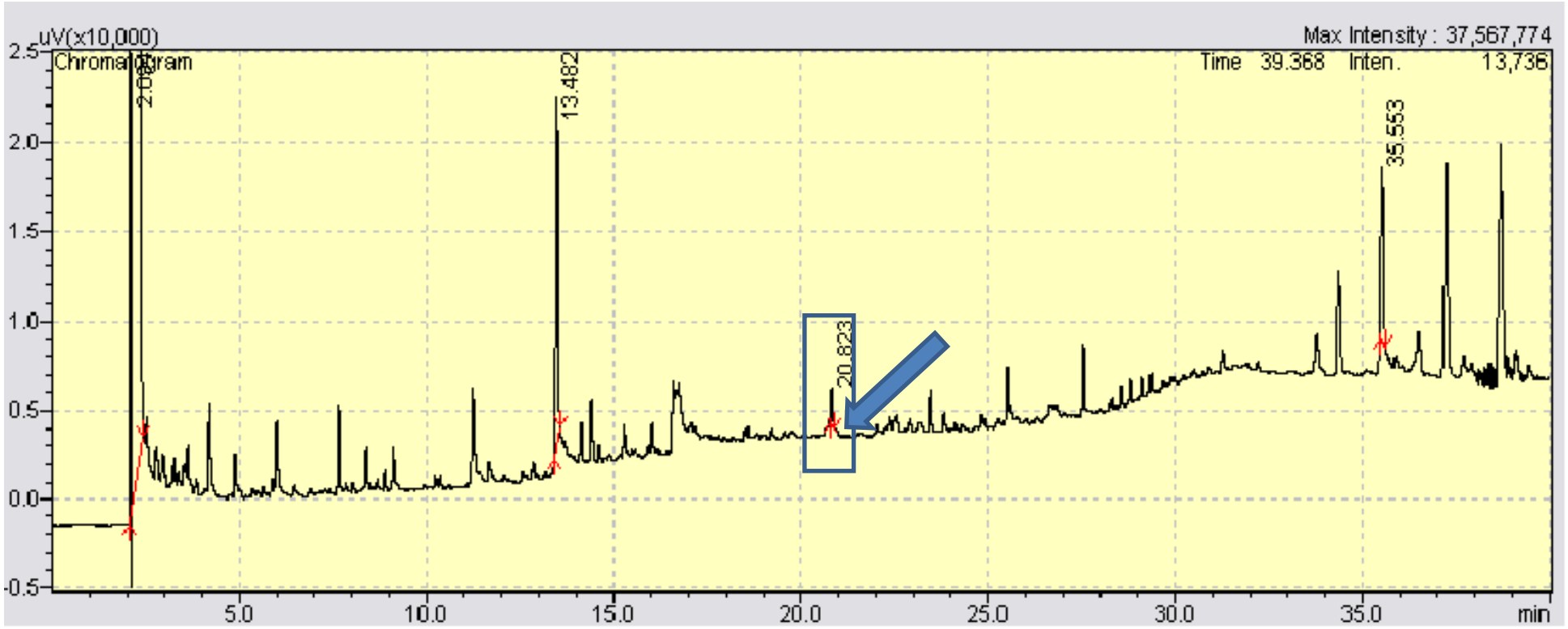

Figure 4.19: GC-FID chromatogram of methanol crude extract from suspension culture cells (21 days)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

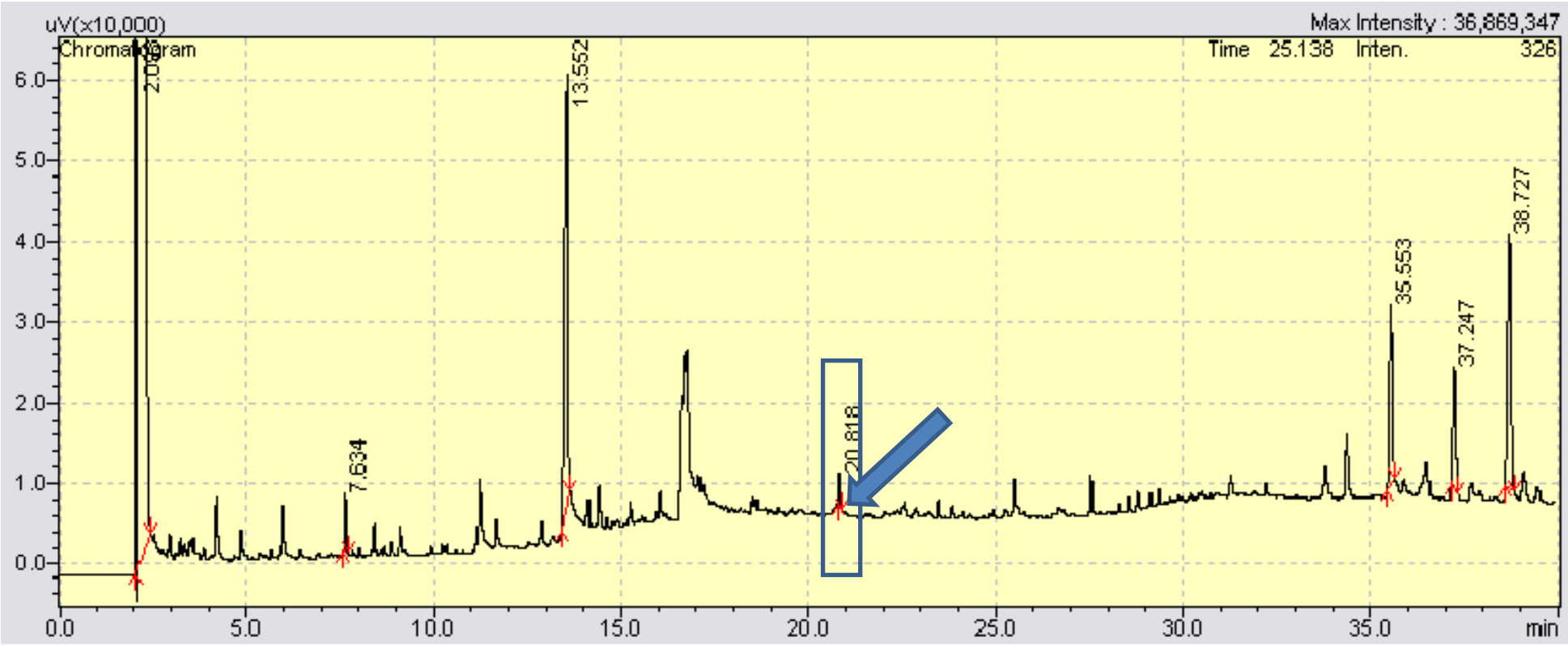

Figure 4.20**:** GC-FID chromatogram of methanol crude extract from suspension culture cells (24 days)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

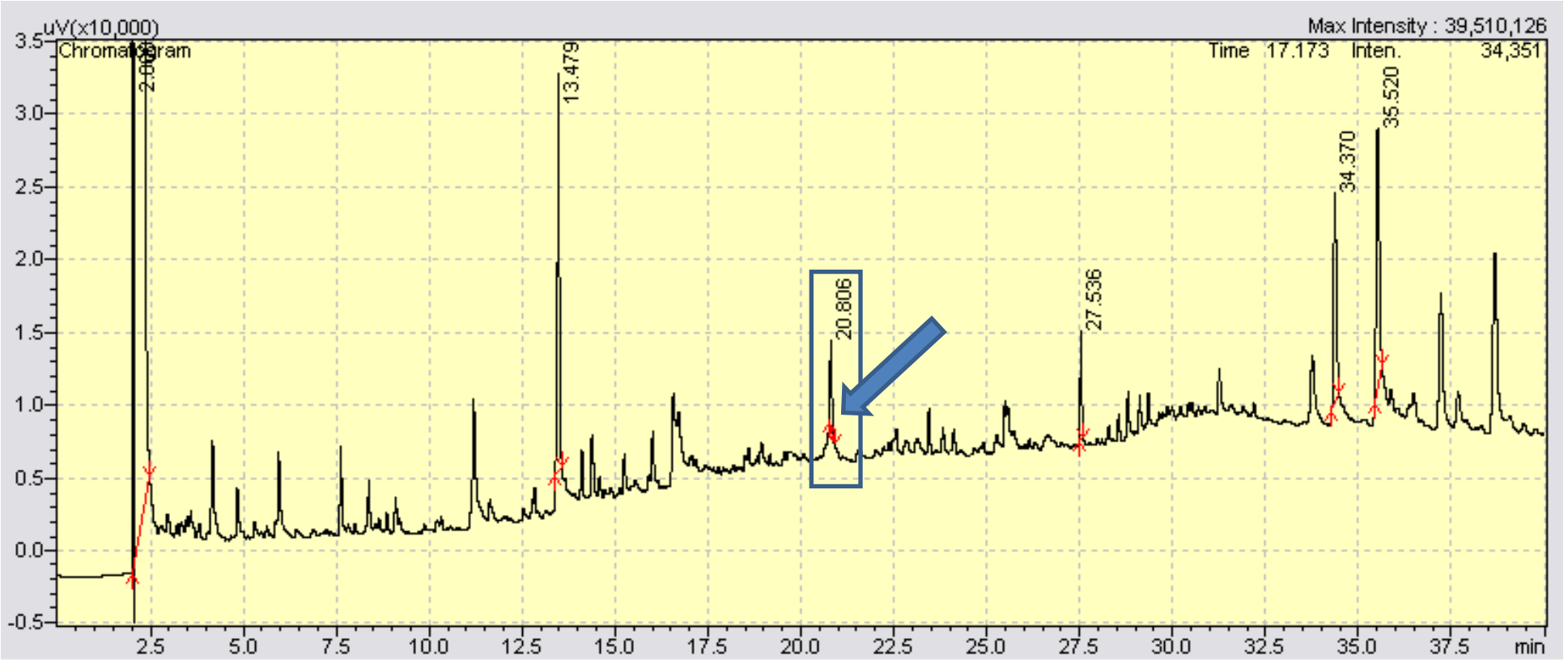

Figure 4.21**:** GC-FID chromatogram of methanol crude extract from suspension culture cells (27 days)

: (*E*)- Labda-8(17),12-diene-15, 16 dial

The production of secondary metabolites in callus or suspension cultures can vary greatly. Secondary metabolites in *in vitro* callus and suspension cell culture, either fails to accumulate or lower than concentrations found *in vivo* samples. Ma *et al*., (2006) reported that this might be due to this callus and cells are undifferentiated entities. Meanwhile, Wink (1990) suggested that the genes that are in control of secondary metabolites production may be turned off or not under specific control in undifferentiated callus or cells.

In this study, the concentration of (*E*)-labda-8(17),12-diene-15,16-dial in suspension cultures and callus were lower compared to the amount found in the *in vivo* rhizomes and shoot buds. Many factors have been reported to affect the production of secondary metabolites in cultures such as environment, plant materials used, medium component, pH, temperature and growth period.

Plant growth regulators such as auxins and cytokinin affected growth and differentiation and thus metabolism of cultured cells in many plants (Dai & Li, 2007; Brown *et al*., 1990). It was reported that high hormone concentration will promote non-productive callus and suspension cells. In the presence of hormones, all the cells are mitotic rather than producing secondary metabolites (Rateb *et al*., 2007; Wu *et al*., 2006). Whitmer *et al*., (1998) and Arvy *et al*., (1994) reported that incoporration of 1 to 2 mg l^{-1}2,4-D in culture medium, usually inhibits secondary metabolites formation in cultures. On the contrary, in compact callus aggregate of *Rhodiola sachalenensis*, the addition of 2,4-D was found to inhibit cellular growth and stimulate salidroside accumulation (Wu *et al*., 2003). In this study, the presence of 2,4-D in culture medium did not inhibit the production of (*E*)-labda-8(17),12-diene-15,16-dial. (*E*)-labda-8(17),12-diene-15,16-dial concentration in *in vitro* cultures were lower when compared with the amount present in *in vivo* rhizomes and shoot buds. (*E*)-labda-8(17),12-diene-15,16-dial was also identified in mucilaginous callus that was initiated on MS medium

with 8% sucrose. Weathers *et al.,* (1997) reported that high sucrose concentration in culture medium increase the concentration of sesquiterpene in *Artemisia annua* culture. In *Morinda elliptica* liquid culture, 8% sucrose yielded higher concentration of anthraquinone (Abdullah *et al*., 1998)

Growth period also effects the production of secondary metabolites in *in vitro* cultures. Results showed that (*E*)-labda-8(17),12-diene-15,16-dial was detected at end of exponential phase (3 months) and during stationary phase (4 months) in *C. mangga* callus culture (Fig 4.5). In suspension culture, (*E*)-labda-8(17),12-diene-15, 16 dial was also identified at the end of exponential phase and during stationery phase (day 18, 21, 24 and 27) (Fig 4.8). The production of secondary metabolites did not necessarily exhibit a positive correlation with the maximal growth rate of the culture (Jacinda *et al*., 2008), while some secondary products were formed maximally during the active growing stage such as in the cells of *Passiflora quangrangularis* (Antonigni *et al*., 2007) and *Saussurea medusa* (Fu *et al*., 2005; Zhao *et al*., 2001). Abbasi *et al*., (2007); Miguel *et al*., (2003) and Arias-Castro *et al*., (1993) reported that most secondary metabolites were produced when the cultures entered the stationery or death phase. During the exponential phase, the cells will be rapidly dividing, thus most of the energy is thought to be used in the mitotic division. In the stationary phase, this energy is redirected towards the production of metabolites.

In vitro sources could be an alternative sources for (*E*)-labda-8(17),12-diene-15,16-dial production. Modification of callus and cell suspension cultures by triggering the production of (*E*)-labda-8(17),12-diene-15,16-dial through the incorporation suitable precursors could be a promising method to produce (*E*)-labda-8(17),12-diene-15,16-dial in significant amounts.

CHAPTER 5: CONCLUSION

Rhizomes of*Curcuma mangga* Val.that were left to sprout at room temperature under dark condition produced the highest amount of shoot buds in 2 weeks. Forty to sixty shoot buds were produced by ten rhizomes under these conditions. A protocol was developed for surface sterilization using young actively growing vegetative shoot buds. The best surface sterilization treatment was by using 0.3% (w/v) $HgCl_2$, 50% chlorox (v/v) and 70% (v/v) ethanol.

Shoot bud explants of *C. mangga* that was surface sterilized were placed on callus induction medium supplemented with various PGRs. Friable callus with highest induction percentage was initiated on MS medium supplemented with 1 or 2 mg l^{-1} 2,4-D and 3% sucrose. Increasing the concentration of sucrose to 8 % in this medium resulted in mucilaginous and wet callus in lumps. Sigmoidal growth pattern was observed in friable and rapidly growing callus culture where three different growth phases namely lag phase (day 0 to day 42), exponential phase (day 42 to day 110) and stationery phase (day 110 to day 120) was distinguished.

Further verification on fast growing callus was shown through the histological structure of the calli induced on MS medium supplemented with various PGR. Groups of large cells with large nuclei and dense cytoplasm with islets of protein compounds stained blue- black and polysaccharide stained red were observed on friable callus initiated on MS medium supplemented with 1 mg l^{-1} 2,4-D and 3 % sucrose.

Cell suspension culture was established for rapid propagation of cells in liquid culture. MS supplemented with 0.3mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 mg l^{-1} malt extract and 5 mg l^{-1} ascorbic acid and citric acid respectively was the best media for maintaining suspension culture. Ascorbic acid (5 mg l^{-1}) and citric acid (5 mg l^{-1}) were used as antioxidant agents to

overcome cell browning. A growth pattern were observed where the cell suspension culture reached a maximum growth at day 21 and subsequently became stationery and started to decline after day 28.

(*E*)-labda-8(17),12-diene-15,16-dial was found in *in vitro* sources. Three months old friable callus showed higher (*E*)-labda-8(17),12-diene-15,16-dial production when compared to callus produced through other treatments. In suspension cells, (*E*)-labda-8(17),12-diene-15,16-dial was found highest in cells extracted at stationery phase (day 24).

The presence of (*E*)-labda-8(17),12-diene-15,16-dial in *in vitro* cultures could be enhanced through elicitation, precursor feeding and metabolic engineering. In metabolic engineering, genes could either be overexpressed or silenced depending on the signalling pathways. Many successful studies been reported.

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APPENDIX

Appendix A

Composition of culture medium, Murashige and Skoog (MS) medium (1962)

CHEMICAL	FORMULA	CONCENTRATION
Macronutrients (10X)		**100 ml l^{-1}**
Ammonium nitrate	NH_4NO_3	16.5
Potassium nitrate	KNO_3	19.0
Calcium chloride	$CaCl_2.2H_2O$	4.4
Magnesium sulfate	$MgSO_4.7H_2O$	3.7
Potassium dihydrogen orthophosphate	KH_2PO_4	1.7
Micronutrients		**10 ml l^{-1}**
Manganese sulphate	$MnSO_4.4H_2O$	2.23
Zinc sulphate	$ZnSO_4.7H_2O$	0.86
Potassium iodide	KI	0.086
Cupric sulphate	$CuSO_4.5H_2O$	0.0026
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.025
Cobalt (ous) chloride	$CoCl_2.6H_2O$	0.0026
Boric acid	H_3BO_3	0.62
Vitamin source (100x)		**10 ml l^{-1}**
Nicotinic acid	$C_6H_5NO_2$	0.05
Thiamine hydrochloride	$C_{12}H_{17}CIN_4OS.HCl$	0.01
Pyridoxine hydrochloride	$C_8H_{12}N_2O_2.2HCl$	0.05
Glycine	$C_6H_{12}O_6$	0.2
Iron source (100x)		**10 ml l^{-1}**
Sodium EDTA	$C_{10}H_{14}N_2O_8Na_2H_2O$	2.78
Ferrous sulphate	$FeSO_4.7H_2O$	3.72
Myo-inositol	$C_2H_5NO_3$	0.1 g (freshly add)

Appendix B

Analysis of surviving explants under different methods of surface sterilization

Replicates	Treatment 1			Treatment 2			Treatment 3		
Trials	Contamination (%)	Necrotic (%)	Survival (%)	Contamination (%)	Necrotic (%)	Survival (%)	Contamination (%)	Necrotic (%)	Survival (%)
Trial 1	80	16	4	13	83	4	19	4	77
Trial 2	76	17	7	10	82	8	20	5	75
Trial 3	79	17	4	11	89	0	25	0	75
Trial 4	83	17	0	4	85	11	26	0	74
Mean	79.5	16.75	3.75	9.5	84.75	5.75	22.5	2.25	75.25
Std. Deviation	2.886751346	0.5	2.87228132	3.872983346	3.09569593	4.78713553	3.511884584	2.6299556	1.2583057
SQRT (Count)	2	2	2	2	2	2	2	2	2
Std. Error	1.443375673	0.25	1.436140662	1.936491673	1.547847968	2.393567769	1.755942292	1.31497782	0.62915287

Appendix C

Analysis on fresh weight of *C. mangga* callus culture in MS medium supplemented with 1mg l^{-1} 2,4-D at different time period

Days	Average	Std. Dev.
0	0.25075	0.169671
14	0.591667	0.096645
28	0.947	0.078352
42	1.6	0.165879
56	3.924667	0.252195
70	6.326667	0.555641
84	7.845	0.950225
98	9.404	1.412001
112	10.71467	1.800289
126	10.59667	1.795645

Appendix D

Analysis on fresh weight of *C. mangga* suspension culture in MS medium supplemented with 0.3 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 100 mg l^{-1} glutamine, 0.1 mg l^{-1} d-Biotin, 30 g l^{-1} sucrose , 0.1 mg l^{-1} malt extract, 5 mg l^{-1} ascorbic acid and 5 mg l^{-1} citric acid

Days	Mean	Std. Dev
0	5	0
3	6.416667	0.144338
6	9.916667	0.144338
9	12.83333	1.040833
12	16.5	1.322876
15	23.66667	3.21455
18	30.33333	6.048416
21	40.83333	4.645787
24	42.66667	2.428134
27	44	0.866025
30	43.16667	1.040833

Appendix E: Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial in rhizomes and shoot buds

Appendix E(i): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g methanol crude extract of rhizomes and shoot buds

Replicate	Shoot (1)	Shoot (2)	Shoot (3)	Rhz (1)	Rhz (2)	Rhz (3)
Trials						
a	39.701	34.159	36.421	99.731	89.546	73.144
b	35.712	31.502	33.721	102.635	72.985	
c	36.714	33.44	37.444	109.29	68.566	
means	37.375667	33.033667	35.862	103.885333	77.0323333	

Appendix E (ii): Analysis on amount of methanol crude (g) obtained in 1 g dried samples of rhizomes and shoot buds

Shoot (1)	Shoot(2)	Shoot(3)	rhz(1)	rhz(2)	rhz(3)
0.243	0.325	0.402	0.276	0.315	0.415

Appendix E (iii): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g dried sample of rhizomes and shoot buds

Replicate	Shoot (1)	Shoot(2)	Shoot(3)	Rhz(1)	rhz(2)	rhz(3)
Trials						
a	9.647343	11.101675	14.641242	27.525756	28.20699	30.35476
b	8.678016	10.23815	13.555842	28.32726	22.990275	29.281
c	8.921502	10.868	15.052488	30.16404	21.59829	28.221
means	9.082287	10.735942	14.416524	28.672352	24.265185	29.2855867
std. deviation	0.5042693	0.4466524	0.7732147	1.35257236	3.48393105	1.06688739
Sqrt (count)	1.7320508	1.7320508	1.7320508	1.73205081	1.73205081	1.73205081
std. error	0.29114	0.2578749	0.4464157	0.78090801	2.01144853	0.61596772

Appendix F: Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial in callus at different growth period

Appendix F(i): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g crude methanol extract of callus at different growth period

Replicates	3mnths (1)	3mnths (2)	3mnths (3)	4mnths (1)	4mnths (2)	4mnths (3)
Trial						
a	9.802	10.301	11.237	5.221	5.304	5.146
b	10.06	8.851	8.05	5.389	4.882	5.591
c	10.111	11.237	8.578	5.101	5.559	5.222
means	9.991	10.129666	9.28833333	5.237	5.2483333	5.31966666

Appendix F(ii): Analysis on amount of methanol crude (g) obtained in 1 g dried samples of callus at different growth period

Replicates	3mnths(1)	3mnths(2)	3mnths(3)	4mnths(1)	4mnths(2)	4mnths(3)
Amount	0.231	0.155	0.254	0.212	0.184	0.249

Appendix F(iii): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g dried sample of callus at different growth period

Replicate	3mnths (1)	3mnths (2)	3mnths (3)	4mnths (1)	4mnths (2)	4mnths (3)
Trials						
a	2.264262	1.596655	2.854198	1.106852	0.975936	1.281354
b	2.32386	1.371905	2.0447	1.142468	0.898288	1.392159
c	2.335641	1.741735	2.178812	1.081412	1.022856	1.300278
means	2.307921	1.570098	2.3592366	1.110244	0.965693	1.324597
std. dev	0.0382659	0.186339	0.4338623	0.0306690	0.062912	0.0592705
sqrt (count)	1.7320508	1.732050	1.7320508	1.7320508	1.7320508	1.7320508
std. error	0.0220928 2	0.107583 3	0.25049055 1	0.0177067 6	0.0363225 4	0.0342198 6

Appendix G: Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial in callus of different morphologies

Appendix G(i): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g crude methanol extract of callus of different morphologies

Replicate	Friable (1)	Friable (2)	Friable (3)	Mucil (1)	Mucil. (2)	Mucil. (3)	Compact(1)	Compac (2)	Compact (3)
Trials									
a	9.802	10.30	11.23	4.353	5.209	4.942	5.549	5.799	5.662
b	10.06	8.851	8.05	3.69	4.89	5.202	5.223	5.432	5.379
c	10.11	11.23	8.578	3.794	5.779	3.904	5.818	5.283	5.571
means	9.991	10.129	9.288	3.945	5.292	4.682	5.53	5.504	5.537

Appendix G(ii): Analysis on amount of methanol crude (g) obtained in 1 g dried samples of callus of different morphologies

Replicate	Friable (1)	Friable (2)	Friable (3)	Mucil. (1)	Mucil. (2)	Mucil. (3)	Compact (1)	Compact (2)	Compact (3)
Amount.	0.231	0.155	0.254	0.279	0.204	0.242	0.214	0.212	0.3

Appendix G(iii): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g dried sample of callus of different morphologies

Replicate	Friable (1)	Friable (2)	Friable (3)	Mucil. (1)	Mucil. (2)	Mucil. (3)	Compact (1)	Compact (2)	Compact (3)
Trial									
a	2.264	1.596	2.854	1.214	1.062	1.195	1.1874	1.2293	1.6986
b	2.323	1.371	2.044	1.029	0.997	1.258	1.1177	1.1515	1.6137
c	2.335	1.741	2.178	1.058	1.178	0.944	1.2450	1.1199	1.6713
means	2.307	1.570	2.359	1.100	1.079	1.133	1.1834	1.1669	1.6612
std. deviation	0.038	0.186	0.433	0.099	0.091	0.166	0.0637	0.0562	0.0433
sqrt (count)	1.732	1.732	1.732	1.732	1.732	1.732	1.7320	1.7320	1.7320
std. error	0.022	0.107	0.250	0.057	0.053	0.095	0.0368	0.0325	0.0250

Appendix H: Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial in suspension culture cells at different growth periods

Appendix H(i): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g crude methanol extract of suspension culture cells at different growth periods

Replicates	18(1)	18(2)	18(3)	21(1)	21(2)	21(3)	24(1)	24(2)	24(3)	27(1)	27(2)	27(3)
Trials												
a	1.796	1.802	1.762	1.794	1.577	1.794	3.081	4.198	5.087	1.604	1.932	1.931
b	1.952	1.603	1.99	2.092	1.317	2.092	3.622	3.46	5.145	1.788	1.849	1.888
c	1.785	1.744	1.833	1.737	1.478	1.737	2.939	4.372	5.412	1.612	1.567	1.569
Means	1.8443	1.716	1.8617	1.8743	1.4573	1.8743	3.214	4.01	5.215	1.668	1.783	1.796

Appendix H(ii): Analysis on amount of methanol crude (g) obtained in 1 g dried samples of suspension culture cells at different growth periods

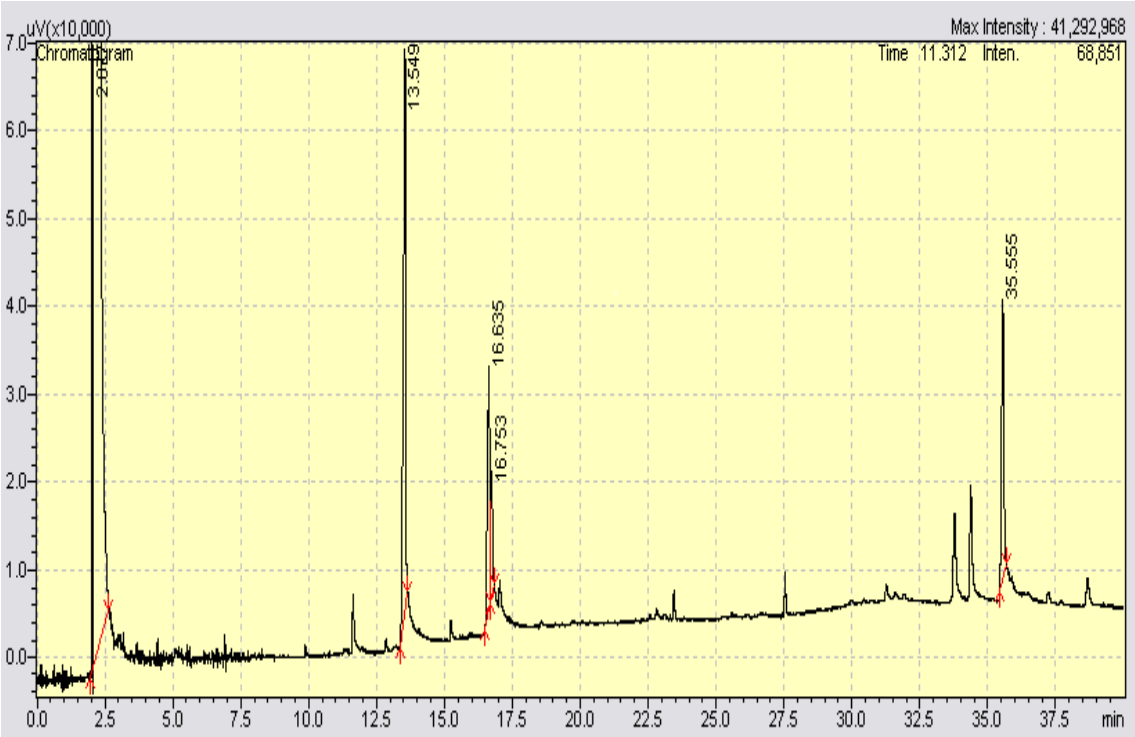
Rep.	18(1)	18(2)	18(3)	21(1)	21(2)	21(3)	24(1)	24(2)	24(3)	27(1)	27(2)	27(3)
Amount	0.222	0.293	0.367	0.193	0.268	0.410	0.202	0.297	0.391	0.180	0.201	0.325

Appendix F(iii): Analysis on amount of (*E*)-labda-8(17),12-diene-15,16-dial (mg) obtained in 1 g dried sample of suspension culture cells at different growth periods

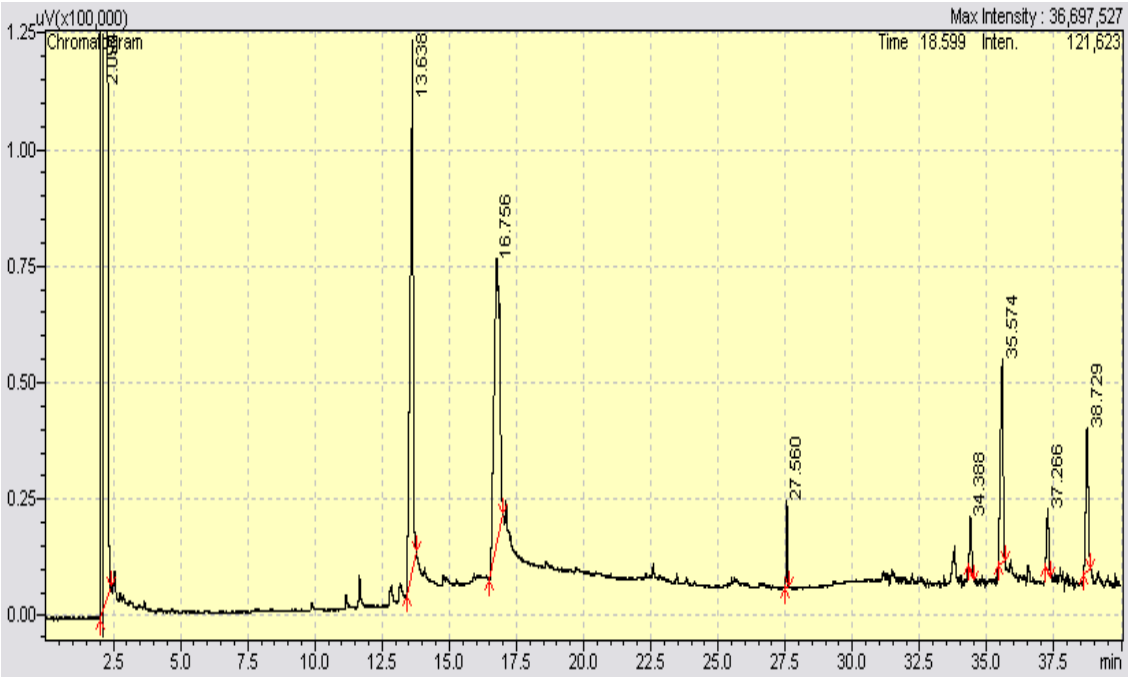
Replicate	18(1)	18(2)	18(3)	21(1)	21(2)	21(3)	24(1)	24(2)	24(3)	27(1)	27 (2)	27(3)
Trial												
a	0.394	0.53	0.648	0.346	0.423	0.736	0.625	1.249	1.989	0.289	0.388	0.629
b	0.435	0.471	0.732	0.404	0.353	0.858	0.735	1.028	2.012	0.322	0.372	0.615
c	0.398	0.512	0.674	0.335	0.396	0.712	0.597	1.298	2.116	0.29	0.315	0.511
Means	0.409	0.504	0.6847	0.3617	0.3907	0.7687	0.652	1.192	2.039	0.3	0.358	0.585
Std. Deviation	0.0226	0.03	0.043	0.0371	0.0353	0.0783	0.073	0.144	0.068	0.019	0.038	0.0645
SQRT (Count)	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321	1.7321
Std. Error	0.0131	0.017	0.0248	0.0214	0.0204	0.0452	0.042	0.083	0.039	0.011	0.022	0.0372

Appendix I: GC-FID chromatograms

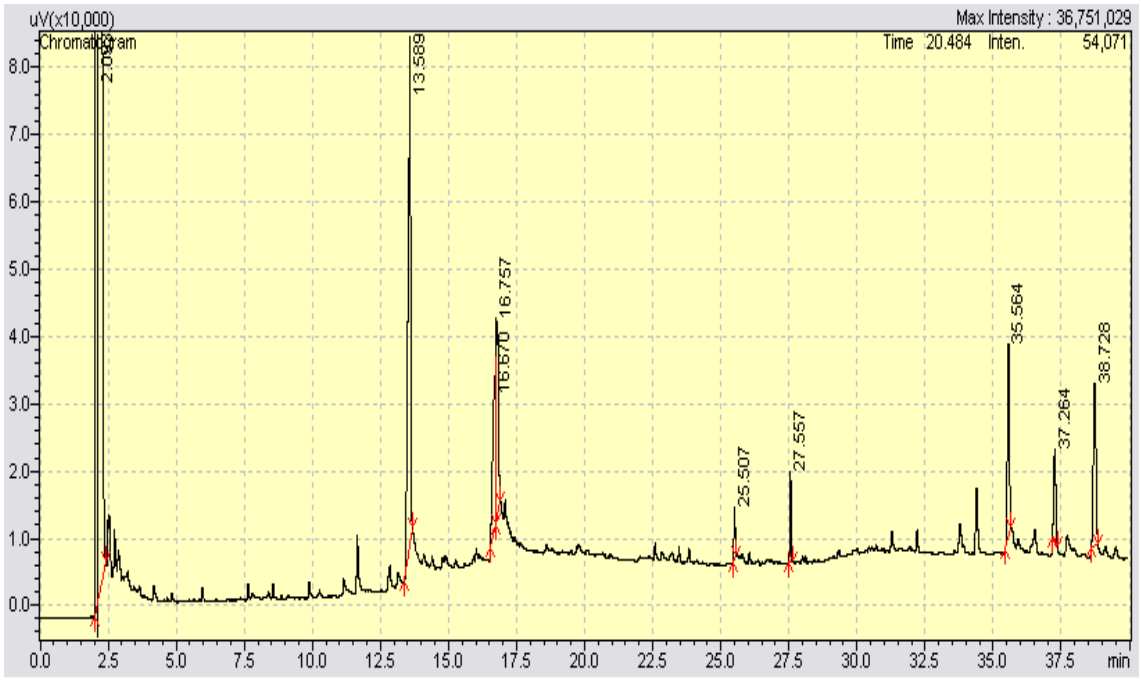
Appendix I (i): GC-FID chromatogram of methanol crude extract from 2 months friable callus.



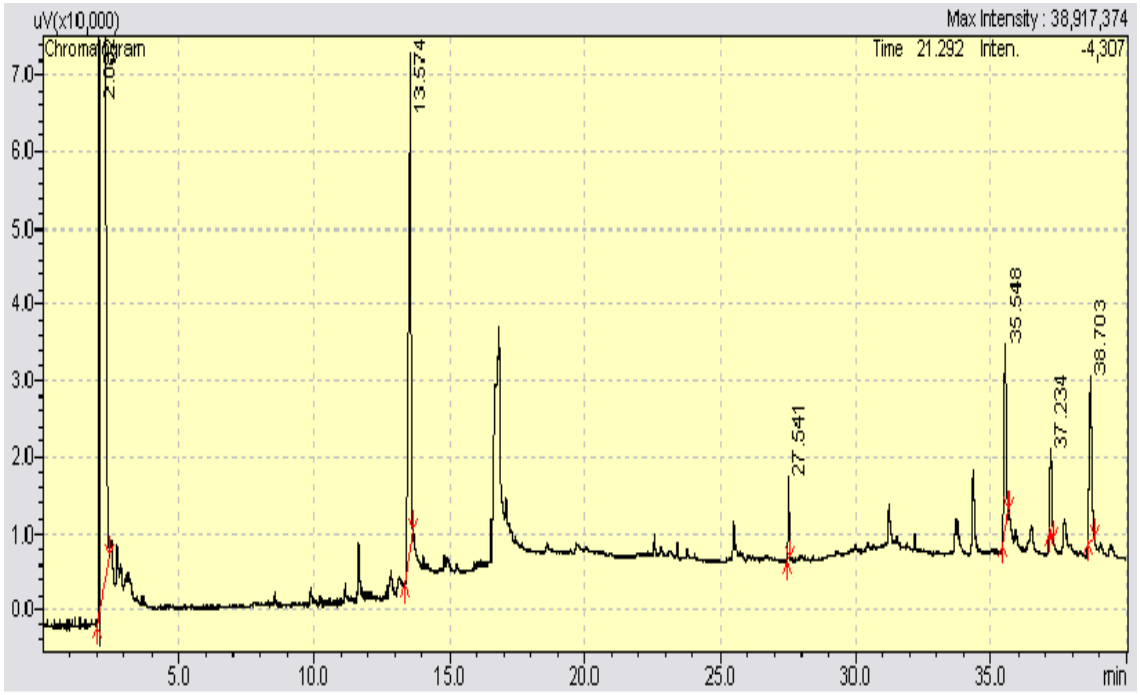
Appendix I (ii): GC-FID chromatogram of methanol crude extract from suspension culture (3 days)



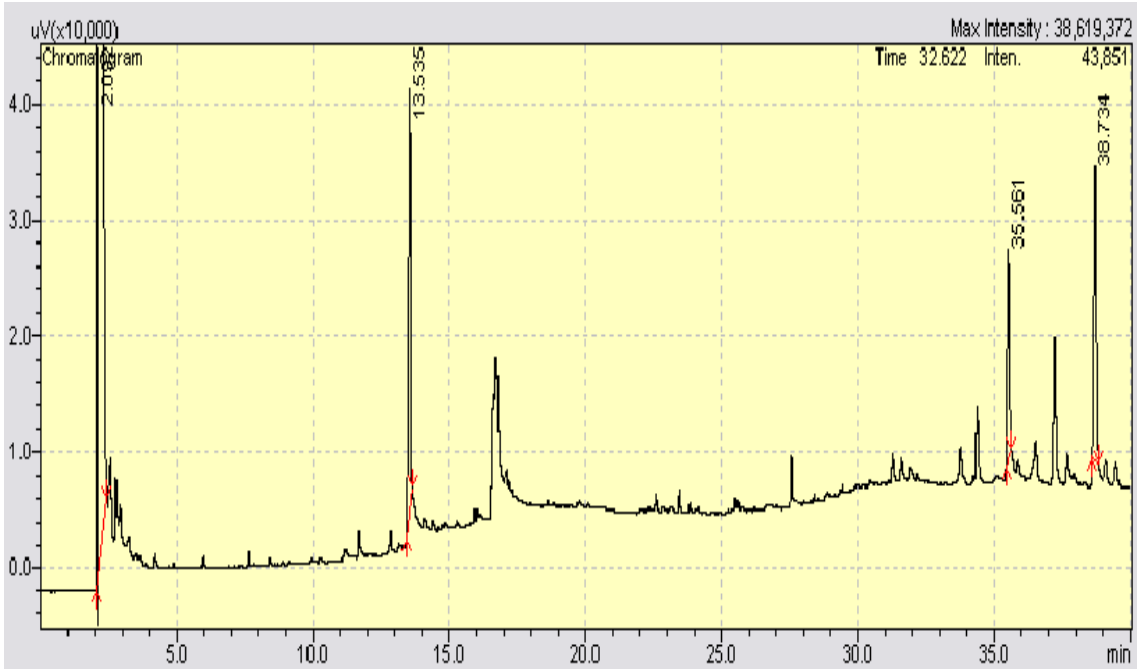
Appendix I (iii): GC-FID chromatogram of methanol crude extract from suspension culture (6 days)



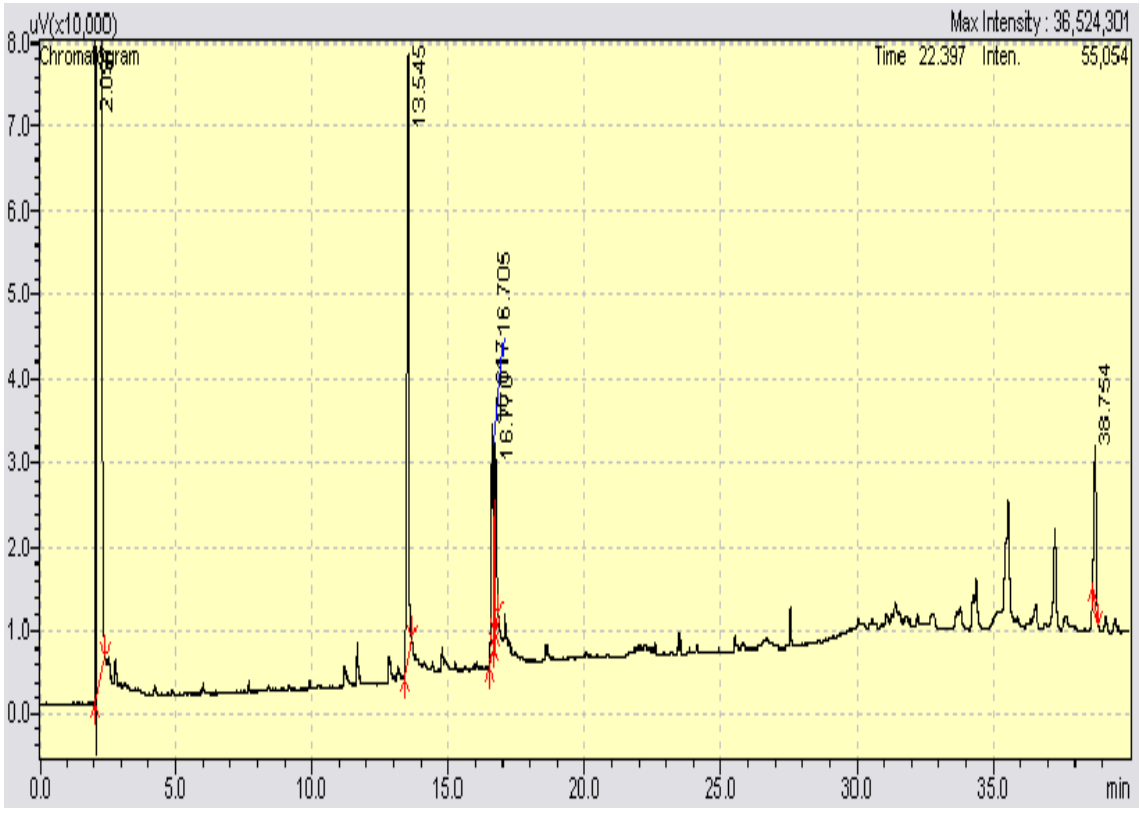
Appendix I (iv): GC-FID chromatogram of methanol crude extract from suspension culture (9 days)



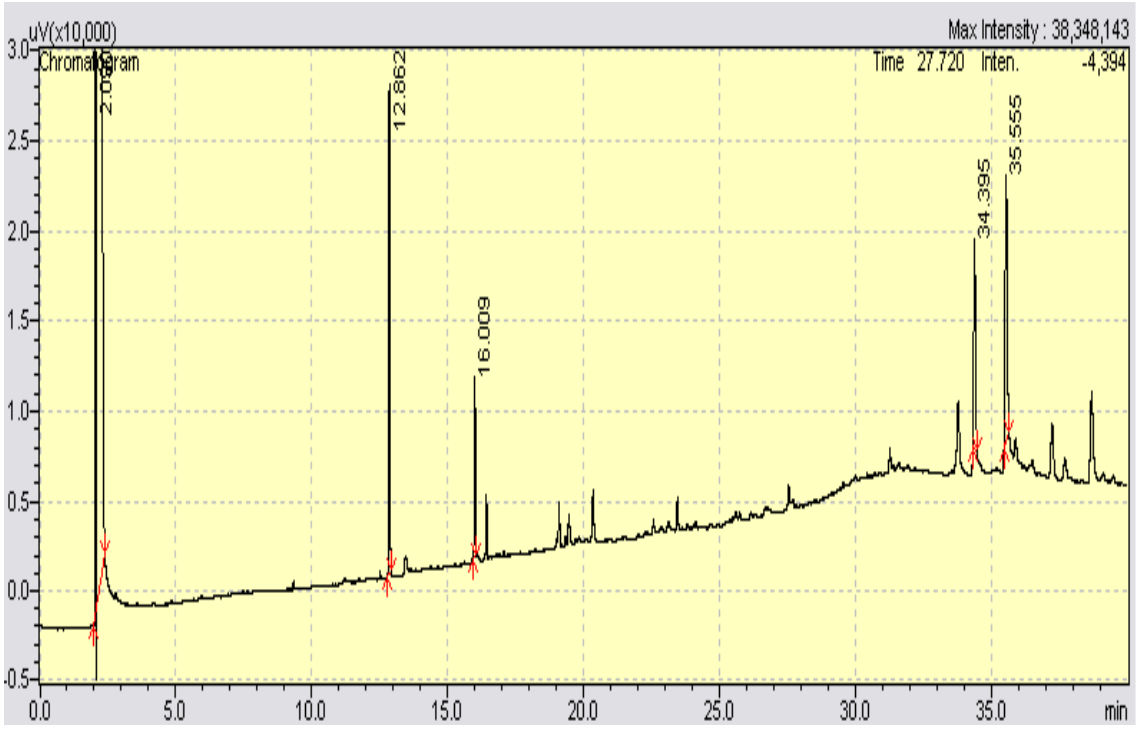
Appendix I (v): GC-FID chromatogram of methanol crude extract from suspension culture (12 days)



Appendix I (vi): GC-FID chromatogram of methanol crude extract from suspension culture (15 days)

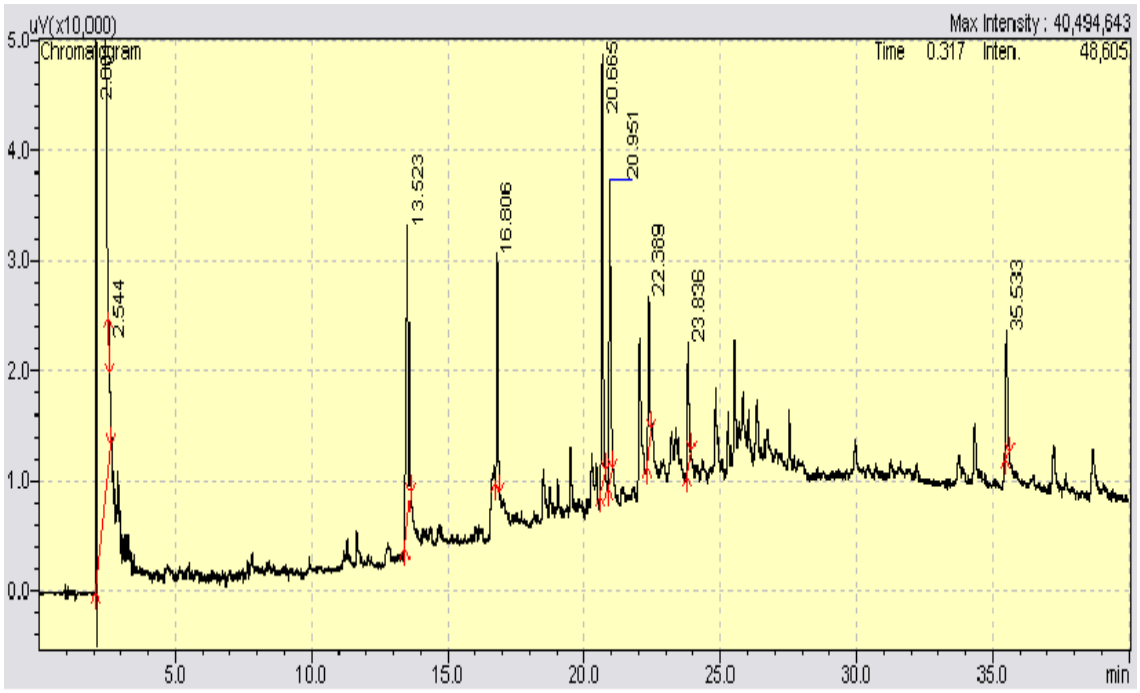


Appendix I (vii): GC-FID chromatogram of methanol crude extract from suspension culture (30 days)

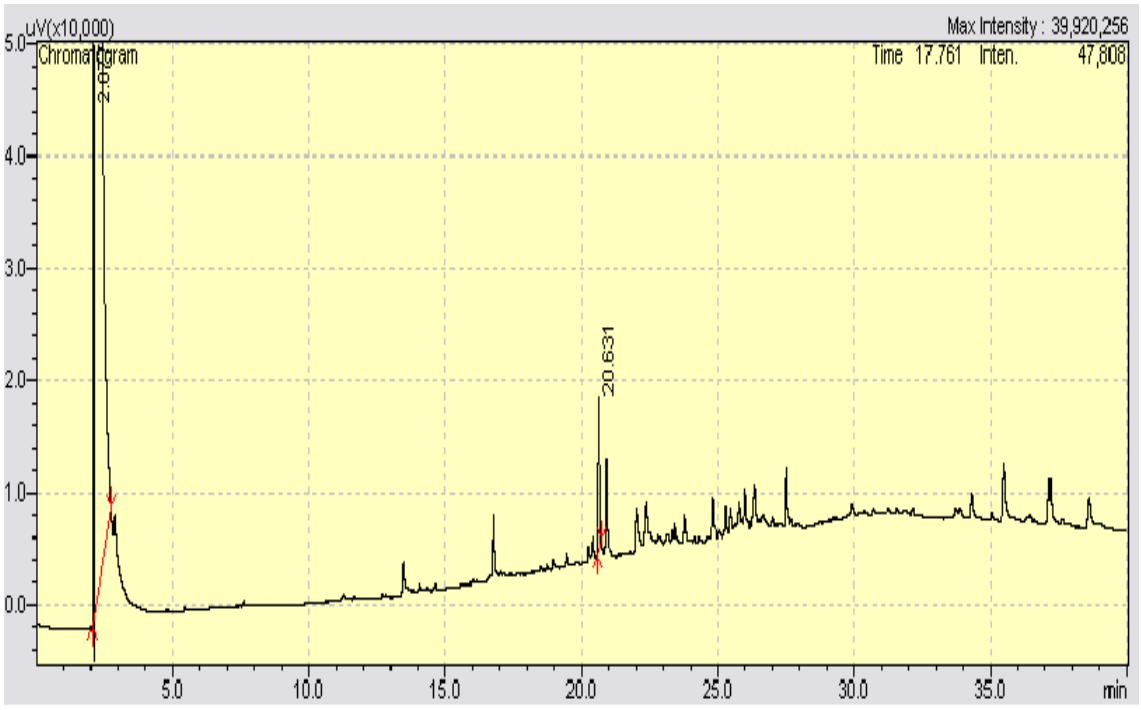


Appendix I (viii): GC-FID chromatogram of methanol crude extract from browning cells of suspension culture

Appendix I (ix): GC-FID chromatogram of methanol crude extract from callus (MS + 5 mg l^{-1} NAA and 5 mg l^{-1} IAA with 3% sucrose)



Appendix I (x): GC-FID chromatogram of methanol crude extract from callus (MS + 1 mg l^{-1} NAA and 1 mg l^{-1} IAA with 3% sucrose)



PUBLICATIONS

Academic Journal

No.	Title
1.	**Tamil C.M. Sundram, M. Suffian M. Annuar and Norzulaani Khalid (2012)** Optimization of culture condition for callus induction from shoot buds for establishment of rapid growing cell suspension cultures of *Curcuma mangga* (Mango ginger), *Australian Journal of Crop Science (AJCS) 6(7),* 1139-1146(ISI-cited publication-Q2*)*

Posters

No.	Title
1.	**Tamil C.M. Sundram, M. Suffian M. Annuar and Norzulaani Khalid** The effect of different culture media on morphology and growth of msngo ginger (*Curcuma mangga*) callus and cell suspension The 14th Biological Sciences Graduate Congress (BSGC), Chulalongkorn University, Bangkok, Thailand (December 2009)
2.	**Tamil C.M. Sundram, Lee Guan Serm, Sri Nurestri Abdul Malek, M. Suffian M. Annuar and Norzulaani Khalid** Production of (E)-labda-8(17),12-dien-15,16 from *in vivo* and *in vitro* source materials of *Curcuma mangga* Val International Conference on Natural Products (ICNP 2011), Putrajaya, Malaysia (November 2011)
3.	**Tamil C.M. Sundram, Lee Guan Serm, Sri Nurestri Abdul Malek, M. Suffian M. Annuar and Norzulaani Khalid** (*E*)-labda-8(17),12-diene-15,16-dial production from *Curcuma mangga* (*Mango ginger*) *in vitro* cultures 4th Australasian Metabolomics Symposium 2012, UiTM, Puncak Alam, Malaysia (September 2012)