IN VITRO STUDIES AND STIGMASTEROL PRODUCTION FROM *Wedelia biflora* (L.) D.C

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KUALA LUMPUR

2017

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

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ABSTRACT

Wedelia biflora Linn. D.C belongs to the family Asteraeceae and one of the potential medicinal plants. In the present study, callus induction procedure from stem and leaf explants of *W. biflora* was established via manipulation of auxins and cytokinins; and further improved by manipulation of abiotic stress conditions (sucrose concentration, incubation temperature, light colours and photoperiod). Combination of 2.0 mg/L NAA and 3.0 mg/L BAP promoted the best callus formation from stem explants (98% callus formation); whereas 1.0 mg/L 2,4-D and 3.0 mg/L BAP was the best combination for leaf explants (96% callus formation). The formation of callus was further improved by application of 4% of sucrose concentration, 25°C incubation temperature and incubated in total darkness for stem callus; and application of 3% sucrose, incubation at 30°C under white light and exposed to 8 h light/16 h darkness improved the growth of leaf callus. Further subculture of selected healthy callus onto 3.0 mg/L BAP and 2.0 mg/L IBA media resulted in complete regeneration of plantlets. Internodal explants of *W. biflora* successfully regenerated whole plantlets by application of 1.0 mg/L BAP and 1.0 mg/L IBA in half-strength MS medium. Consequently, indirect organogenesis was established by using the previously obtained healthy callus and direct organogenesis accomplished by using internodal segments of *W. biflora*. The methanolic extracts from the callus were compared with *in vivo* and *in vitro* grown explants extracts in terms of antimicrobial and antioxidant activities; and the stigmasterol content was screened in all those extracts by using spectrophotometer method and clarified by HPLC analysis. Stem callus treated with 4% sucrose and *in vivo* leaf as well as *in vivo* stem extracts gave potent antimicrobial activity, especially towards *Bacillus subtilis* (13 mm, 12 mm and 11 mm, respectively) and *Candida albicans* (16 mm, 14 mm and 13 mm, respectively). Those three extracts also showed higher antioxidant ability when assayed with DPPH

radical scavenging assay (IC_{50}: 250, 251 and 297 mg/L, respectively), H_2O_2 radical scavenging assay (IC_{50}: 272, 299, and 301 mg/L, respectively), reducing power activity assay (absorbance at 700 nm: 2.21, 2.00 and 1.96, respectively) and ferric reducing antioxidant power assay (antioxidant power: 720.82, 639.00 and 520.82 mM Fe (II)/g dry weight, respectively). Estimation of stigmasterol in stem callus grown with 4% sucrose, *in vivo* stem and *in vivo* leaf showed higher stigmasterol content; 0.088, 0.078 and 0.066 mg/g, respectively. Results from this research enable callus derived from stem explants and improved with 4% sucrose concentration to be used in mass production of bioactive compound (stigmasterol) which is responsible for the antimicrobial and antioxidant activities in this plant. This study is the first report in callus induction and regeneration of *W. biflora* with *in vitro* stigmasterol production.

ABSTRAK

Wedelia biflora Linn. D.C tergolong dalam famili Asteracea dan merupakan salah satu tumbuhan ubatan yang berpotensi. Dalam kajian ini, prosedur penghasilan kalus daripada eksplan batang dan daun *W. biflora* dicapai melalui manipulasi hormon auksin dan sitokinin; dan dipertingkatkan lagi dengan manipulasi pelbagai tekanan abiotik seperti kepekatan sukrosa, haba eraman, warna cahaya dan fotokala. Gabungan antara 2.0 mg/L NAA dan 3.0 mg/L BAP menghasilkan kalus terbanyak daripada batang (98% pembentukan kalus); manakala 1.0 mg/L 2,4-D dan 3.0 mg/L BAP merupakan gabungan terbaik untuk kalus dari daun (96% pembentukan kalus). Penghasilan kalus dipertingkatkan lagi dengan penggunaan 4% kepekatan sukrosa dan dieram pada 25°C tanpa sebarang cahaya untuk kalus batang; manakala penggunaan 3% kepekatan sukrosa dan eraman pada 30°C, berlampu putih selama 8 jam cahaya/16 jam gelap meningkatkan pertumbuhan kalus daun. Kalus yang sihat dikulturkan semula ke dalam media MS mengandungi 3.0 mg/L BAP dan 2.0 mg/L IBA untuk menghasilkan plantlet. Eksplan daripada internod *W. biflora* berjaya menghasilkan plantlet dengan menggunakan 1.0 mg/L BAP dan 1.0 mg/L IBA dalam media MS (separuh kepekatan). Seterusnya, organogenesis secara tidak langsung dihasilkan menggunakan kalus sihat yang terhasil sebelum ini, manakala organogenesis secara langsung dihasilkan pada keratan batang *W. biflora*. Ekstrak metanol daripada kalus dibandingkan dari segi antimikrobial dan antioksidan dengan ekstrak daripada eksplan yang ditanam secara *in vivo* dan *in vitro*. Kandungan stigmasterol di dalam ekstrak tersebut juga disaring menggunakan kaedah spektrofotometer dan disahkan melalui analisis HPLC. Ekstrak daripada batang kalus yang dihasilkan menggunakan 4% sukrosa, eksplan daun daripada *in vivo* dan eksplan batang daripada *in vivo* mempunyai kebolehan antimikrobial terutamanya terhadap *Bacillus subtilis* (13 mm, 12 mm, dan 11 mm) dan

Candida albicans (16 mm, 14 mm, dan 13 mm). Ketiga-tiga ekstrak tersebut juga menunjukkan aktiviti antioksidan yang tinggi bila di uji menggunakan kaedah radikal bebas DPPH (IC_{50}: 250, 251 dan 297 mg/L), kaedah radikal bebas H_2O_2 (IC_{50}: 272, 299, dan 301 mg/L), esei penurunan kuasa (penyerapan pada 700 nm: 2.21, 2.00 dan 1.96) dan kaedah FRAP (kuasa antioksida: 720.82, 639.00 dan 520.82 mM Fe (II)/g berat kering). Penyaringan jumlah stigmasterol di dalam ketiga-tiga ekstrak tersebut menunjukkan kandungan stigmasterol yang tinggi iaitu 0.088 mg/g (kalus batang 4% sukrosa), 0.078 mg/g (batang *in vivo*) dan 0.066 mg/g (daun *in vivo*). Data yang diperolehi daripada kajian ini membolehkan kalus yang dihasilkan menggunakan 4% kepekatan sukrosa digunakan untuk penghasilan stigmasterol secara besar-besaran di mana stigmasterol tersebut mempunyai potensi antimikrobial dan antioksida daripada *W. biflora*. Kajian ini merupakan yang pertama dalam penghasilan kalus dan pertumbuhan semula bagi pokok *W. biflora* dengan penghasilan stigmasterol secara *in vitro*.

ACKNOWLEDGEMENTS

Praises to Allah the Almighty, under His willing and blesses, I am able to accomplish all the experiments with reliable results and finish this theses accordingly. A special thanks to University of Malaya for giving me the opportunity to further my study here, and providing me with Postgraduate Research Fund (PG099-2013B). Million thanks to my supervisor Prof Dr Rosna Mat Taha for allowing me to use her tissue culture lab and facilities in it to run my experiments, for her guidance and supports; also to my co-supervisor Dr. Bakrudeen Ali Abdul for his helps, guidance and advices. Thanks to Dr. Ahmad Faris, for letting me using the HPLC systems to analyze my samples. In addition, I really appreciated the helps and opinions from my lab mates which without them it's impossible to successfully completed my works.

On the other hand, I am grateful to my parents for their supports and understanding while I am far away from them, pursuing my study. I would like to also thank my siblings for giving me a helping hand when I am in need. Last but not least, a very special thanks to the one who strongly encouraged me to further my study; which is my lovely husband, Muhamad Azri Jabar. In times when I feel like giving up, he is the one who cheered me up and providing a lot of space for me to concentrate on my works. Thank you very much to all of those who involved.

This work is especially dedicated to my family, my husband and our children; Muhamad Azmi and Shazya Noraida. They are the reasons I keep holding on even at some points quitting is already on my mind. I want to be successful for them.

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LIST OF SYMBOLS AND ABBREVIATIONS

µg/mL	:	Microgram per milliliter
µL	:	Microliter
2,4-D	:	2,4-Dichlorophenoxyacetic acid
ADAMTS-4	:	A disintegrin and metalloproteinase with thrombospondin motifs 4
BAP	:	6-Benzylaminopurine
Caco-2	:	Heterogeneous human epithelial colorectal adenocarcinoma cells
CFU/mL	:	Colony formation unit per milliliter
cm	:	Centimeter
CNC	:	Creamy and nodular callus
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
FRAP	:	Ferric Reducing Antioxidant Power
g/L	:	Gram per liter
GCC	:	Green and compact callus
GFC	:	Green and friable callus
GNC	:	Green and nodular callus
h	:	Hour
H_2O_2	:	Hydrogen Peroxide
$HgCl_2$	:	Mercury Chloride
HL-60	:	Human promyelocytic leukemia cells
HPLC	:	High-Performance Liquid Chromatography
IAA	:	Indole-3-acetic acid
IBA	:	Indole-3-butyric acid
IC_{50}	:	The concentration of an inhibitor where the response (or binding) is reduced by half
KN	:	Kinetin

mg/L	:	Milligram per liter
MIC	:	Minimum inhibition concentration
min	:	Minute
mL	:	Milliliter
mm	:	Millimeter
mM Fe (II)/g	:	Millimolar Ferum (II) ion per gram
MMP-13	:	Matrix metalloproteinase-13
MMP-3	:	Matrix metalloproteinase-3
MS medium	:	Murashige and Skoog medium
NA	:	Nutrient Agar
NAA	:	1-Naphthaleneacetic acid
NaOH	:	Natrium hydroxide
nm	:	Nanometer
ºC	:	Degree Celcius
PDA	:	Potato Dextrose Agar
PGR	:	Plant Growth Regulator
SE	:	Standard error
v/v	:	Volume per volume
w/v	:	Weight per volume
ZI	:	Zone of inhibition

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Wedelia biflora (L.) D.C belongs to the Asteraceae family which is widely distributed in Western and Eastern sea coasts in India and other Southern Asia (Biswas et al., 2013). It is a creeping herb with yellow flowers possessing various ethnobotanical and medicinal applications in cytotoxicity, anti-inflammatory, antimicrobial etc. (Thu et al., 2013; Biswas et al., 2013). It has been used as a traditional medicine to treat stomach ache, ulcer, fever, varicose veins etc. (Yoganandam et al., 2009). *W. biflora* contains sesquiterpenoids, frideline, epifriedelanol, quercetin derivatives, grandifloric acid and steroid (Thu et al., 2013; Huang et al., 2013).

The importance of this plant is centered on its medicinal properties. Since ancient times, this plant has been used as traditional medicine to cure wounds and ulcers (Kasahara & Hemmi, 1986), soothe swelling and inflamed areas, heal appendicitis and eczema and when its leaves are soaked in coconut oil, it can be used to massage joint and muscle (Cambie, 1986). *W. biflora* was reported to have antifeedant and antifungal activities (Miles et al., 1993), analgesic activity and antimicrobial and antihelmintic properties (Meena et al., 2010) which make it more beneficial to pharmaceutical industries.

Plant tissue culture applications offer an alternative for production of bioactive compounds other than providing an efficient conservation method. Most of the bioactive compounds production can be induced via external factors. Manipulation of plant growth regulators (PGRs) is one of the factors which could increase or decrease the production of plants constituents. In addition, mass production of bioactive compounds could be obtained from callus derived from parts of the plants. Regulation of cytokinins and auxins in parts of the plants can induce callus of different colour, amount and nature

(Zand et al., 2014). Therefore, mass production of callus could be an alternative for extraction of important constituents from plants (Castro et al., 2016).

Organogenesis is the formation of organs that leads to regeneration of plant parts (shoots, roots, flowers) from explants by *in vitro* method. This process could occur directly or indirectly based on whether the formation of callus is observed or not. Direct organogenesis took place when the inoculated explants directly produce shoots or roots without formation of callus. Formation of callus from inoculated explants before the formation of roots or shoots is an indirect organogenesis. Normally, cytokinins is used to induce shoot formation (Borzabad et al., 2010), while auxins is used for roots induction (Paduchuri et al., 2010). Plantlets derived from organogenesis often display similar characteristic as wild population; and sometimes provided superior benefits than intact plants due to manipulation that could be done during its growth via *in vitro* methods.

Limited publications have focused on tissue culture application on *Wedelia* spp., especially *W. biflora*. Plant regeneration and callus induction had been reported only in *W. chinensis* and *W. trilobata* (Martin et al., 2003; Agarwala et al., 2010; Rahman and Bhadra, 2011). To our knowledge, the present study is the first report on callus induction and *in vitro* plant regeneration of *W. biflora* and stigmasterol production. Optimization of callus induction and plant regeneration procedure is very crucial to study the cellular behaviour of plant and to increase the propagation of the plant as well as its secondary metabolite production via manipulation of plant growth regulators (PGRs). Therefore, the proposed objectives of this study are as follows:

i. to establish a laboratory procedure (*in vitro*) for regeneration of *W. biflora*;
ii. to optimize the culture conditions for *in vitro* callus production from stem and leaf explants of *W. biflora* as well as the production of stigmasterol

under abiotic stress conditions (sucrose concentration, temperature, light colours and photoperiod);

iii. to study the bioactivities (antimicrobial and antioxidant) of the extracts from *W. biflora*; and

iv. to identify and quantify stigmasterol from callus as well as *in vitro* and *in vivo* stem and leaf through spectrophotometric and HPLC studies.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to *Wedelia* spp.

Plants from *Wedelia* genus gained a lot of attention since six decades ago due to their ethnobotanical properties. Long ago before the plants had been studied scientifically, the ancient people used these plants to treat some kinds of illnesses from skin disorders, bacterial infections and even inner body sickness such as heart diseases and liver failure.

Leaves of *W. chinensis* were reported to be effective to be used as grey hair dyeing and promoting hair growth. The juice from the leaves could be served as a tonic for several illnesses such as coughs, skin diseases, alopecia and cephalagia. The decoction from seeds, flowers and leaves can be used in treatment of uterine haemorrhage and menorrhagia (Meena et al., 2011).

W. biflora leaves had also been widely used as a traditional medicine. The leaves juice, when combined with ginger juice could serve as a great prescription for flatulence (Meena et al., 2011). The crushed leaves of *W. biflora* can be used to treat ulcers, cut, sore, and varicose veins in poultice form (Biswas et al., 2013).

Crushed leaves of *W. trilobata* Linn. were also used as poultice to treat cuts, ulcers and sore. The decoction from leaves was able to alleviate the symptoms of colds and fever. Apart from that, the decoction was also useful in treatment of hepatitis, microbial infections and able to clear the uterus after birth (Huang et al., 2006).

2.2 Phytochemical studies of *Wedelia* spp.

Phytoconstituent of plants play crucial role in determining the functions and benefits of the plants. Most of the phytoconstituent are very beneficial remedy and could serve as new alternatives to the existing chemical-based medicines. The phytoconstituent and their chemical structures present in *Wedelia* spp. were summarized in Table 2.1.

A study carried out by Mishra et al. (2011) revealed that the ethanolic extract of whole *W. chinensis* plant contains glycosides, alkaloids and flavonoids while the petroleum ether extract consists of steroids and the aqueous extract contains glycosides and saponins. The chemical components of *W. chinensis* aerial parts were isolated and identified as (3β)-oleanolic acid 3-(β-D-glucopyranosiduronic acid 6-methyl ester); and (3β)-3-hydroxy-30-noroleana-12,20(29)-dien-28-oic acid 3-(β-D-glucopyranosiduronic acid) 6-methyl ester and structures elucidated using the spectral values (Li et al., 2012). Another study by Banu & Nagarajan (2013) revealed that the bioactive compounds present in *W. chinensis* (Osbeck) Merrill leave extract are 2-tridecanone, n-(methyoxyphenylmethylene) carbamic acid ethyl ester and 9,12,15-octadecatrienoic acid methyl ester.

In a previous study carried out by Zhu et al. (2012) the chemical composition of essential oils from different parts of *W. urticifolia* were quantified. The most abundant compounds were α-Pinene and D-limonene in flower essential oils, while the leaf essential oils contained α-pinene, D-limonene, α-phellandrene, ϒ-terpinene and germacrene D. The major compound in stems essential oils were D-limonene, ϒ-terpinene, germacrene D, and α-pinene.

Table 2.1: Phytochemicals present in *Wedelia* spp.

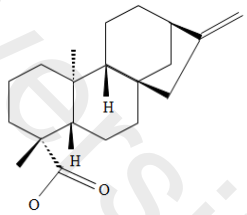
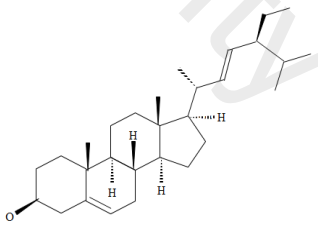
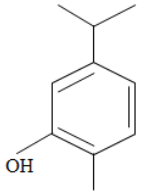
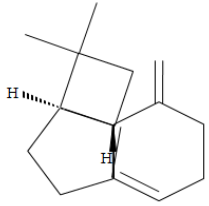
No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
1.	Kaurenoic acid		Antinociceptive, anti-inflammatory, antimicrobial, hypoglycemic and antioxidant activities	*W. paludosa*	Block et al. (1998); Sartori et al. (2003); & Bresciani et al. (2004)
2.	Stigmasterol		Antinociceptive, anti-inflammatory, antimicrobial and antioxidant activities	*W. prostrata, W. biflora, and W. paludosa*	Block et al. (1998); & Filho et al. (2004)
3.	Carvacrol		Antimicrobial and anti-inflammatory activities	*W. biflora*	Manjamalai et al. (2012)
4.	t-caryophyllene		Antimicrobial and anti-inflammatory activities	*W. biflora*	Manjamalai et al. (2012)

Table 2.1, continued.

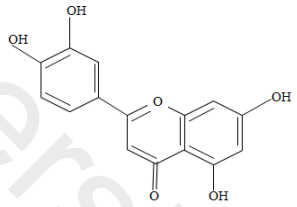
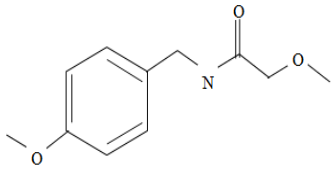
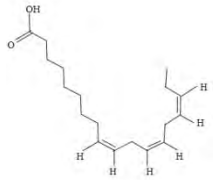
No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
5.	Luteolin		Antifungal activity	*W. paludosa*	Sartori et al. (2003)
6.	2-tridecanone		Antimicrobial, anti-tumor and antioxidant properties	*W. chinensis*	Banu and Nagarajan (2013)
7.	n-(methoxyphenyl methylene) carbamicacidethyl ester		Antimicrobial, anti-tumor and antioxidant activities	*W. chinensis*	Banu and Nagarajan (2013)
8.	9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)		Antimicrobial, anti-inflammatory and antioxidant activities	*W. chinensis*	Banu and Nagarajan (2013)

Table 2.1, continued.

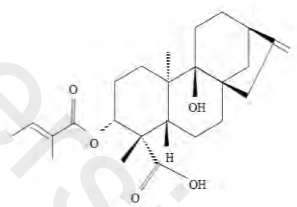
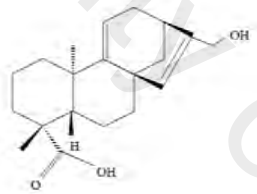
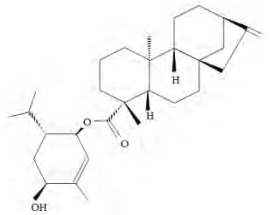
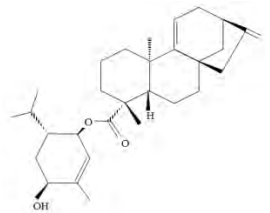
No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
9.	3α-tigloyloxypterokaurene L_3		Antimicrobial, anti-inflammatory and antioxidant activities	*W. trilobata*	Ma et al. (2013)
10.	*ent*-17-hydroxy-kaura-9(11),15-dien-19-oic acid		Antimicrobial, anti-inflammatory and antioxidant activities	*W. trilobata*	Ma et al. (2013)
11.	Wedelobatins A		Antimicrobial, anti-inflammatory and antioxidant activities	*W. trilobata*	Ma et al. (2013)
12.	Wedelobatins B		Antimicrobial, anti-inflammatory and antioxidant activities	*W. trilobata*	Ma et al. (2013)

Table 2.1, continued.

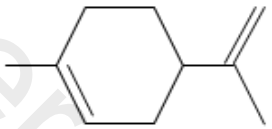
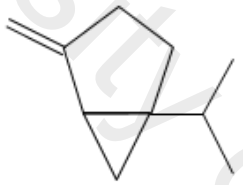
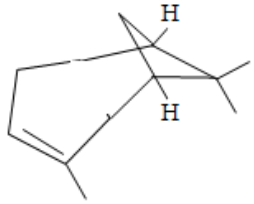
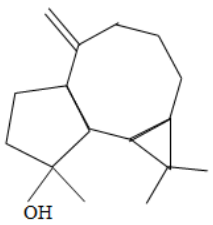
No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
13.	Limonene		Antimicrobial and antioxidant activities	*W. biflora, W. urticifolia, W. glauca* (Ort.) Hoffman ex Hickens, *W.*	Bailac et al. (2005) & Garg et al. (2005)
14.	Sabinene		Antimicrobial and antioxidant activities	*W. glauca* (Ort.) Hoffman ex Hickens	Bailac et al. (2005)
15.	α-pinene		Antimicrobial and antioxidant activities	*W. glauca* (Ort.) Hoffman ex Hickens, *W. biflora, W. chinensis*	Bailac et al. (2005) & Garg et al. (2005)
16.	Spathulenol		Antimicrobial and antioxidant activities	*W. chinensis*	Garg et al. (2005)

Table 2.1, continued.

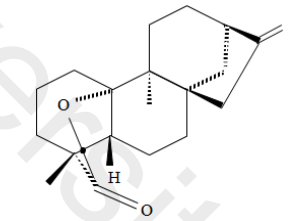
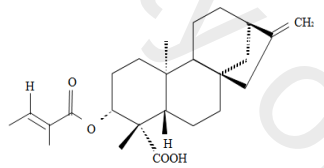
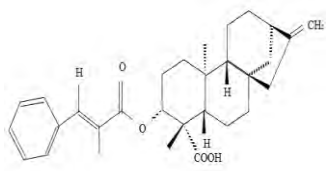
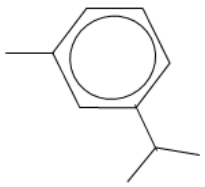
No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
17.	Tetrachyrin		Anti-inflammatory and antioxidant activities	*W. paludosa*	Vieira et al. (2001)
18.	3α-tigloyloxykaur-16-en-19-oic acid		Anti-inflammatory and antioxidant activities	*W. paludosa*	Vieira et al. (2001)
19.	3α-cinnamoyloxykaur-16-en-19-oic acid		Anti-inflammatory and antioxidant activities	*W. paludosa*	Vieira et al. (2001)
20.	1-methyl-3-(1-methylethyl)-benzene		Antimicrobial and anti-inflammatory activities	*W. biflora*	Yang et al. (2013)

Table 2.1, continued.

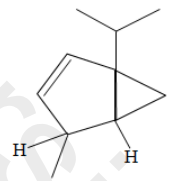
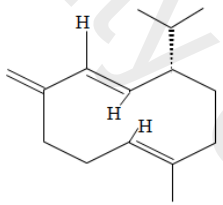
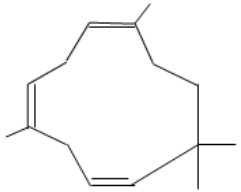
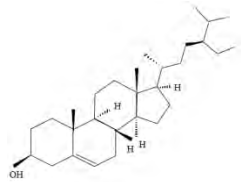
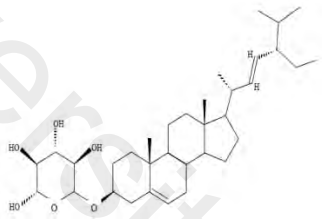
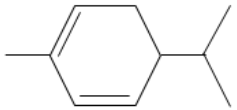
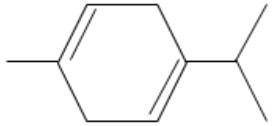
No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
21.	4-methyl-1-(1-methylethyl-didehydroderiv.)bicyclo [3.1.0]hexane		Antimicrobial and anti-inflammatory activities	*W. biflora*	Yang et al. (2013)
22.	Germacrene D		Antimicrobial and anti-inflammatory activities	*W. biflora, W. urticifolia*	Zhu et al. (2012)
23.	1,1,4,8-tetramethyl-cis, cis, cis-4,7,10-cycloundecatriene		Antimicrobial and anti-inflammatory activities	*W. biflora*	Yang et al. (2013)
24.	β-sitosterol		Antinociceptive, anti-inflammatory, antimicrobial and antioxidant activities	*W. prostrata*	Zhang et al. (2011)

Table 2.1, continued.

No.	Phytochemicals	Chemical structures	Pharmacological effects	Species	References
25.	Stigmasterol 3-O-beta-D-glucoside		Antinociceptive, anti-inflammatory, antimicrobial and antioxidant activities	*W. prostrata*	Zhang et al. (2011)
26.	n-hexacosanol		Antinociceptive, anti-inflammatory, antimicrobial and antioxidant activities	*W. prostrata*	Zhang et al. (2011)
27.	α-phellandrene		Antimicrobial and anti-inflammatory activities	*W. urticifolia*	Zhu et al. (2012)
28.	γ-terpinene		Antimicrobial and anti-inflammatory activities	*W. urticifolia*	Zhu et al. (2012)

2.3 Stigmasterol

Stigmasterol is a type of phytosterol along with β-sitosterol, ergosterol, campesterol and brassicasterol. Phytosterol is a naturally occurring phytoconstituent in plants which is referred as plant sterols. Stigmasterol is chemically similar to animals' cholesterol. This plant sterol is different from β-sitosterol as it has an additional double-bond in the side chain of its molecules (Fig. 2.1). Stigmasterol is utilized as a precursor in the manufacturing of synthetic and semi-synthetic compounds such as in hormone production of progesterone and acts as an intermediate in the biosynthesis of other hormones such as androgens, estrogens, and corticoids in pharmaceutical industry. Limited information is available on the *W. biflora* stigmasterol content but stigmasterol was found from *W. biflora* stem parts (Khare, 2007), and from *W. biflora* flower parts (Thu et al., 2012).

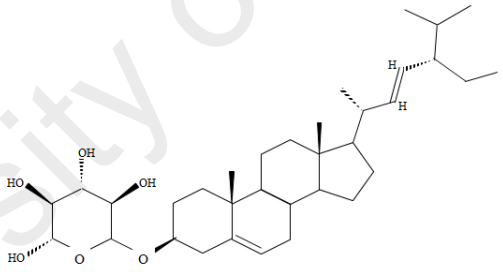


Figure 2.1: Structural formula of stigmasterol (Antwi et al., 2017).

Stigmasterol is one of the potential phytomedicine that has been isolated from many plants species and investigated for numerous pharmacological and biological activities. A number of researchers have indicated that stigmasterol could be used in prevention of certain diseases (Antwi et al., 2017, Ramu et al., 2016).

i. Reducing cholesterol level

Previous studies reported that stigmasterol possess significant effect in cholesterol lowering activity. According to Batta et al. (2006), this plant sterol has bee proven to compete with cholesterol during intestinal absorption and therefore result in lowering the plasma cholesterol level. The results showed that rats fed with stigmasterol exhibited lower plasma cholesterol level, suppressed intestinal cholesterol and plant sterol absorption together with hepatic cholesterol and classic bile acid synthesis. It was reported that stigmasterol were able to suppress the cholesterol biosynthesis by inhibiting the sterol Δ24-reductase in human Caco-2 and HL-60 cell lines.

ii. Reducing blood glucose level

Stigmasterol together with β-sitosterol in the chloroform extract of *Parkia speciosa* seeds was found to possess hypoglycemic activity. The extract was administered orally to the alloxan-induced diabetic rats and it showed significant depression in blood glucose levels (Jamaluddin et al., 1994). In a study conducted by Panda et al. (2009), they isolated stigmasterol from *Butea monosperma* and administered it to mice for 20 days. It showed a significant reduction on serum triiodothyronine, thyroxin, glucose concentrations and hepatic glucose-6-phosphate. An increase in insulin was reported that indicates it possess both thyroid inhibiting and hypoglycemic activity.

iii. Anti-tumor effect

Ghosh et al. (2011) investigated the antitumor activity of stigmasterol from aerial parts of *Bacopa monnieri* Linn. . This study was carried out against Ehrlich Ascites Carcinoma (EAC) in Swiss Albino mice. The results revealed that stigmasterol was able to decrease the tumor volume, packed cell volume and viable tumor cell count. It was also found to increase mean survival time thus increasing the life span of stigmasterol

treated mice. The hematological profile was reverted to near normal level and histopathological profile of liver tissues showed protective effect against the cancer cells by decreasing the level of lipid peroxidation and increasing the level of glutathione, superoxide dismutase and catalase in the liver.

iv. Cytotoxicity ability

A study on the cytotoxic activity from the chloroform extract of *Achillea ageratum* revealed that stigmasterol inhibited the growth of Hep-2 and McCoy cells cultures when compared with the control group, 6-Mercaptopurine (Gomez et al., 2001). Further, the cytotoxicity activity of stigmasterol with another two isolated compounds, namely frideline and rotenone from *Cacalia tangutica* extract was studied against *Musca domestica* L., *Aedes albopictus* and *Spodoptera litura* cells *in vivo* and *in vitro*. It was found that stigmasterol exhibited greater cytotoxicity action against *Spodoptera litura* comparable to the other active phytoconstituents (Huang et al., 2009).

v. Anti-osteoarthritis activity

A study to investigate the anti-osteoarthritic activity of stigmasterol was carried out by Gabay et al. (2010). This study was conducted by using newborn mouse chondrocytes and human osteoarthritis chondrocytes in primary culture. They were incubated with or without the interleukin-1 beta (1L-1β) for 18 hours. 1L-1β increased the expression of various genes responsible in the cartilage turn over, MMP-3, MMP-13 and ADAMTS-4. These cells were then further incubated with stigmasterol for 48 hours and results obtained were compared with untreated group. It showed that stigmasterol decreased the effect of interleukin-1 beta and thus possess an anti-osteoarthritic effect.

2.4 Pharmacological studies of *Wedelia* spp.

2.4.1 Antimicrobial properties

W. biflora is useful in the treatment of microbial and wounds infections. Several studies have been conducted to prove this traditional claim. For instance, a study was conducted on the antifungal activity of essential oil of *W. biflora* against *Candida albicans* in Indonesia. The essential oils from the leaves of *W. biflora* with other plant species found in Aceh Province were tested against *C. albicans* at concentrations of 1%, 5% and 10%. The results for *W. biflora* showed no zone of inhibitions for antifungal activity (Ginting, 2012). On a contrary, a recent study by Biswas et al. (2013) on the evaluation of antimicrobial and wound healing property of ethanolic extract of *W. biflora* was conducted. The results of ethanolic extracts on tested microorganisms showed maximum inhibition with higher range of zone of inhibition (ZI); which was 11.3-21.6 mm. Best result for antimicrobial study was achieved against *Candida albicans* with MIC values of 39 µg/mL as compared to the control; which was 4.8-19.50 µg/mL.

The methanolic extract of *W. chinensis* leaves exhibited a potent antibacterial activity against Gram positive bacteria (*Bacillus cereus*, *Bacillus subtilis* and *Staphylococcus aureus*) as compared to the Gram negative bacteria (*Escherichia coli*, *Proteus rettgeri* and *Pseudomonas aeruginosa*). This study showed that higher concentration of methanolic extracts exhibited significant inhibition of bacterial cells growth (Darah et al., 2013). The antibacterial and antifungal activities of ethanolic and hexane extracts of *W. chinensis* leaves were investigated against pathogenic bacteria and fungi. Both extracts exhibited significant result in antibacterial and antifungal study. For antifungal activity evaluation, the extract was found to be effective against four strains of fungi (*Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* and *Alternaria alternate*) (Das et al., 2013).

The phytochemistry and antimicrobial effect of the essential oil from *W. prostrata* were studied against 10 different strains of microorganisms by Dai et al. (2013). The results showed that the essential oil possess a potent antimicrobial activity against the tested microorganisms. The antimicrobial activity of the main bioactive compounds, limonene and α-pinene was also evaluated and α-pinene exhibited higher antimicrobial activity compared to limonene compound. The antimicrobial activity of *W. trilobata* from different plant parts was investigated against 7 bacterial strains using agar diffusion method by Jayakumar et al. (2011). The flower part showed maximum inhibition against *Salmonella pratyphi* and *Bacillus cereus*.

2.4.2 Antioxidant properties

The antioxidant activity of the essential oils from *W. chinensis* was investigated against *in vitro* and *in vivo* Lung Cancer Bearing c57BL/6 in mice (Manjamalai & Grace, 2013) and it showed a significant correlation between concentrations of *W. chinensis* essential oils and percentage inhibition of free radicals. In a previous study by Senthilkumar et al. (2008), treatment with ethanolic extract of *W. chinensis* enhanced the antioxidant activity in alloxan induced rats. *W. chinensis* can be extensively studied to treat diseases related to free radicals scavenging activities.

The antioxidant activity of methanolic extract from different plant parts of *W. trilobata* was also studied *in vitro*. The result showed that the flower part exhibited the highest antioxidant activity when compared to the other plant parts (Jayakumar et al., 2011). The methanolic flower extract of *W. trilobata* (L.) contains antioxidant activity with IC_{50} value of 90 µg/mL for DPPH assay and IC_{50} value of 80 µg/mL for radical scavenging assay. They displayed different values due to different mechanism of radical antioxidant reaction in both assays (Chetan et al., 2012). Apart from that, water extract

of leaf and flower of *W. trilobata* had also shown some free radicals scavenging activity (Govindappa et al., 2011).

Essential oil from *W. prostrata* is another source of potential antioxidant agent. Dai et al. (2013) studied the antioxidant properties of this essential oil by determining the free radicals-scavenging activity. It was shown that essential oil of *W. prostrata* had a moderate antioxidant activity and could be developed further for production of useful medicine.

2.5 Application of plant tissue culture of *Wedelia* spp.

Plant tissue culture technique is the best method to study the cellular behavior of plants and also for mass propagation of important species. In this technique, only small portions of plants are needed for mass propagation. Micropropagation to produce a whole new plant is useful in bulk production of plants in a short period. Meanwhile, callus culture is very useful in secondary metabolites production and analysis.

In *W. chinensis*, micropropagation studies were carried out by using nodal segments as explants; with Murashige and Skoog (MS) medium supplemented with 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) as the best for axillary bud multiplication and rooting, respectively (Martin et al., 2003). Meanwhile, Rahman and Bhadra (2011) had established a rapid and reproducible propagation method for *W. chinensis* by using different type of explants. They obtained the highest number of multiple shoots from nodal segment in MS basal medium fortified with BAP (3.0 mg/L) and indole-3-acetic acid (IAA, 0.5 mg/L). Rooting of generated shoots was best observed on half strength MS medium supplemented with IBA (2.0 mg/L).

In a study by Agarwala et al., (2010), they used shoot tips and nodes as explants for clonal propagation on MS medium supplemented with different concentrations of auxins, cytokinins and gibberellic acid. Best response for shoot elongation was observed on shoot tips cultured in MS medium containing combination of BAP (1.0 mg/L) and α-napththaleneacetic acid (NAA, 1.5 mg/L) and best response for shooting and rhizogenesis was observed in MS medium fortified 1.0 mg/L BAP and 1.5 mg/L NAA.

In vitro W. trilobata calli were obtained using leaf, shoot tips and nodal segments as explants. Best result for callusing was achieved in leaf explants supplemented with 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and shoot tips explants with 1 mg/L 2,4-D (Thakur et al., 2010). A new potential method for *in vitro* propagation of *W. trilobata* was developed using extracellular products (EP) and biomass water extracts (BWE) of *Phormidium subincrustatum*. This cyanobacterial extracts serves as an organic source to the MS media. Best medium for callus growth was observed on MS medium supplemented with BWE as compared to the control. It showed a significant increase in shoot length and callus volume within 15 days of culture. It was found that MS medium fortified with cyanobacterial extracellular product is cost effective and can replace the widely used chemically synthesized plant growth regulators in MS media (Keerthiga et al., 2012).

2.6 The effects of abiotic stresses on callus induction and secondary metabolites production

2.6.1 Temperature

Temperature plays important role in plant growth. Most of plants in Malaysia can grow well in temperature around 25℃, thus most of incubation chambers are set with 25℃ as default incubation temperature. Incubation temperature could affect the morphology of callus as reported by Summart et al. (2015). They found that callus

incubated at 25℃ are dry and compact whereas callus incubated at 30℃ are soft and friable. Warmer temperature creates higher humidity which caused the callus to absorb more water and thus make it softer and friable. Different incubation temperatures could also affect the callus metabolites production. Kumar et al. (2012) reported that various temperatures considerably altered the total phenolics and flavonoids content in *Heliotropium indicum* L. Higher temperature (30℃) caused the callus to produce more phenolic compound while lower temperature (20℃) remarkably reduced the total phenolic synthesis. However, they also found that flavonoids content was reduced in callus incubated under 30℃. Their results indicate that the synthesis of different metabolites is influenced by various incubation temperatures.

2.6.2 Light colours and photoperiod

The presence of light is another factor to be considered in *in vitro* cultures. In most cases, callus can form well under adequate light intensity but may have lower content of metabolites (Siddique & Islam, 2015). Siddique & Islam (2015) reported callus from tobacco initiated earlier and more green under light condition compared to total dark condition. The callus developed under dark conditions are watery, glossy silver in colour and less embryogenic features. Despite the presence of light, the colour of lights also could affect the callus formation and secondary metabolites production. Nhut et al. (2015) found that yellow light yield highest biomass for *Panax vietnamensis* callus compared to white, blue and red lights. Meanwhile, Kishima et al. (1995) found that blue light has the potential to accumulate betalain in *Portulaca* callus.

2.6.3 Sucrose concentration

In this study, Murashige and Skoog medium was used as the culture medium. The main carbon source used in MS medium is sucrose. Carbon source is essential for the growth of callus and plantlets in *in vitro* culture. Many studies used different

concentration of sucrose to obtained maximum formation of callus (Shahnewaz & Bari, 2004 & Saikia et al., 2012). Shahnewaz & Bari (2004) found that 4% of sucrose yielded high formation of callus and greener plant regeneration. Sucrose concentration higher than 4% could also yield high formation of callus, but with lesser chlorophyll content (less green). This may be due to the cells rapidly proliferated without proper pigment synthesis because of excess supply of carbon source. Most of callus grow well in MS medium supplemented with 3% of sucrose (Pande and Khetmalas, 2012).

CHAPTER 3: MATERIALS AND METHODS

3.1 Callus Induction from Stem and Leaf Explants of *W. biflora*

3.1.1 Plant materials and its preparation

W. biflora plants were collected from the Botanical Garden of University of Malaya, Kuala Lumpur, Malaysia. In this study, the young stem and leaf of *W. biflora* plants were used as the starting materials for callus induction. Prior to inoculation, the explants were washed in 5% (v/v) Teepol for 2 minutes, followed by 20% (v/v) sodium hypochlorite for 2 minutes. Subsequently, the explants were surface sterilized with 0.1% (w/v) mercury chloride for 1 minute followed by 0.001% (w/v) carbendazim in 70% (v/v) ethanol for 1 minute and thoroughly rinsed in sterile distilled water three times. The explants were air-dried on sterile tissue papers in a laminar air flow chamber and cut into 5 to 8 mm long before inoculation onto fresh MS medium.

3.1.2 Chemical purchase

MS basal salt, Teepol, mercury chloride, carbendazim, ethanol, IAA, NAA, IBA, 2,4-D, KN, BAP, sucrose, agar, acetonitrile, methanol, chloroform, acetic anhydride, sulphuric acid, sodium phosphate buffer, 2,4-6-tri (2-pyridyl)-s-triazine (TPTZ), ferric chloride hexahydrate, ferrous sulphate, potassium ferricyanide, ferric chloride, phosphate buffer, and sodium hydroxide were purchased from Duchefa Biochemie, The Netherlands through Malaysian suppliers. Stigmasterol (99%), and 2,2-diphenyl-1-picrylhydrazil (DPPH) were purchased from Sigma, Malaysia. Sodium hypochlorite was purchase from Clorox, Malaysia.

3.1.3 Optimization of callus induction with auxins and cytokinins

The selection of auxin and its concentration was using MS medium (Murashige and Skoog, 1962) with 3% sucrose supplemented with various concentrations of IAA, NAA, IBA and 2,4-D from 0.5 to 4.0 mg/L. The best auxin and its concentration to induce

callus was selected and combined with various concentrations of BAP and Kinetin from 0.5 to 4.0 mg/L. All media were adjusted to pH 5.7 with 1 N NaOH and autoclaved at 121 ºC for 20 minutes. An antifungal, 0.001% (w/v) carbendazim in 70% (v/v) ethanol was added into the media and the media was dispensed into 60mm (in diameter) specimen containers in the laminar air flow chamber under aseptic condition. The sterile explant was inoculated horizontally onto the media for callus induction. All cultures were incubated at 25±2 ºC under 16 h photoperiod. Biomass of callus (fresh and dry weights) from both explants was taken at day 14, 21, 28, 35 and 42 post inoculation. The optimum auxins and cytokinins combinations to yield highest biomass were recorded. Another batch of samples was investigated using the optimum auxins and cytokinins combinations respectively, for stem and leaf explants to determine the period of optimum stigmasterol production. Callus from day 14, 21, 28, 35 and 42 were taken randomly, extracted with methanol and the production of stigmasterol was estimated by using spectrophotometer method (procedure explained in Section 3.6.1). This method was chosen because it is the simplest method to estimate stigmasterol content in shortest time by using limited amount of extracts (Fujiwara et al., 2013).

3.1.4 Optimization of callus induction under abiotic stress conditions

The MS media with the best auxin and cytokinin combination were prepared. Four abiotic stresses were selected for this experiment; temperature (20°C, 25°C, 30°C, 35°C), sucrose concentration (1%, 2%, 3%, 4%, 5%), lighting colours (red, blue, green, white) and photoperiod (0, 4, 8, 12, 16, 20, 24 h photoperiod). The explants were inoculated onto the prepared MS media and incubated under various abiotic stresses conditions. The cultures placed in the culture room were adjusted according to their stress conditions. The observations and biomass of the callus were taken at 14, 21, 28, 35 and 42 days. The best parameters for callus induction from each stress conditions were selected to undergo quantification of stigmasterol content.

3.2 Indirect regeneration of *W. biflora*

3.2.1 Plant materials

Indirect regeneration of *W. biflora* was accomplished by using selected callus that showed promising organogenesis from previous experiment (Exp. 3.1). The callus were separated carefully from the explants and inoculated onto MS media with appropriate hormones as described later in this text.

3.2.2 Shoots induction and shoots elongation

After 45 days, all callus were inspected in terms of health, nature and colour. Callus that showed healthy appearance (not dry), compact and green in colour were separated from the explants and transferred onto shoot induction medium which were composed of MS basal medium fortified with BAP (1.0, 2.0, 3.0, 4.0 mg/L). The cultures were incubated for 30 days before multiple shoots emerged and separated one by one to be transferred into MS medium without any hormones for 15 days to promote single shoots elongation.

3.2.3 Roots induction

Elongated single shoots were transferred into MS medium containing different concentrations of IBA (1.0, 2.0, 3.0, 4.0 mg/L). The cultures were incubated in the culture room for 30 days until roots emergence could be seen. After 30 days, rooting response, mean roots per shoot and mean length of roots were recorded. The regenerated plantlets were allowed to elongate *in vitro* up to 8-10cm shoot length and 2-3cm root length.

3.2.4 Plants hardening and acclimatization

The appropriate elongated plantlets were carefully removed from MS media and the roots were washed with sterile distilled water to remove the excess agar. The cleaned plantlets were transferred into polycups containing black soil with one plantlet per

polycup. The polycups were covered with a punctuated plastic bag to allow air exchange for 30 days and the plantlets were watered with soft mist in every two days to maintain the humidity. Within these 30 days, the plantlets were kept under 25±2 °C, with 16 h light/ 8 h dark cycle. Then, the hardened plantlets were transferred to a greenhouse and the plastic bag was removed after 7 days. Watering was carried out every day.

3.3 Direct regeneration of *W. biflora*

3.3.1 Plant materials and sterilization

Direct regeneration of *W. biflora* was obtained by using internode as the starting material. *W. biflora* was collected from Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. A voucher specimen with no. 47746 has been deposited at University of Malaya Herbarium (KLU). The explants were washed thoroughly under running tap water for 30 min, followed by treatment in 3 drops of Teepol for 2 min. After that, the explants were rinsed with tap water to remove the excess Teepol and washed with hypochlorite (20% v/v) for 5 min and surface sterilized with $HgCl_2$ (0.1% w/v) for 2 min. After each step, the explants were washed 3 times for 4 min with sterilized distilled water to remove excess of bleach and $HgCl_2$. Finally, the explants were treated with carbendazim as an antifungal (0.001% w/v) by dilution in ethanol (70% v/v) in a laminar flow chamber. The internodes were approximately excised around 1.0-1.5 cm in size and then cultured on Murashige-Skoog (MS) medium. The cultures were kept in the culture room at 25±2°C, with a photoperiod of 16 h daylight under white fluorescent light and 8 h dark.

3.3.2 Induction of shoots

Internode explants were cultured on Murashige-Skoog (MS) medium supplemented with cytokinins (BAP and KN) and auxins (2,4-D, IAA, IBA and NAA) either singly or

in combinations. The concentrations of plant growth regulators being used were between 0.5 to 4.0 mg/L. The media contained 3% sucrose (Duchefa Biochemie) and 0.8% agar (Oxoid). The pH was adjusted to pH 5.8 before the media was heated up, dispensed in test tubes and then finally autoclaved at 121°C for 15 min. The number of shoots, length of shoots and height of plantlets were being recorded after 6 weeks.

3.3.3 Induction of roots

For rooting purposes, the *in vitro* shoots were cultured on MS medium with different strength (1/4 to full-strength) without any PGRs. Then, the best MS strength was selected and supplemented with various concentrations of IBA from 0.5 to 5.0 mg/L. Data were recorded based on the percentage of rooting and the root number and length after 6 weeks to culture.

3.3.4 Acclimatization of plantlets

After 6 weeks of culture, recovered plantlets were washed under running tap water to remove excess agar sticking on the roots. Developed plantlets (about 5 cm in height) with fully expanded leaves and well-developed roots were transferred to plastic cups containing three different growth substrates; which are black soils, red soils and combinations of black soil and red soil in 1:1 ratio. The plantlets were grown under culture room conditions of regulated humidity and temperature for 2 weeks before being transferred to soil under full sunlight. The condition of culture room was kept constant at 50% humidity level, temperature 25±2°C, 16 h light and 8 h dark.

3.4 Sample extraction

The plant samples included *in vivo*- , *in vitro*-, *ex vitro*-grown plants and callus tissues were oven-dried at 40 ºC for 2 days. After that, samples were ground to produce fine homogenous powder using a mortar and pestle. The fine powder was soaked in 50 mL methanol at room temperature for three days, before it was centrifuged at 9000 rpm

for 5 minutes. The supernatant was evaporated to dryness at room temperature for 3 days. The plant extracts was dissolved in methanol (50 mg plant/mL methanol) and kept in glass vial at 4 ºC until further use.

3.5 Examination of extracts bioactivities

3.5.1 Antimicrobial activity assay

3.5.1.1 Disc diffusion method

Two common bacteria (*Escherichia coli* and *Bacillus subtilis*) and two common fungi (*Aspergillus niger* and *Candida albicans*) were used for antimicrobial activity assay. These microbes were obtained from Division of Biotechnology, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. The bacteria were grown on Nutrient Agar (NA) plate while fungi were grown on Potato Dextrose Agar (PDA) plates. NA and PDA were autoclaved at 121ºC for 20 minutes and dispensed into petri dishes after cooled to 60ºC. Twety µL of inoculum ($1.0x10^5$ CFU/mL) were transferred onto the fresh medium and spread evenly by using sterile cotton bud. Sterile paper disks were put in the middle of the plate and 5 µL of plant extracts or controls were dropped on top of the paper disk. The plates were sealed with parafilm and incubated at 37 ºC for bacteria and 27ºC for fungi. Results of the antimicrobial screening were recorded as the average diameter of zone of inhibition (ZI) surrounding the paper disc containing the test solution in comparison to control. Ampicillin and fluconazole were used as positive controls for bacteria and fungi, respectively and methanol was used as negative control.

3.5.2 Antioxidant activity assays

3.5.2.1 DPPH free radicals scavenging assay

DPPH (2,2-diphenyl-1-picrylhydrazil) free radical scavenging activity assay was established using the protocol described by Kikuzaki et al. (2002). DPPH (1 mL) with concentration of 0.4 mM was mixed with 50 µL of the plant extracts (50 g/L) and the

volume was adjusted to 5 mL using 98% methanol before incubation at room temperature in the dark for 30 min. Scavenging activity of the plant extracts reduced the colour of DPPH from purple to yellow and was measured using a spectrophotometer at 517 nm. Comparison of the reduction of colour in the examined samples with the control (solution without plant extracts) was used to measure the scavenging activity of the plant extracts using the following equation (Kikuzaki et al., 2002).

$$Radical\ scavenging\ activity(\%) = \left(1 - \frac{As}{Ac}\right) X\ 100$$

'As' referred to absorbance of sample at 517 nm, while 'Ac' indicated absorbance of control at 517 nm. IC_{50} value (the concentration necessary to obtain half of the maximum scavenging activity) of each samples were calculated based on scavenging activity percentage curve.

3.5.2.2 H_2O_2 scavenging assay

This experiment was carried out by adapting methods by Ruch et al. (1989). Firstly, 2 mM of H_2O_2 was prepared in 50 mM phosphate buffer (pH 7.4). Then, 0.1 mL extracts were transferred into micro tubes and 0.3 mL methanol was added. Consequently, 0.6 mL H_2O_2 solution was added, the solution was mixed thoroughly and incubated at room temperature for 10 min. Absorbance was taken at 230 nm against blank. Percentage of H_2O_2 scavenging ability was calculated as follow:

$$Percentage\ of\ scavenging\ (\%) = \left(1 - \frac{Ac}{Ao}\right) x\ 100$$

where; Ac was absorbance of sample and Ao was absorbance of control (without sample).

3.5.2.3 Reducing power assay

Reducing power assay was carried out by method described by Oyaizu (1986) with some modifications. The extracts (100 µL) was added to 400 µL phosphate buffer (0.2 M, pH 6.6) and 400 µL potassium ferricyanide (1% w/v) and incubated in water bath (50 °C) for 20 min. Consequently, 500 µL of trichloroacetic acid was added to the mixture and centrifuged for 10 min at 1000 rpm in room temperature. The supernatant (1400 µL) was taken and added to 1400 µL deionized water and 280 µL ferric chloride (0.1% w/v) and incubated in an oven at 37 °C for 10 min. Finally, the absorbance was taken at 700 nm. Ascorbic acid was used as positive control. A higher absorbance of the reaction mixture indicated greater reducing power.

3.5.2.4 Ferric reducing antioxidant power (FRAP) assay

Ferric Reducing Antioxidant Power (FRAP) assay was carried out using method described by Wong et al. (2006) with some modifications. Sample or ascorbic acid (200 µL) was added into 3 mL FRAP reagent that was freshly prepared. FRAP reagent consisted of 300 mM sodium phosphate buffer (pH 3.6), 10 mM 2,4-6-tri (2-pyridyl)-s-triazine (TPTZ) solution and 20 mM $FeCl.6H_2O$ with 10:1:1 ratio, respectively. The reaction mixture was incubated in water bath (37 °C) for 30 min. After 30 min, blue colour developed and the absorbance was taken at 593 nm. Absorbance of samples or ascorbic acid was compared with ferrous sulfate standard curve (200 – 1000 mM) and results were expressed in mM Fe (II)/g dry mass. The relative activity of the samples was compared to ascorbic acid.

3.6 Identification and estimation of stigmasterol

3.6.1 Estimation of stigmasterol content via spectrophotometric method

Fujiwara et al. (2013) described quantification of stigmasterol by using Liebermann-Burchard (LB) reagent and detected using UV-vis spectrophotometer at 640nm. LB

reagent was made of 60 mL acetic anhydride and 3 mL sulphuric acid. Two mL of each extracts was mixed with 2 mL of LB reagent. The mixture was then added with 5 mL chloroform and incubated in the dark at room temperature for 15 min. After that, the absorbance was taken at 640 nm by using spectrophotometer. The results were compared with stigmasterol standard curve and stigmasterol content was estimated in each sample.

3.6.2 HPLC analysis

HPLC analysis was performed using JASCO HPLC System Manager equipped with UV-vis detector. Chromatographic separation was carried out using a C18 reversed phase column under isocratic conditions. The mobile phase was a mixture of acetonitrile:methanol (70:30) at the flow rate of 1 mL/min. The operative wavelength was set at 205 nm and the injection volume was 20 µL. Each sample was run for 16 min. The amount of stigmasterol in samples was calculated using standard curve of stigmasterol standard with known concentration.

3.7 Statistical analysis

All experiments were repeated three times with 20 replicates each. The effect of different treatment was quantified as mean ± SE and the data were subjected to statistical analysis using Duncan's Multiple Range Test (DMRT) at 5% significance level.

CHAPTER 4: RESULTS

4.1 Optimization of callus induction with auxins and cytokinins

4.1.1 The effects of auxins (NAA, 2,4-D, IBA and IAA) on stem and leaf callus induction.

Callus induction from stem and leaf explants of *W. biflora* has been successfully achieved. Callus initiation was observed from the cut end surface of leaf and stem explants within two months in all media tested with the exception of MS media without hormones and MS media supplemented with IAA alone. Explants in both MS media without hormones and MS media supplemented with IAA alone showed no active growth and remained green even after three months of culture.

MS media supplemented with 2.0 mg/L NAA resulted in the highest frequency and fastest response for callus induction followed by application of 2.0 mg/L IBA and 0.5 mg/L 2,4-D for stem explants. Whereas, application of 1.0 mg/L 2,4-D provided the highest frequency and fastest response for leaf explants followed by 3.0 mg/L NAA and 1.5 mg/L IBA. The highest frequency of callus formation was observed in MS media supplemented with 2.0 mg/L NAA for stem explants (85%) and 1.0 mg/L 2,4-D for leaf (88%) explants, respectively (Table 4.1).

There were two types of callus observed in this experiment which were green and compact as well as green and nodular. Most of the green and compact callus were from stem explants while green and nodular callus were from leaf explants (Fig. 4.1). The best single hormone concentration (2.0 mg/L NAA for stem explants; 1.0 mg/L 2,4-D for leaf explants) was then supplemented in MS media together with various concentrations of BAP or Kinetin to induce more callus formation.

4.1.2 The effects of combinations of auxins (NAA and 2,4-D) with cytokinins (BAP and KN) on stem and leaf callus induction.

Application of auxins alone (NAA or 2,4-D) produced callus from stem and leaf explants of *W. biflora*. However, the size of callus produced was very small (less than 1.5 cm for both explants). Therefore, the size of callus production was improved by addition of cytokinins (BAP or KN) in MS media for both explants.

For stem explants, combination of 2.0 mg/L NAA with either Kinetin or BAP (0.5 – 4.0 mg/L) resulted in faster callus formation (in 10-12 days) and producing callus with greater than 1.5 cm in size. This suggested that combination of auxins and cytokinins accelerated the cell division and proliferation of the cells into callus. Even though addition of either BAP or KN showed higher percentage of callus formation (Table 4.1), only stem explants incubated in MS media supplemented with 2.0 mg/L NAA and various concentrations of BAP showed the highest biomass readings. Among all BAP concentrations (from 0.5 – 4.0 mg/L), 2.5 mg/L BAP gave the best results in terms of callus nature and biomass when combined with 2.0 mg/L NAA (98% callusing). This result proved that BAP as a cytokinin played a role to modulate callus induction and growth when combined with NAA which was an auxin. In this experiment, higher concentration of cytokinins as compared to auxins in a growth medium promotes more callus production. Further subculture of callus from this hormone combinations resulted in more green and compact callus with mixture of shoots formation.

The leaf explants of *W. biflora* did not show prominent responses as stem explants. Longer inoculation period (17-20 days) was required for the callus to form in MS media supplemented with combinations of 1.0 mg/L 2,4-D and BAP or Kinetin. Leaf explants inoculated in MS medium supplemented with 3.5 mg/L and BAP showed intense formation of green and compact callus as compared to Kinetin (Fig. 4.2). The nature of

callus formed from leaf explants changed from green and nodular into green and compact when cytokinins (3.5 mg/L BAP) was added into the media together with auxins (1.0 mg/L 2,4-D). This study showed that combination of auxins with BAP will produced green and compact callus for both stem and leaf explants. This is perhaps due to rapid cell proliferation occurred by the combinations of auxins and cytokinins, thus forcing the proliferated cell to segregating more compact to each other.

In conclusion, stem and leaf explants of *W. biflora* were best inoculated in MS media supplemented with combination of one auxin and one cytokinin. Combination of 2.0 mg/L NAA with 2.5 mg/L BAP was the best for stem explants; while combination of 1.0 mg/L 2,4-D and 3.5 mg/L BAP was the best for leaf explants. These respective combinations for stem and leaf explants will be used in further experiments.

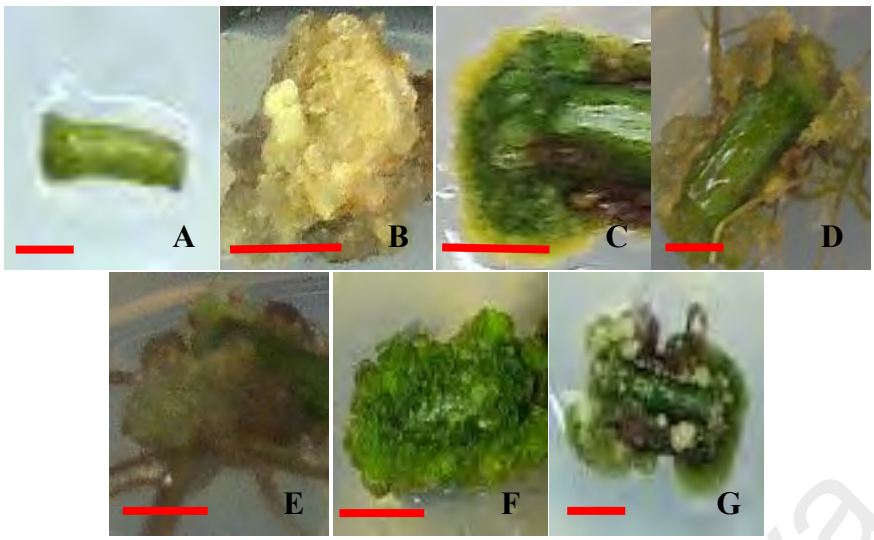


Figure 4.1: Nature of stem callus inoculated in MS media fortified with various PGRs after 45 days. A) No callus formation in MS basal medium; B) Yellow and nodular callus in MS medium with 2,4-D; C) Brown and friable callus in MS medium with NAA; D) Transparent and friable callus, with roots in MS medium with IBA; E) Transparent and friable callus, with roots in MS medium with IAA; F) Green and compact callus in MS medium with NAA and BAP; and G) Green and compact callus in MS medium with NAA and KN. Bars represent 0.5 cm.

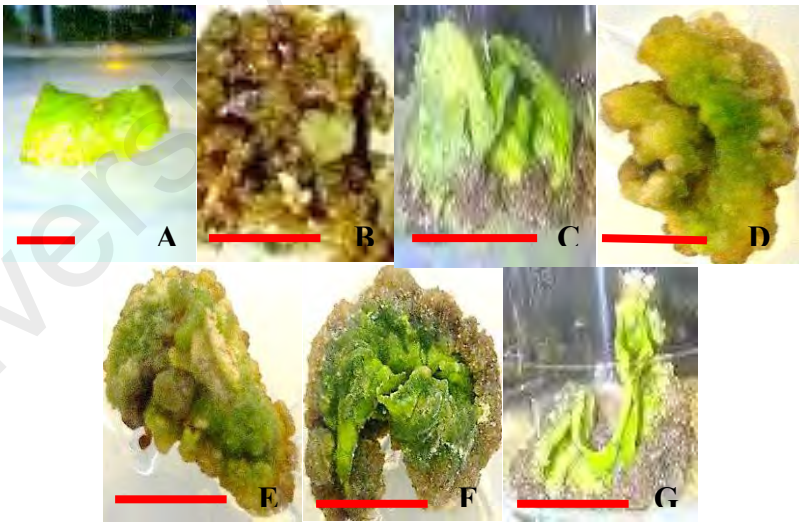


Figure 4.2: Nature of leaf callus inoculated in MS medium supplemented with various plant hormones after 45 days. A) No callus growth in MS basal medium; B) Green and nodular callus in MS medium with 2,4-D; C) Brown and compact callus in MS medium with NAA; D) Yellow and compact callus in MS medium with IAA; E) Green and compact callus in MS medium with IBA; F) Green and compact callus in MS medium with 2,4-D and BAP; and G) Brown and friable callus in MS medium with 2,4-D and KN. Bars represent 0.5 cm.

Table 4.1: Effect of different PGRs concentrations on callus induction of stem and leaf explants of *W. biflora*.

Hormones (mg/L)	Intensity of callus		Percentage of callus formation (%)		Days of callus formation		Nature of callus		Callus dry weight (mg)		Callus fresh weight (mg)	
	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
MS basal	--	--	0	0	--	--	--	--	11±2.5	21 ± 2.2	56±3.2	80 ± 4.8
NAA												
0.5	+	+	78.1 ± 2.1	69.3 ± 1.5	18	18	GCC	GCC	39 ± 1.5	13 ± 2.3	121 ± 2.5	78 ± 2.1
1.0	+	+	73.3 ± 1.7	66.4 ± 1.9	18	18	GCC	GCC	38 ± 2.5	17 ± 2.1	392 ± 3.9	94 ± 2.5
1.5	+	+	77.0 ± 2.5	68.1 ± 1.8	18	18	GCC	GCC	33 ± 1.6	15 ± 2.2	327 ± 4.5	105 ± 3.1
2.0	++	+	**85.1 ± 3.3**	64.7 ± 1.5	18	18	GFC	GCC	**48 ± 2.8**	10 ± 1.7	**559 ± 5.5**	60 ± 1.4
2.5	++	+	81.5 ± 2.8	62.3 ± 2.8	18	18	GFC	GCC	43 ± 1.8	12 ± 2.5	328 ± 2.6	76 ± 2.6
3.0	++	+	79.1 ± 2.5	70.8 ± 3.1	18	18	GFC	GCC	30 ± 3.1	19 ± 2.8	166 ± 4.1	122 ± 1.8
3.5	++	+	76.1 ± 2.1	59.5 ± 2.5	18	18	GFC	GCC	36 ± 1.9	22 ± 1.4	471 ± 2.7	141 ± 1.2
4.0	++	+	82.3 ± 2.3	63.2 ± 1.6	18	18	GFC	GCC	46 ± 1.5	24 ± 1.2	385 ± 2.1	150 ± 2.3
IBA												
0.5	+	+	64.5 ± 2.1	57.8 ± 2.3	21	21	GFC	GCC	36 ± 2.5	20 ± 1.6	102 ± 1.4	128 ± 2.3
1.0	+	+	76.5 ± 2.9	63.2 ± 1.2	21	21	GFC	GCC	35 ± 2.6	18 ± 1.5	376 ± 3.7	165 ± 1.2
1.5	++	+	75.1 ± 2.4	65.5 ± 1.8	21	21	GFC	GCC	40 ± 2.1	9 ± 1.9	259 ± 3.5	37 ± 1.8
2.0	++	+	82.1 ± 2.5	49.6 ± 2.6	18	21	GFC	GCC	40 ± 3.9	18 ± 2.5	533 ± 3.2	96 ± 2.6
2.5	++	+	80.1 ± 2.6	58.8 ± 1.4	18	21	GFC	GCC	38 ± 1.4	10 ± 3.1	421 ± 3.1	67 ± 1.4
3.0	++	+	71.6 ± 2.5	55.1 ± 3.1	18	21	GFC	GCC	31 ± 1.8	9 ± 1.7	335 ± 2.5	53 ± 3.1
3.5	++	+	73.5 ± 2.8	62.0 ± 2.5	18	21	GFC	GCC	28 ± 0.9	15 ± 1.9	298 ± 2.1	119 ± 2.5
4.0	++	+	80.6 ± 2.5	56.5 ± 2.1	18	21	GFC	GCC	26 ± 1.5	7 ± 2.8	410 ± 2.6	49 ± 2.1

GCC - Green compact callus; GFC - Green friable callus; CFC - Creamy friable callus; CNC - Creamy nodular callus; GNC - Green nodular callus; IBA - Indole-3-butyric acid; IAA - Indole-3-acetic acid; NAA - α-naphthalene acetic acid; 24D - 2,4-dichlorophenoxyacetic acid; BAP - Benzylaminopurine; KN - Kinetin; + - Meager (0.5-1.0 cm); ++ - Moderate (1.0-1.5 cm); +++ - Intense (>1.5 cm). Values are mean ± SE of 10 replicates per treatment and repeated thrice.

Table 4.1, continued

Hormones (mg/L)	Intensity of callus		Percentage of callus formation (%)		Days of callus formation		Nature of callus		Callus dry weight (mg)		Callus fresh weight (mg)	
	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
IAA					--							
0.5	--	--	0	0	--	--	--	--	21 ± 1.2	7 ± 2.5	39 ± 2.4	55 ± 2.6
1.0	--	--	0	0	--	--	--	--	16 ± 2.6	9 ± 1.5	39 ± 2.8	53 ± 1.4
1.5	--	--	0	0	--	--	--	--	19 ± 3.1	18 ± 1.2	92 ± 2.1	117 ± 3.5
2.0	--	--	0	0	--	--	--	--	19 ± 2.1	21 ± 1.6	69 ± 2.9	112 ± 2.2
2.5	--	--	0	0	--	--	--	--	16 ± 2.5	17 ± 1.0	63 ± 3.3	79 ± 3.3
3.0	--	--	0	0	--	--	--	--	17 ± 1.4	13 ± 1.7	57 ± 1.5	53 ± 2.5
3.5	--	--	0	0	--	--	--	--	29 ± 1.8	10 ± 1.9	71 ± 2.7	50 ± 3.1
4.0	--	--	0	0	--	--	--	--	15 ± 2.3	12 ± 2.1	74 ± 1.8	61 ± 2.1
2,4-D												
0.5	+	++	68.2 ± 2.1	81.8 ± 2.5	18	18	CNC	GNC	45 ± 1.5	37 ± 1.8	241 ± 3.6	221 ± 2.5
1.0	+	++	59.1 ± 2.8	**88.0 ± 1.5**	18	18	CNC	GNC	33 ± 1.2	**41 ± 2.4**	221 ± 2.4	**241 ± 2.1**
1.5	+	++	61.2 ± 2.5	81.0 ± 2.6	18	18	CNC	GNC	35 ± 0.9	25 ± 2.8	172 ± 2.6	172 ± 2.9
2.0	+	++	67.5 ± 2.9	73.4 ± 2.1	18	18	CNC	GNC	28 ± 1.9	28 ± 2.7	202 ± 1.8	202 ± 2.8
2.5	+	++	43.9 ± 2.1	66.7 ± 0.9	18	18	CNC	CNC	27 ± 2.1	24 ± 1.5	167 ± 2.0	167 ± 2.4
3.0	+	++	51.3 ± 2.1	61.6 ± 1.8	18	18	CNC	CNC	26 ± 1.7	25 ± 2.1	137 ± 2.9	137 ± 2.5
3.5	+	++	55.6 ± 2.0	52.7 ± 1.4	18	18	CNC	CNC	26 ± 1.6	24 ± 3.3	158 ± 1.5	158 ± 2.6
4.0	+	++	47.0 ± 2.3	56.4 ± 2.9	18	18	CNC	CNC	31 ± 2.5	23 ± 2.9	95 ± 1.6	75 ± 3.1

GCC - Green compact callus; GFC - Green friable callus; CFC - Creamy friable callus; CNC - Creamy nodular callus; GNC - Green nodular callus; IBA - Indole-3-butyric acid; IAA - Indole-3-acetic acid; NAA - α-naphthalene acetic acid; 24D - 2,4-dichlorophenoxyacetic acid; BAP - Benzylaminopurine; KN - Kinetin; + - Meager (0.5-1.0 cm); ++ - Moderate (1.0-1.5 cm); +++ - Intense (>1.5 cm). Values are mean ± SE of 10 replicates per treatment and repeated thrice.

Table 4.1, continued

Hormones (mg/L)	Intensity of callus		Percentage of callus formation (%)		Days of callus formation		Nature of callus		Callus dry weight (mg)		Callus fresh weight (mg)	
	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
NAA+BAP												
2.0+0.5	++	--	82.3 ± 2.5	--	12	--	GFC	--	49 ± 1.5	--	413 ± 3.4	--
2.0+1.0	++	--	87.1 ± 3.2	--	12	--	GFC	--	36 ± 2.1	--	385 ± 3.6	--
2.0+1.5	++	--	89.1 ± 2.1	--	12	--	GFC	--	39 ± 1.8	--	387 ± 3.1	--
2.0+2.0	++	--	91.5 ± 2.4	--	12	--	GFC	--	20 ± 0.7	--	266 ± 2.6	--
2.0+2.5	+++	--	**96.2 ± 2.8**	--	10	--	GFC	--	**55 ± 1.9**	--	**563 ± 3.2**	--
2.0+3.0	+++	--	95.1 ± 3.5	--	10	--	GCC	--	45 ± 1.6	--	439 ± 3.9	--
2.0+3.5	+++	--	92.4 ± 2.8	--	10	--	GCC	--	25 ± 1.2	--	269 ± 2.8	--
2.0+4.0	+++	--	93.1 ± 2.1	--	10	--	GCC	--	25 ± 2.3	--	227 ± 2.6	--
NAA+KN												
2.0+0.5	++	--	80.5 ± 2.4	--	10	--	GFC	--	22 ± 3.6	--	171 ± 1.9	--
2.0+1.0	++	--	86.2 ± 2.1	--	10	--	GFC	--	50 ± 3.2	--	294 ± 2.5	--
2.0+1.5	++	--	85.3 ± 3.6	--	10	--	GFC	--	58 ± 2.4	--	365 ± 1.6	--
2.0+2.0	+++	--	93.3 ± 1.8	--	10	--	GFC	--	41 ± 2.9	--	260 ± 1.4	--
2.0+2.5	+++	--	90.1 ± 2.8	--	10	--	GCC	--	52 ± 2.6	--	318 ± 2.9	--
2.0+3.0	+++	--	87.5 ± 2.5	--	10	--	GCC	--	34 ± 2.7	--	206 ± 3.1	--
2.0+3.5	+++	--	89.5 ± 2.6	--	10	--	GCC	--	52 ± 1.5	--	319 ± 3.7	--
2.0+4.0	++	--	83.1 ± 2.1	--	10	--	GCC	--	53 ± 1.6	--	314 ± 3.2	--

GCC - Green compact callus; GFC - Green friable callus; CFC - Creamy friable callus; CNC - Creamy nodular callus; GNC - Green nodular callus; IBA - Indole-3-butyric acid; IAA - Indole-3-acetic acid; NAA - α-naphthalene acetic acid; 24D - 2,4-dichlorophenoxyacetic acid; BAP - Benzylaminopurine; KN - Kinetin; + - Meager (0.5-1.0 cm); ++ - Moderate (1.0-1.5 cm); +++ - Intense (>1.5 cm). Values are mean ± SE of 10 replicates per treatment and repeated thrice.

Table 4.1, continued

Hormones (mg/L)	Intensity of callus		Percentage of callus formation (%)		Days of callus formation		Nature of callus		Callus dry weight (mg)		Callus fresh weight (mg)	
	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf
2,4-D+BAP												
1.0+0.5	--	++	--	76.5 ± 2.6	--	14	--	GCC	--	46 ± 3.7	--	301 ± 1.6
1.0+1.0	--	++	--	71.2 ± 3.4	--	14	--	GCC	--	56 ± 3.2	--	285 ± 2.3
1.0+1.5	--	++	--	79.6 ± 2.8	--	14	--	GCC	--	42 ± 2.5	--	261 ± 1.2
1.0+2.0	--	++	--	81.2 ± 3.6	--	14	--	GCC	--	59 ± 1.9	--	231 ± 2.1
1.0+2.5	--	++	--	86.3 ± 3.9	--	14	--	GCC	--	38 ± 3.1	--	244 ± 1.5
1.0+3.0	--	+++	--	84.6 ± 3.1	--	14	--	GCC	--	58 ± 2.9	--	321 ± 1.8
1.0+3.5	--	+++	--	**87.3 ± 3.2**	--	14	--	GCC	--	63 ± 1.6	--	365 ± 0.7
1.0+4.0	--	+++	--	83.1 ± 2.6	--	14	--	GCC	--	58 ± 1.4	--	335 ± 1.9
2,4-D+KN												
1.0+0.5	--	++	--	77.1 ± 2.2	--	14	--	GCC	--	27 ± 1.6	--	178 ± 1.7
1.0+1.0	--	++	--	72.1 ± 2.5	--	14	--	GCC	--	25 ± 3.6	--	162 ± 3.5
1.0+1.5	--	++	--	79.4 ± 2.7	--	14	--	GCC	--	22 ± 1.5	--	153 ± 1.8
1.0+2.0	--	++	--	67.9 ± 2.3	--	14	--	GCC	--	26 ± 3.2	--	174 ± 3.1
1.0+2.5	--	++	--	71.0 ± 2.6	--	14	--	GCC	--	31 ± 2.7	--	217 ± 2.7
1.0+3.0	--	++	--	73.6 ± 3.7	--	14	--	GCC	--	32 ± 2.4	--	218 ± 2.3
1.0+3.5	--	++	--	78.8 ± 2.9	--	14	--	GCC	--	28 ± 2.6	--	174 ± 2.9
1.0+4.0	--	++	--	76.4 ± 1.8	--	14	--	GCC	--	29 ± 2.9	--	203 ± 1.6

GCC - Green compact callus; GFC - Green friable callus; CFC - Creamy friable callus; CNC - Creamy nodular callus; GNC - Green nodular callus; IBA - Indole-3-butyric acid; IAA - Indole-3-acetic acid; NAA - α-naphthalene acetic acid; 24D - 2,4-dichlorophenoxyacetic acid; BAP - Benzylaminopurine; KN - Kinetin; + - Meager (0.5-1.0 cm); ++ - Moderate (1.0-1.5 cm); +++ - Intense (>1.5 cm). Values are mean ± SE of 10 replicates per treatment and repeated thrice.

4.2 Optimization of callus induction under abiotic stress conditions

4.2.1 Manipulation of incubation temperature

In this experiment, both of the leave and stem explants were maintained at four different temperatures; which were 20, 25 (control), 30 and 35 ºC. Some of the species including *Corchorus olitorius* (Ali and Jones, 2000) were able to produce more callus when incubated at higher temperature (35 ºC). Therefore, 35 ºC was also being tested in this experiment to see whether explants from *W. biflora* can produce the same results. However, the results turn out that both stem and leaf explants of *W. biflora* could not withstand temperature of 35 ºC and turning black (died) after second week of culture (Fig. 4.3).

At 30 ºC, callus formation was observed in both explants after second week of culture. At 30 ºC, the intensity of callus production was quite higher; which was 1.2 cm (but lower than callus intensity at 25 ºC; which was 1.6 cm) and the callus was covering the whole explants. However, the colour of callus was brown indicating the accumulation of phenolic compound and initiated the death of callus. Much lower temperature; which was 20 ºC reduced the callus production rate and turned the callus into yellow colour (Table 4.2). There was only little callus observed at this temperature (0.6 cm).

On the other hand, incubation of stem and leaf explants at 25 ºC gave the optimum results. The callus proliferated into green and compact in nature. The production of callus from both explants at this temperature (25 ºC) was begun from the edge of the explants and finally covered the whole explants. Most of the callus incubated at this temperature was sized more than 1.5 cm.

4.2.2 Different light colours for cultures incubation

Different light colours emitted different wavelengths to be absorbed by plants phytochrome. Exposure to certain light colours will stimulate the production of secondary metabolites in plants. Four types of light colours were used in this study; which were green, blue, red and white (control).

In both explants, normal light colour (white light) resulted in the optimum callus production with green and compact in nature. The callus was observed from the cut ends of the explants and eventually surrounded the perimeter of the explants. The size of the callus incubated under white light was 1.1 and 1.3 cm for leaf and stem explants, respectively.

Exposure to other light colours (blue, red and green) did not improve the formation of callus. In fact, the production of callus was reduced when incubated under blue, red or green light with size less than 0.5 cm, averagely. Furthermore, the callus produced was yellow in colour, which indicated the presence of phenolic compound (Fig. 4.4).

4.2.3 Sucrose concentration in MS medium

Sucrose is the source of carbon (energy) needed by the explants to grow in a particular growth medium. Scarcity in carbon source will cause the explants to grow slower or even no growth at all, while too much amount of carbon source will become toxic to the explants. This study used MS medium fortified with sucrose with 1%, 2%, 3% (control), 4% and 5% of concentration. In this experiment, addition of extra sucrose was expected to produce more callus than application of normal sucrose concentration (3%).

For stem explants, the higher the concentration of sucrose stimulated more callus formation with optimum production at 4% of sucrose concentration (Table 4.2). The callus formed from stem explants inoculated with 4% of sucrose was green in colour, compacted and sized more than 1.0 cm (Fig. 4.5). Application of 5% of sucrose concentration was also stimulating the production of intense callus from stem explants. However, the browning effect was observed after 21 days of culture which suggested the necrosis of the callus cells. Therefore, application of 4% sucrose concentration on MS media concluded as the optimum condition for callus production from stem explants.

In contrast with stem explants, the intensity of callus formation from leaf explants was very low with either decreased or increased concentration of sucrose. The formation of callus from leaf explants showed higher intensity in normal sucrose concentration; which is 3% of sucrose (Table 4.2). Inoculation of leaf explants in other concentrations of sucrose (1%, 2%, 4% and 5%) seems to slow the production of callus. The formation of callus was only observed at the edge of the explants; while the middle part of the explants was eventually turned to black in colour and died (Fig. 4.5). Therefore, it was concluded that addition or reduction of sucrose from normal concentration (3%) was not necessary for optimum callus production leaf explants.

4.2.4 The effects of photoperiod on callus induction

Photoperiod is the duration of light being exposed to the explants to support the growth. In this experiment, normal light colour (white light) was being used. For stem explants, the longer the duration of light exposure, the slower the callus induction; where the callus was smaller in size and lesser in biomass (Table 4.3). Total darkness (0 h photoperiod) was the best to induce callus from stem explants; which resulted in

higher dry weight (91 mg) and more than 1.0 cm of callus. However, the callus produced was yellow to brown in colour.

As for leaf explants, the callus induction was increasing as the duration of light exposure increased until 8 h, and gradually decreased after 12h (Fig. 4.6). Therefore, maximum photoperiod for leaf explants was 8 h; which resulted in bigger callus (more than 1.0 cm) and highest biomass (78 mg dry weight).Despite providing bigger callus and highest biomass, incubation under 8 h photoperiod resulted in yellow to brown colour callus. In fact, all explants incubated under all tested photoperiods; except for 16 h photoperiod, resulted in yellow to brown colour callus. In order to obtain green colour callus, it was suggested to incubate the explants under normal photoperiod (16 h).

Table 4.2: Dry weight and fresh weight of stem and leaf callus incubated under different temperature and sucrose concentration after 35 days.

Abiotic stress treatment		Colour of callus		Callus dry weight (mg)		Callus fresh weight (mg)	
		Stem	Leaf	Stem	Leaf	Stem	Leaf
Temp. (°C)	20	yellow	yellow	22±2.6	49±2.3	135±4.6	365±5.6
	25	green	green	55±3.3	68±3.1	363±5.5	550±6.8
	30	brown	brown	34±3.5	81±3.7	246±4.9	616±4.1
	35	black	black	die	die	die	die
Sucrose conc. (%)	1	green	brown	37±4.1	24±1.9	474±6.2	84±2.8
	2	green	yellow	55±3.8	26±2.4	433±7.3	124±7.7
	3	green	green	56±2.6	63±3.6	363±7.1	550±5.9
	4	green	brown	196±5.3	31±1.7	1186±10.3	152±6.4
	5	green	brown	161±4.9	22±2.1	1048±9.8	83±3.2

* Values are mean ± SE of 10 replicates per treatment and repeated thrice.

Table 4.3: Dry weight and fresh weight of stem and leaf callus incubated under different light colours and photoperiod after 35 days.

Abiotic stress treatment		Colour of callus		Callus dry weight (mg)		Callus fresh weight (mg)	
		Stem	Leaf	Stem	Leaf	Stem	Leaf
Colour light	Blue	yellow	yellow	27±2.1	63±3.3	165±4.3	495±8.7
	Red	yellow	yellow	24±3.2	40±2.9	143±3.6	198±7.6
	Green	yellow	yellow	17±2.9	58±3.0	124±2.5	423±5.4
	White	green	green	55±3.2	63±4.1	363±4.8	550±9.9
Photo Period (hour)	0	brown	yellow	91±4.1	68±2.7	673±6.6	587±7.5
	4	brown	brown	78±3.6	72±3.6	604±5.4	680±8.2
	8	brown	brown	70±2.7	78±3.1	563±5.9	738±6.6
	12	brown	yellow	42±1.7	68±4.2	324±3.1	537±8.5
	16	green	green	55±2.8	63±2.8	363±2.9	550±7.5
	20	brown	brown	47±1.4	60±2.3	334±3.9	452±5.4
	24	brown	brown	50±2.2	72±2.6	276±2.8	652±9.1

* Values are mean ± SE of 10 replicates per treatment and repeated thrice.

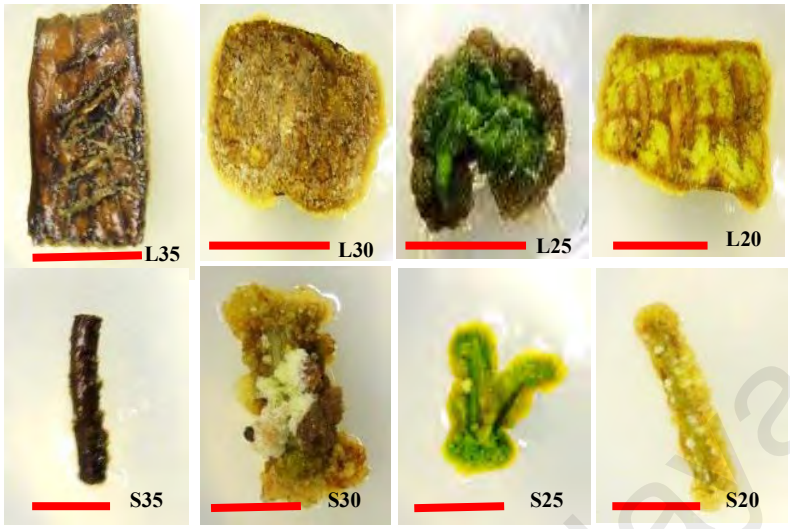


Figure 4.3: Callus growth from stem and leaf explants incubated in different temperature levels after 45 days. Above (L): Leaf callus; Below (S): Stem callus and the numbers represented the temperature levels in degree celcius, eg. 35 = 35°C. Bars represent 0.5 cm.

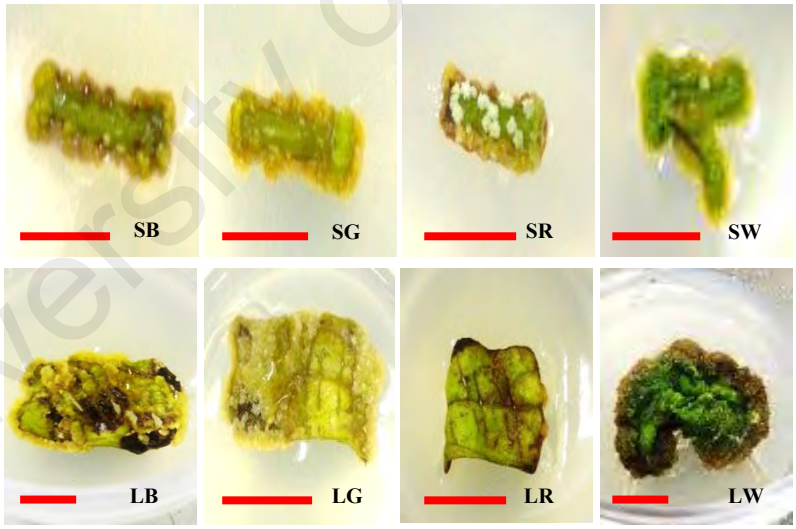


Figure 4.4: Condition of leaf and stem callus after 45 days incubation in different light colours. LB) Leaf callus in blue light; LG) Leaf callus in green light; LR) Leaf callus in red light; LW) Leaf callus in white light; SB) Stem callus in blue light; SG) Stem callus in green light; SR) Stem callus in red light; and SW) Stem callus in white light. Bars represent 0.5 cm.

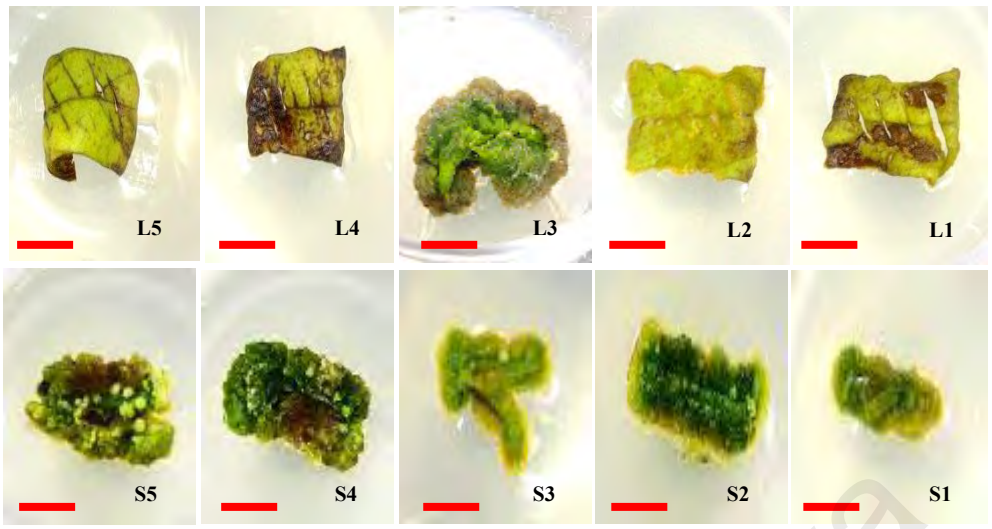


Figure 4.5: Formation of leaf and stem callus inoculated in MS medium fortified with PGRs and various concentration of sucrose after 45 days. L5-L1) Leaf callus inoculated in MS media with sucrose concentration from 5% to 1%; and S5-S1) Stem callus inoculated in MS media with sucrose concentration from 5% to 1%. Bars represent 0.5 cm.

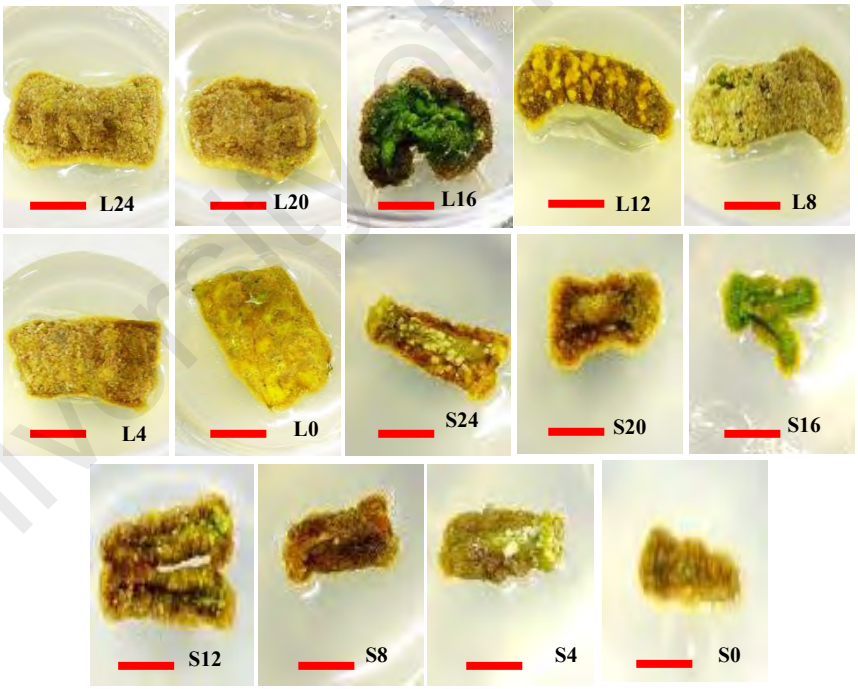


Figure 4.6: Growth of leaf and stem callus incubated in different photoperiod after 45 days. L represents leaf; S represents stem and the numbers represent the photoperiod exposed to the explants in hour. Bars represent 0.5 cm.

4.3 Growth curve and biomass analysis

Generally, the growth curve of callus biomass was in sigmoid type which consists of lag phase, exponential phase, stationary phase and decline phase (App. A-C). The lag phase was observed until 21 days in incubation followed by the exponential phase from day 21 until day 35. There was only brief period of stationary phase followed by decline phase occurred simultaneously until day 42 onwards. Therefore, the callus biomass reached the peak at day 35.

The production of stigmasterol was directly proportional to the growth of callus. Little amount of stigmasterol (less than 0.03 mg/g in both explants) detected during the lag phase (day 14) in both stem and leaf callus. Its production began to increase at the beginning of exponential phase (day 21) and was optimum (more than 4 mg/L in both explants) during the late exponential phase (day 28). The production of stigmasterol gradually decreased during the stationary phase (day 35) and remained low during the decline phase onwards (Fig. 4.7). Based on the results obtained, callus that were 28-day old, which was at its exponential phase was chosen for subsequent experiments because the cells at this age were healthy, rapidly dividing and produced higher stigmasterol content compared to callus of other ages.

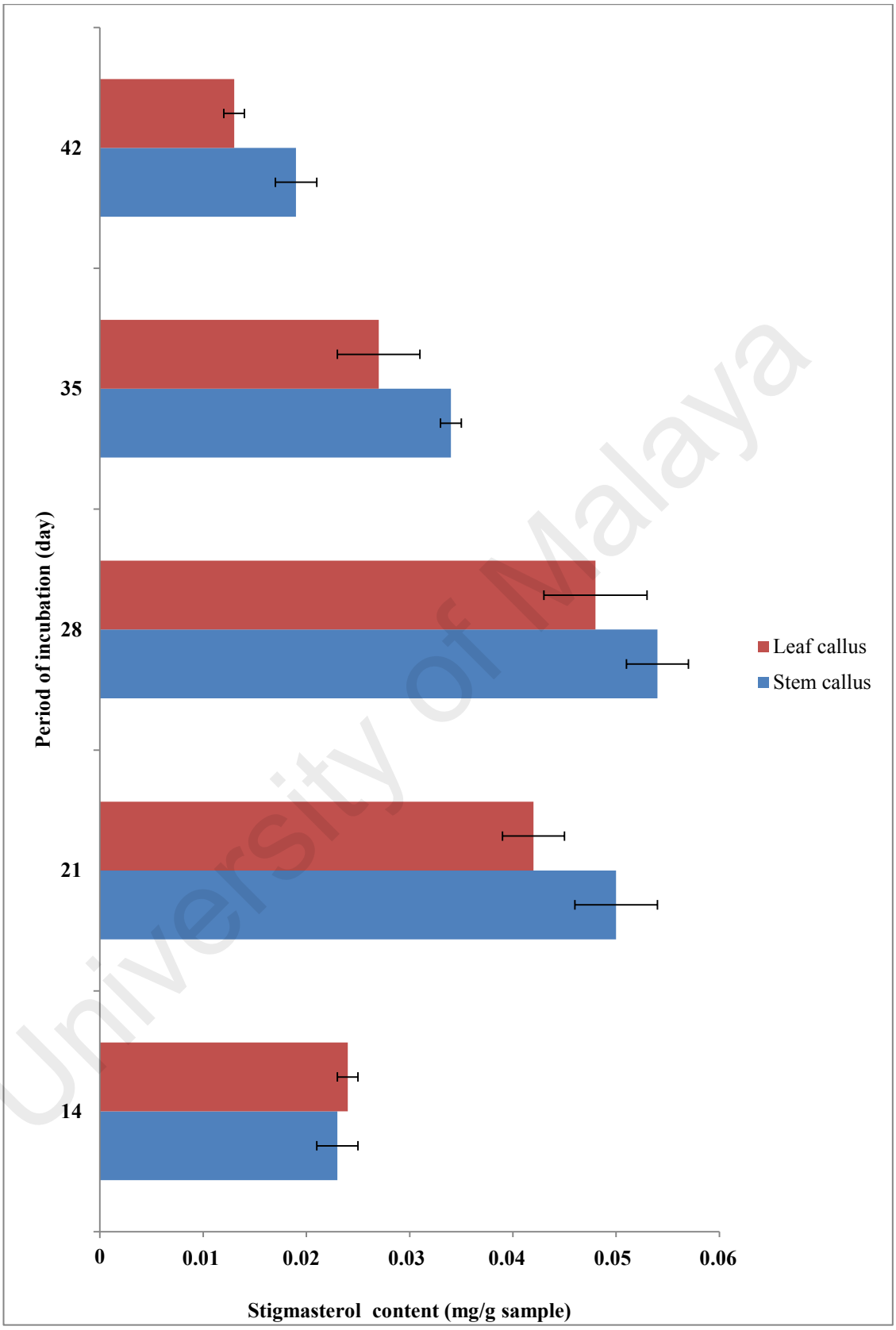


Figure 4.7: Estimation of stigmasterol content at different period of incubations for stem and leaf callus.

4.4 Indirect regeneration of *W. biflora*

4.4.1 The effects of different concentrations of cytokinins on multiple shoots induction from callus of *W. biflora*

After two months of callus induction, the healthy and green colour callus originated from stem explants were separated carefully from the explants and further incubated in MS media fortified with combinations of 2.0 mg/L NAA and 2.5 mg/L BAP for one month to mass produce and ensure the viability of the callus. Consequently, the callus were transferred into MS medium supplemented with various concentrations of BAP (1.0 – 4.0 mg/L) for shoot induction.

Table 4.4 and Fig. 4.8 show higher concentrations of BAP resulted in the higher shoot formation frequency per callus and length of shoots per callus. This experiment showed 3.0 mg/L BAP resulted in 90% shoots formation frequency with 40.5 shoots per callus and 7.8 cm length of shoots. The second best results were obtained from application of 4.0 mg/L BAP with 70% shoots formation, 27.3 shoots per callus and 5.7 cm shoot length; followed by application of 2.0 mg/L BAP and 1.0 mg/L. The multiple shoots from callus were observed after one month of incubation with tiny leaves (Fig. 4.9C).

4.4.2 The effects of different concentrations of IBA on roots induction

One month old shoots on MS media supplemented with 3.0 mg/L BAP were carefully separated and transferred onto MS medium devoid of any hormones to promote shoot elongation. The individual shoots were allowed to elongate for about 5.0 to 8.0 cm in length (Fig. 4.8) before they were transferred into rooting medium which contained various concentrations of IBA (1.0 – 4.0 mg/L). The incubated developed roots after 2 weeks in culture.

Table 4.4: Multiple shoots formation on callus inoculated in different concentrations of BAP.

BAP conc. (mg/L)	Shoot formation frequency (%)	Mean shoots per callus
1.0	20	11.2 ± 3.14^{d}
2.0	60	22.8 ± 5.66^{c}
3.0	90	40.5 ± 9.87^{a}
4.0	70	27.3 ± 4.31^{b}

Values represent mean±SE of 20 replicates treatment and repeated thrice. The different letters within the column indicated that the values are significantly different at p=0.05.

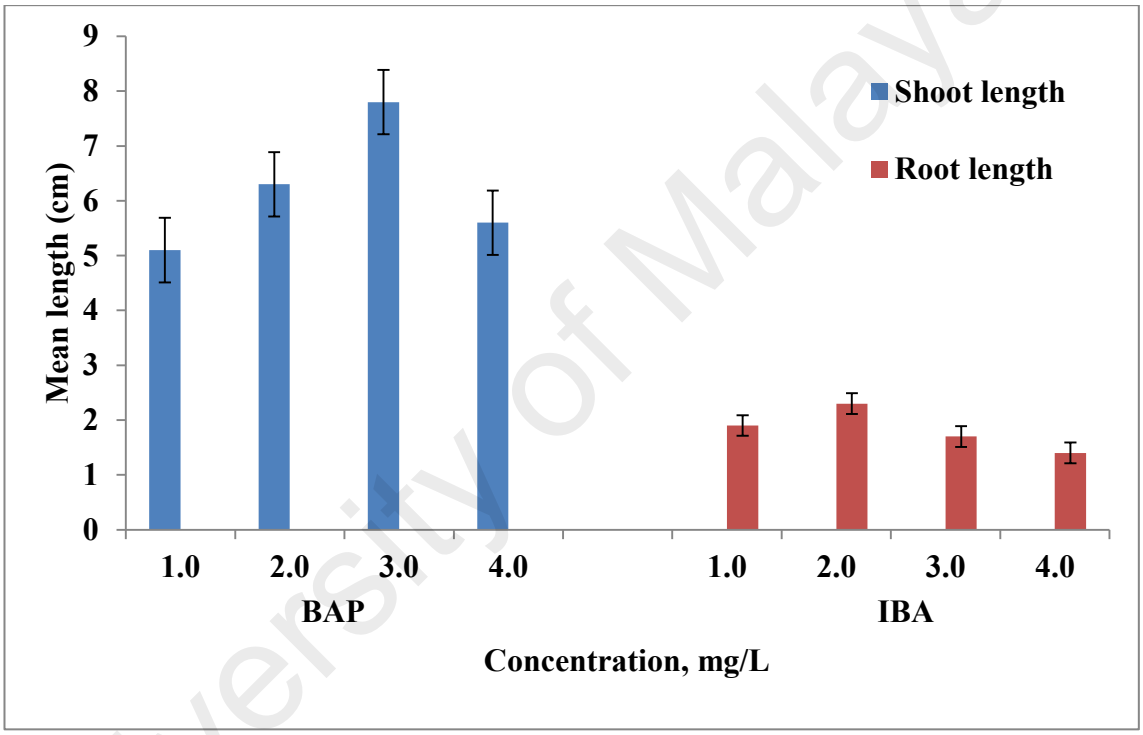


Figure 4.8: Mean length of shoots and roots after elongation.

Almost all concentrations of IBA gave positive rooting response; with 90 to 100% of rooting response (Table 4.5). Application of 2.0 mg/L and 3.0 mg/L IBA resulted in 100% of rooting response. However, shoots incubated in 2.0 mg/L IBA produced more roots (13.4 roots per shoot) as compared to application of 3.0 mg/L IBA (12.7 roots per shoot).Application of 1.0 mg/L and 4.0 mg/L IBA provided 90% rooting responses with 10.1 and 12.9 roots per shoot, respectively.

Table 4.5: Formation of roots on single shoot cultured in MS medium supplemented with various concentration of IBA.

IBA conc. mg/L	Root formation frequency (%)	Mean roots per shoot
1.0	90	10.1±2.21[d]
2.0	100	13.4±3.87[a]
3.0	100	12.7±1.66[c]
4.0	90	12.9±3.12[b]

Values represent mean±SE of 20 replicates treatment repeated thrice. The different letters within the column indicated that the values are significantly different at p=0.05.

The rooting process was taking place until one month to allow the roots to elongate further and matured enough for nutrient uptake. Fig. 4.8 showed the shoots inoculated on MS media supplemented with 2.0 mg/L IBA produced the longest root (2.7 cm) followed by 1.0 mg/L IBA (2.2 cm), 3.0 mg/L IBA (1.9 cm) and 4.0 mg/L IBA (1.6 cm).

4.4.3 Acclimatization of complete regenerated plantlets

The regenerated plantlets (after one month of rooting process) were allowed to incubate further (another one month) to promote roots elongation and preparing the plantlets for acclimatization (Fig. 4.9). The incubation took almost three months to generate whole plantlets that were ready for acclimatization.

For acclimatization purpose, the plantlets were washed thoroughly to remove excess agar and transferred into pots filled with black soil. The acclimatization of the plantlets was for about 30 days in black soil and 100% of the plantlets survived the acclimatization process. During acclimatization process, the leaves developed bigger in size and the number of leaves were also increased (Fig. 4.9E). After the plantlets become more rigid and healthy (after one month), the plantlets were then transferred to the greenhouse and allowed to grow normally.

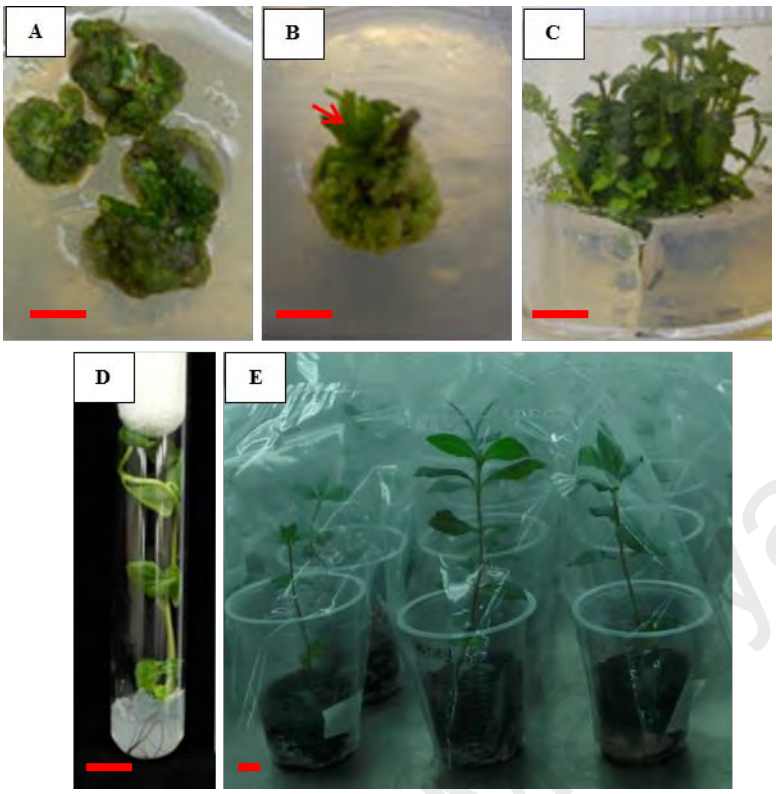


Figure 4.9: Indirect regeneration of *W. biflora*. A: Formation of green and compact callus from stem explants; B: Formation of shoot from callus; C: Formation of multiple shoots from callus after further incubation of callus; D: Rooting and elongation of regenerated shoot; D: Acclimatization of regenerated plantlets. Arrow showed the formation of shoot. Bars represent 0.5 cm.

4.5 Direct regeneration of *Wedelia biflora* from nodal segment

4.5.1 The effects of cytokinins and auxins on multiple shoots induction

In this experiment, cytokinins (BAP & KN; ranged from 0.5 – 4.0 mg/L) were used singly and in combination with auxins (NAA, IAA, & IBA). Nodal segment of *W. biflora* was chosen for starting material. Multiple shoots began to emerge after three weeks in culture from the nodes and cut ends of the explants.

For single application of cytokinins, application of BAP produced more multiple shoots as compared to application of KN. The highest multiple shoots obtained was 35.4 shoots per explant from MS medium supplemented with 1.0 mg/L BAP (Fig. 4.10). However, the number of shoots produced decreased as the concentration of BAP was increased. Application of single KN could only produce less shoots from 1.3 to 3.14 shoots per explant.

Being the best hormone to induce multiple shoots from nodal explant, 1.0 mg/L BAP was selected to be combined with auxins (NAA, IAA & IBA; ranged from 1.0 to 4.0 mg/L). However, these combinations did not result in more multiple shoots production. The highest multiple shoots produced was from combination of 1.0 mg/L BAP and 2.0 mg/L NAA, which was 6.0 shoots per explant (Fig. 4.11). This number was far less than multiple shoots produced from 1.0 mg/L BAP alone. Therefore, single application of BAP (particularly 1.0 mg/L) was the best method to induce multiple shoots from nodal explants of *W. biflora*.

4.5.2 The effects of different MS medium strength and IBA concentrations on root induction

After one month of shoot elongation, the shoots from MS medium supplemented with 1.0 mg/L BAP were transferred to other tubes with different MS medium strength and IBA concentrations as discussed below to study the rooting response.

Four different concentrations of MS medium which were used to examine production of callus were ¼, ½, ¾ and full strength (Table 4.6). The first two strength levels; ¼ and ½ showed better roots induction with 90% rooting response. However, ½ strength MS medium produced the highest number of roots per shoot (9.8 roots/shoot) and the highest length of roots per shoot (2.1 cm) as compared to ¼ strength MS medium. Higher strength levels; ¾ and full strength MS medium produced roots with some callus at the base of the nodal explants. Therefore, these two strength levels were rejected for roots induction as formation of basal callus could interfere with roots production.

Half strength MS medium was then supplemented with different concentration of IBA from 0.5 to 5.0 mg/L to induce more roots formation (Table 4.7). Almost all concentrations resulted in good rooting response with 90-100% rooting response. Application of 1.0 mg/L IBA accelerated the production of roots and resulted in 14.0 roots formation per shoots with average length of 1.67 cm. The production of roots was reduced as the concentration of IBA increased; with the lowest results was from application of 5.0 mg/L IBA (5.6 roots per shoot, 1.63 cm root length).

4.5.3 Acclimatization of the plantlets

After rooting process, the regenerated plantlets were further incubated for two weeks. The plantlets incubated with 1.0 mg/L IBA were chosen because of better root formation (as mentioned in section 4.5.2). Prior to acclimatization, the plantlets were carefully taken out of the tubes and washed thoroughly to remove the excess agar.

The plantlets were acclimatized in three different growth substrates, which were red soil, black soil and combinations of red soil and black soil in 1:1 ratio. The plantlets were able to survive in all types of growth substrates tested. The highest survival rate was obtained from plantlets grown in combination of red soil and black soil (96.67%), followed by black soil alone (86.67%) and red soil alone (73.33%) (Table 4.9).

The leaves from the plantlets grown in combination of black soil and red soil were bigger and healthier than those in either red soil or black soil alone. Plantlets grown in red soil cannot elongate further and the stems were weak (not rigid). Plantlets grown in black soil were able to elongate further but produced smaller leaves than plantlets grown in combination of red soil and black soil. The plantlets were acclimatized for two weeks before being exposed to natural environment (Fig. 4.12).

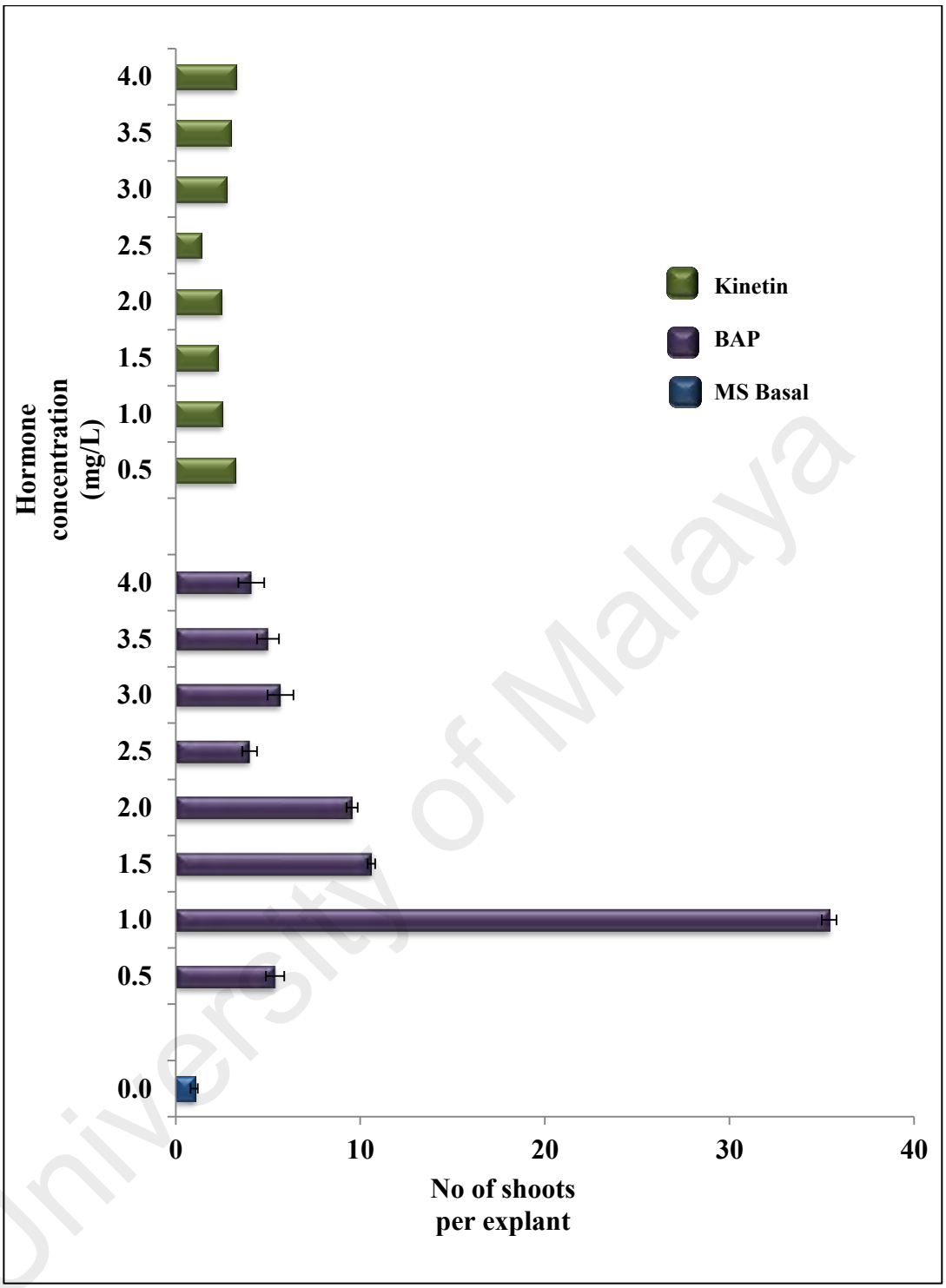


Figure 4.10: Production of multiple shoots from nodal explants inoculated in MS medium supplemented with BAP or KN alone. MS medium supplemented with 1.0 mg/L BAP resulted in more multiple shoots production (35.4 shoots/explant) as compared to other treatments.

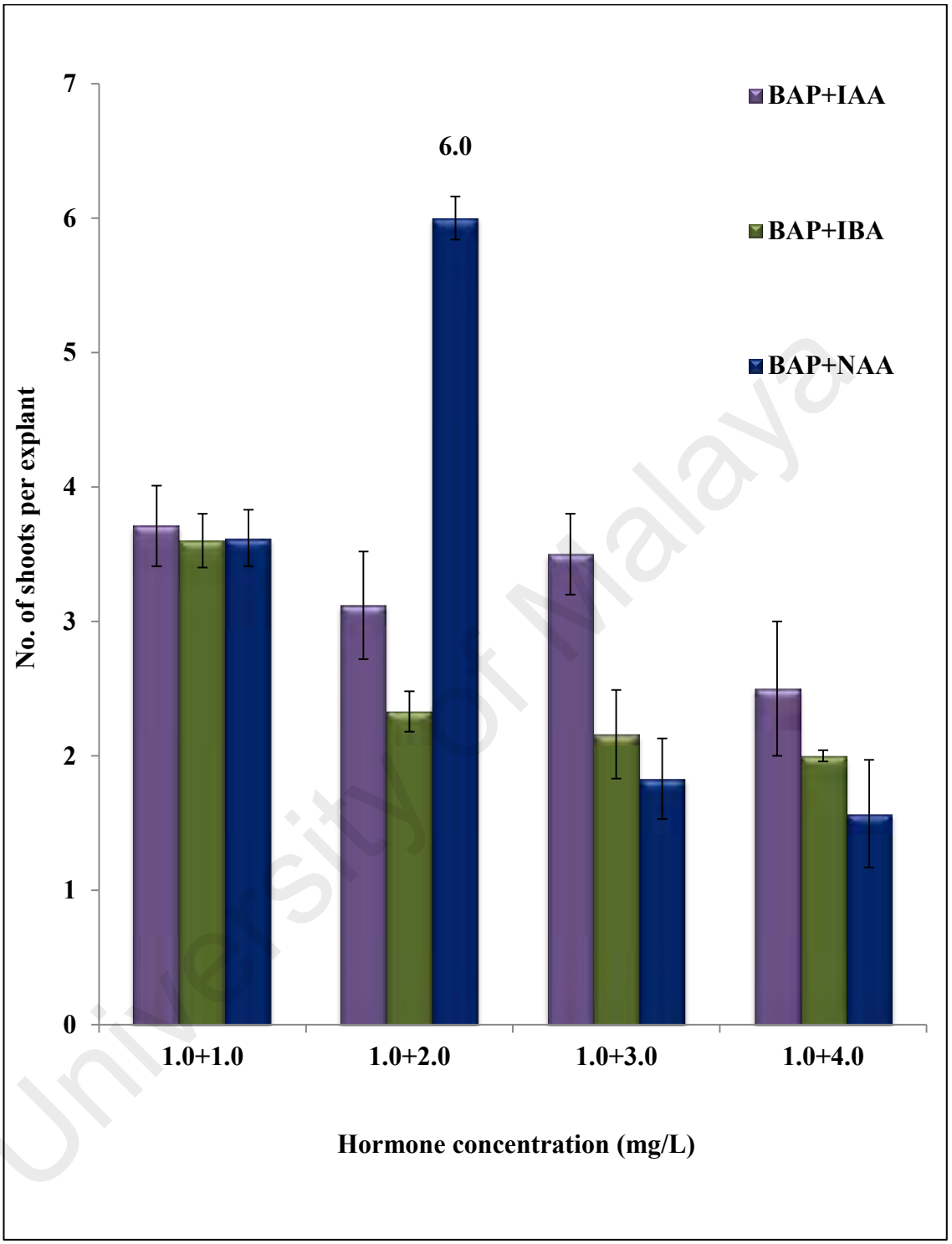


Figure 4.11: Application of cytokinins (BAP) and auxins in combinations. Combination of 1.0 mg/L BAP and 2.0 mg/L NAA yielded highest multiple shoots formations (6 shoots per explant) as compared to the other combinations; but less than application of BAP alone (35.4 shoots per explant).

Table 4.6: Effects of different MS medium strengths on root formation after two weeks of subculture.

MS medium strength	Rooting response (%)	No. of root per shoots (#)	Length of root (cm)	Observations
¼	90	7.3 ± 1.1^{c}	0.8 ± 0.3^{d}	Formation of roots
½	90	9.8 ± 2.1^{a}	2.1 ± 1.4^{a}	Formation of roots
¾	80	6.7 ± 0.8^{d}	1.2 ± 0.5^{b}	Formation of roots and basal callus
1	80	6.3 ± 1.3^{b}	1.1 ± 0.4^{c}	Formation of roots and basal callus

*Mean values with different letters within a column are significantly different at p = 0.05

Table 4.7: Effects of different concentrations of IBA on root formation.

Growth regulator (mg/L)	Percent. response (%)	No. of roots/shoot (#)	Length of root (cm)	Length of shoot (cm)	No. of leaves/shoot (#)
½ MS + IBA					
0.5	90	12.1 ± 1.52^{cd}	1.58 ± 0.11^{d}	6.98 ± 0.63^{ac}	10.5 ± 1.21^{ad}
1.0	100	14.0 ± 1.29^{a}	1.67 ± 0.16^{b}	7.17 ± 0.77^{a}	11.1 ± 1.18^{a}
1.5	100	13.0 ± 1.22^{ac}	1.41 ± 0.16^{ac}	6.22 ± 0.75^{d}	9.6 ± 1.36^{bc}
2.0	100	11.0 ± 1.87^{b}	1.37 ± 0.13^{c}	5.15 ± 0.61^{b}	7.4 ± 1.08^{cd}
2.5	100	11.2 ± 1.63^{bd}	1.52 ± 0.22^{ad}	3.65 ± 0.55^{ad}	8.8 ± 0.52^{ac}
3.0	90	10.0 ± 1.96^{ab}	1.82 ± 0.29^{a}	1.75 ± 0.30^{cd}	7.7 ± 0.81^{c}
3.5	90	9.6 ± 1.41^{ad}	1.63 ± 0.17^{bd}	4.11 ± 0.69^{bc}	7.1 ± 1.85^{d}
4.0	100	8.7 ± 1.61^{c}	1.36 ± 0.13^{cd}	4.92 ± 0.77^{ab}	7.7 ± 1.00^{c}
4.5	100	6.8 ± 1.33^{bc}	1.22 ± 0.26^{bc}	3.87 ± 0.78^{bd}	7.6 ± 1.44^{b}
5.0	100	5.6 ± 1.20^{d}	1.63 ± 0.31^{ab}	2.89 ± 0.60^{c}	8.6 ± 1.95^{b}

*Mean values with different letters within a column are significantly different at p = 0.05

Table 4.8: Survival rates of *in vitro* propagated plantlets on different types of growth substrates after one month being acclimatized.

Type of growth substrates	Survival rate (%)
Black soil	86.67 ± 6.31^{b}
Red soil	73.33 ± 8.21^{a}
Black + Red soil (1:1)	96.67 ± 3.33^{c}

*Mean values with different letters within a column are significantly different at p = 0.05

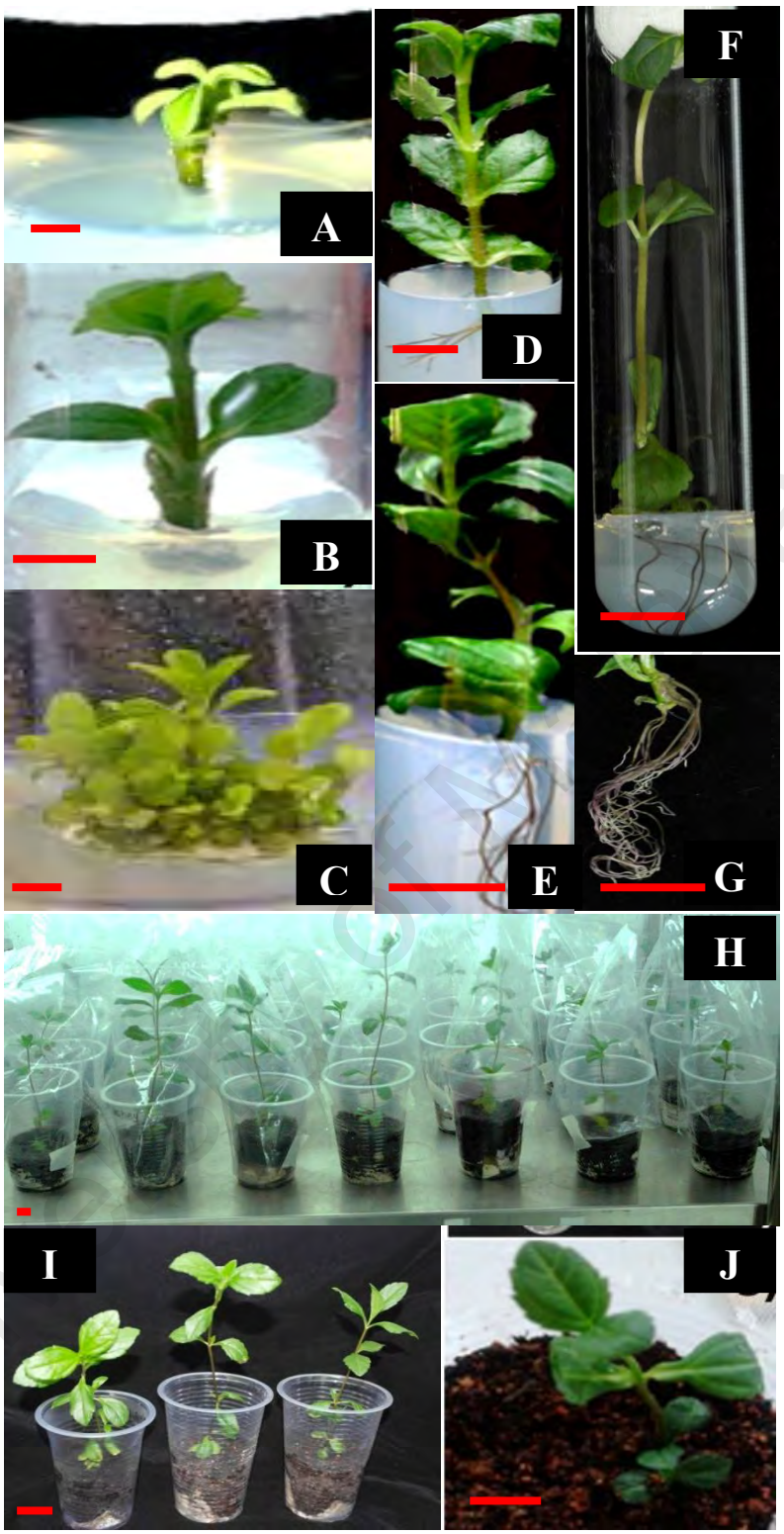


Figure 4.12: Overview of shoot regeneration, root formation and acclimatization of direct regeneration. A) Shoot initiation; B) Single shoot formation; C) Multiple shoot formation; D) Root initiation; E) Root elongation; F) Shoot elongation after rooting; G) *In vitro* root closed-up view; H) Hardening of *in vitro* plantlet in culture room; I) Acclimatized plant maintained in green house; and J) Acclimatized plant maintained in natural environment. Bars represent 0.5 cm.

4.6 Examination of bioactivities in methanolic extracts

4.6.1 Antimicrobial activity assay

4.6.1.1 Disc diffusion method

The antimicrobial activities of methanolic extracts of *in vivo* and *in vitro* leaves and stems as well as leaf and stem callus gave different zones of inhibition on the organisms tested. Zone of inhibition in Table 4.9 showed that stem callus treated with 4% sucrose and *in vivo* leaf as well as *in vivo* stem extracts gave potent antimicrobial activity, especially towards *B. subtilis* (13 mm, 12 mm and 11 mm, respectively) and *C. albicans* (16 mm, 14 mm and 13 mm, respectively). The rest of the callus extracts from stem and leaf with various treatments, as well as *in vitro* leaf and stem showed lowest zone of inhibition than *in vivo* parts of *W. biflora*.

These results indicate that *in vivo* parts were slightly more resistant towards microbes as compared to callus and *in vitro* extracts. However, extract from stem callus inoculated in 4% sucrose provided competitive results which suggested that production of callus from this plant can be used as an efficient alternative method for mass production of antimicrobial agent with proper stress stimulation (in this case was treatment with 4% sucrose). Almost all extracts showed lower antimicrobial activities against *E. coli* and *A. niger* (Table 4.9) which showed that the extracts were selectively resistant against certain microbes.

Table 4.9: Zone of inhibition (mm) of the extracts against tested microorganisms.

Extracts/microbes	***Bacillus subtilis***	***Escherichia coli***	***Aspergillus niger***	***Candida albicans***
Stem callus NAA+BAP*	10 ± 0.25^{b}	8 ± 0.14^{j}	9 ± 0.21^{d}	11 ± 0.11^{c}
Stem callus 25°C**	10 ± 0.25^{b}	8 ± 0.14^{j}	9 ± 0.21^{d}	11 ± 0.11^{c}
Stem callus white light**	10 ± 0.25^{b}	8 ± 0.14^{j}	9 ± 0.21^{d}	11 ± 0.11^{c}
Stem callus 4% sucrose	13 ± 0.29^{l}	11 ± 0.11^{c}	10 ± 0.32^{b}	16 ± 0.40^{i}
Stem callus 0h p/period	8 ± 0.22^{i}	10 ± 0.23^{d}	8 ± 0.12^{e}	9 ± 0.10^{h}
Leaf callus 2,4-D+BAP*	9 ± 0.26^{h}	9 ± 0.12^{h}	7 ± 0.16^{i}	8 ± 0.22^{j}
Leaf callus 30°C	8 ± 0.14^{j}	8 ± 0.20^{k}	7 ± 0.17^{j}	7 ± 0.18^{k}
Leaf callus white light**	9 ± 0.26^{f}	9 ± 0.12^{g}	7 ± 0.16^{i}	8 ± 0.22^{j}
Leaf callus 3% sucrose**	9 ± 0.26^{f}	9 ± 0.12^{g}	7 ± 0.16^{i}	8 ± 0.22^{j}
Leaf callus 8h p/period	8 ± 0.12^{k}	8 ± 0.15^{f}	8 ± 0.13^{h}	9 ± 0.21^{g}
In vivo stem	11 ± 0.21^{d}	9 ± 0.11^{i}	8 ± 0.15^{f}	13 ± 0.18^{d}
In vivo leaf	12 ± 0.31^{c}	10 ± 0.24^{d}	9±0.15c	14 ± 0.36^{a}
In vitro stem	10 ± 0.22^{e}	11 ± 0.16^{a}	8 ± 0.11^{g}	10 ± 0.13^{e}
In vitro leaf	9 ± 0.21^{g}	9 ± 0.14^{e}	7 ± 0.13^{k}	10 ± 0.21^{f}
Ampicillin	23 ± 1.21^{a}	15 ± 0.92^{b}	-	-
Fluconazole	-	-	21 ± 1.93^{a}	26 ± 1.33^{b}

Values represent mean±SE; including 6mm of disc diameter. Mean values with different letters within a column are significantly different at p = 0.05.

*Control treatments for respective stem and leaf callus.

**The treatments yielded the same results as "*" because those were the same treatments as controls for respective stem and leaf callus.

4.6.2 Antioxidant activity assays

The antioxidant capacity of the extracts was tested by using DPPH and H_2O_2 free radical scavenging as well as by using reducing power assay (RPA) and ferric reducing antioxidant power (FRAP) assay. Ascorbic acid was used as the positive control in this experiment. Overall, all of the extracts showed lower antioxidant ability as compared to ascorbic acid in all the four assays.

Nevertheless, extracts from stem callus treated with 4% sucrose showed competitive antioxidant activity as ascorbic acid. The IC_{50} values of the extract (stem callus incubated with 4% sucrose) from DPPH and H_2O_2 scavenging assays were 250 µg/mL and 272 µg/mL, respectively; as compared to ascorbic acid with 163 µg/mL and 235 µg/mL, respectively (Figs. 4.13-4.14; Tables 4.10-4.11). The extract was also shown the second highest absorbance (2.21) after ascorbic acid (294) at 700 nm for reducing power assay; which higher absorbance represented higher reducing power of the extracts (Fig. 4.15; Table 4.12). The same result was obtained when the extract was tested with ferrous sulphate for ferric reducing power of the extract; where extract from stem callus treated with 4% sucrose resulted in 720.82 mM Fe (II)/g dry mass and ascorbic acid with 1320.82 mM Fe (II)/g dry mass (Fig. 4.16; Table 4.13). The extracts from leaf parts; regardless of *in vivo*, *in vitro* grown or callus showed lower antioxidant activity as compared to stem parts.

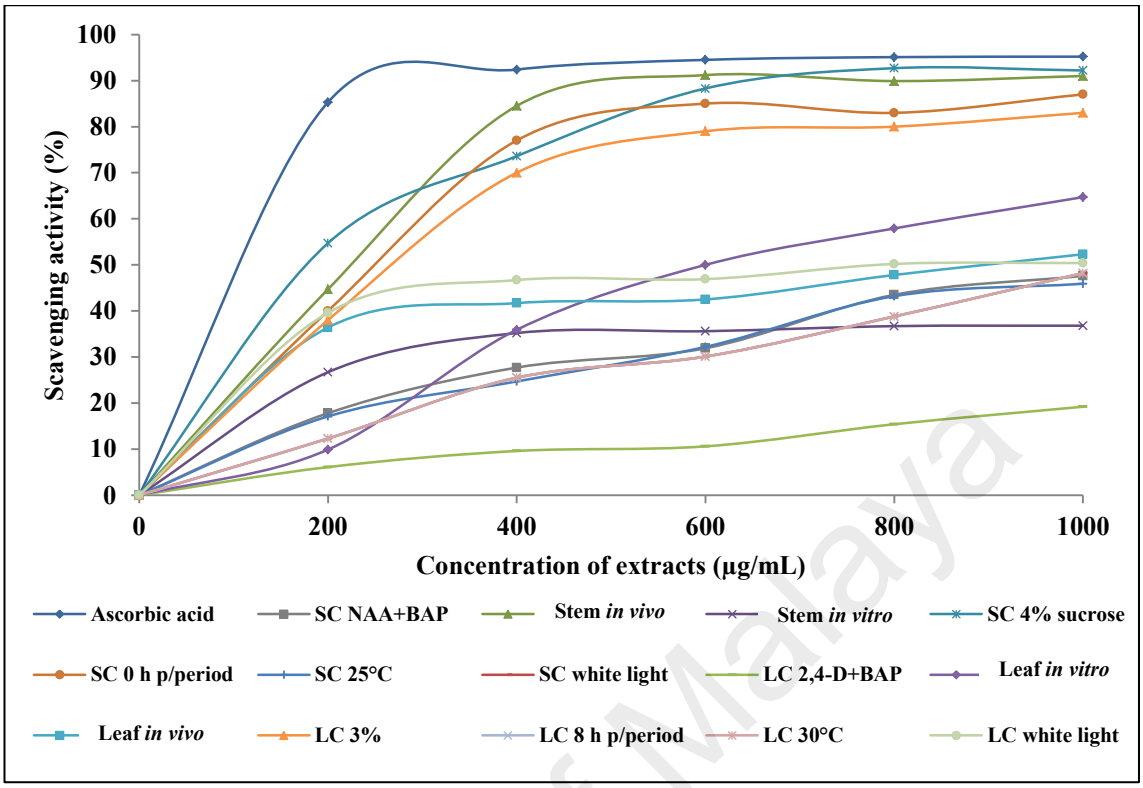


Figure 4.13: Scavenging activity (in percentage) of the extracts against DPPH radicals. SC represents Stem Callus; LC represents Leaf Callus.

Table 4.10: IC_{50} values of all the extracts tested against DPPH free radicals.

Extracts	**IC_{50} values (µg/mL)**
Ascorbic acid	163
SC 4% sucrose	250
Stem *in vivo*	251
SC 0 h p/period	277
Leaf *in vivo*	297
LC 2,4-D+BAP	449
LC white light	471
SC white light	486
LC 3% sucrose	506
SC NAA+BAP	590
SC 25°C	603
LC 8 h p/period	607
LC 30°C	611
Stem *in vitro*	700
Leaf *in vitro*	1446

*SC: Stem callus; LC: Leaf callus; IC_{50}: concentration needed to reduce the radicals activity by 50%.

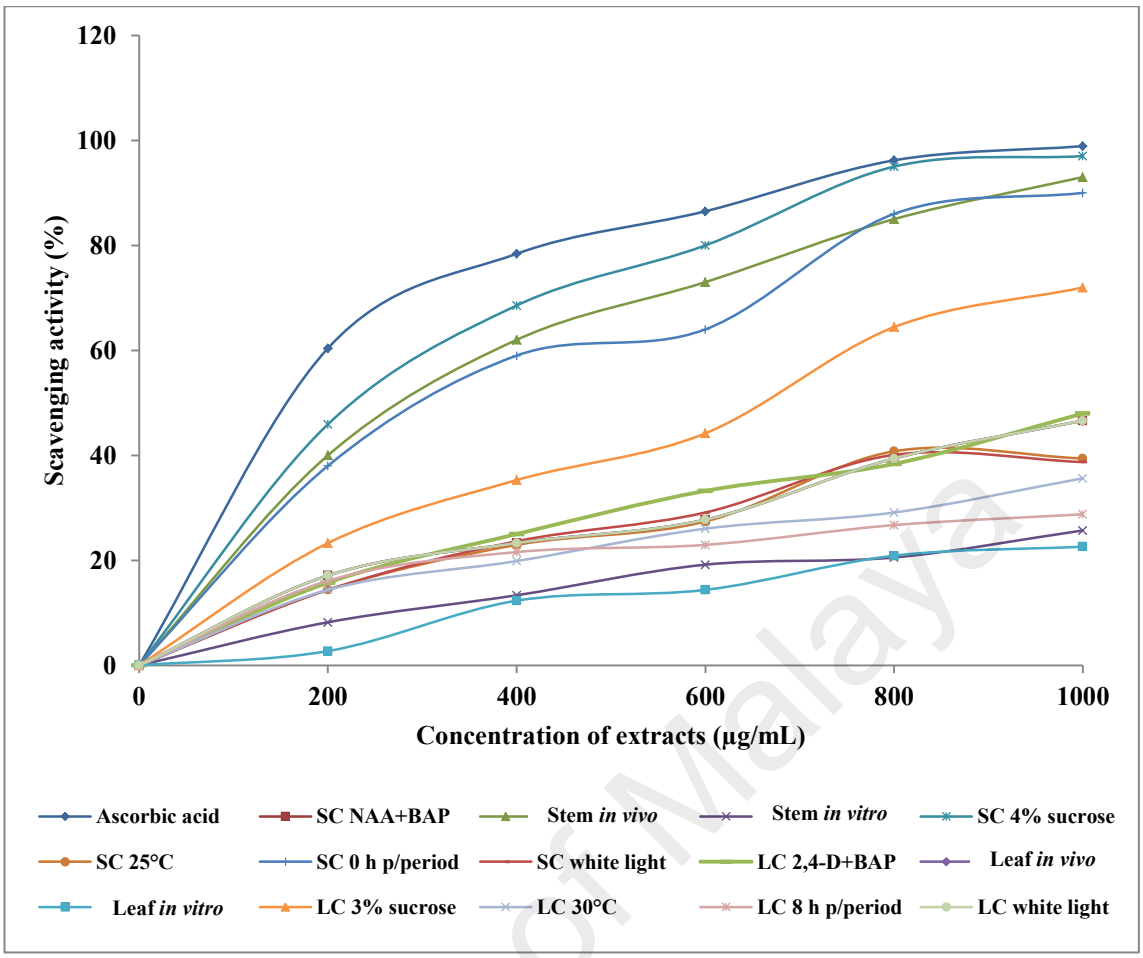


Figure 4.14: Scavenging activity (in percentage) of the extracts against H_2O_2 radicals. LC = Leaf callus; SC = Stem callus.

Table 4.11: IC_{50} values of ascorbic acid and the extracts tested against H_2O_2 radicals.

Extracts	IC_{50} values (µg/mL)
Ascorbic acid	235
SC 4% sucrose	272
Stem *in vivo*	299
SC 0 h p/period	314
LC 3% sucrose	422
LC 2,4-D+BAP	608
SC NAA+BAP	630
Leaf *in vivo*	301
LC white light	630
SC 25°C	672
SC white light	676
LC 30°C	797
LC 8 h p/period	956
Stem *in vitro*	1076
Leaf *in vitro*	1132

*SC: Stem callus; LC: Leaf callus; IC_{50}: concentration needed to reduce the radicals activity by 50%.

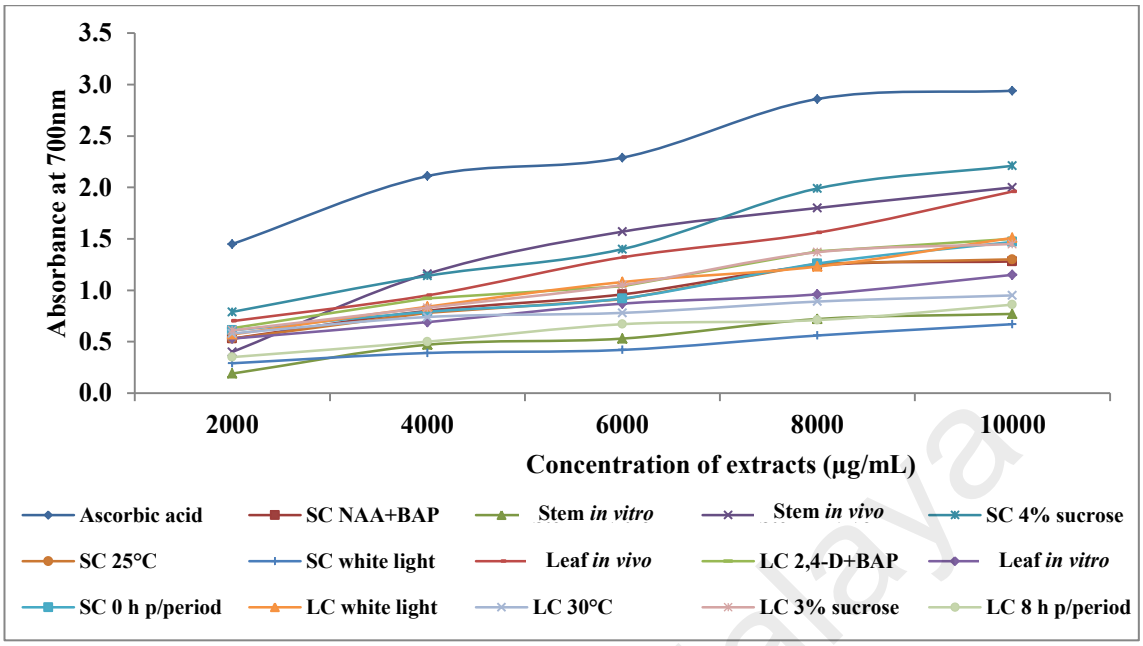


Figure 4.15: Absorbance of different concentration of extracts at 700 nm for reducing power assay. Higher absorbance indicated higher reducing power. LC = Leaf callus; SC = Stem callus.

Table 4.12: Absorbance of ascorbic acid and all the extracts to determine the antioxidant power.

Extracts	Absorbance at 700 nm
Ascorbic acid	2.94
SC 4% sucrose	2.21
Stem *in vivo*	2.00
SC 0 h p/period	1.47
LC 3% sucrose	1.45
LC 2,4-D+BAP	1.50
SC NAA+BAP	1.28
Leaf *in vivo*	1.96
LC white light	1.51
SC 25°C	1.30
SC white light	0.67
LC 30°C	0.95
LC 8 h p/period	0.86
Stem *in vitro*	0.77
Leaf *in vitro*	1.15

*SC: Stem callus; LC: Leaf callus.

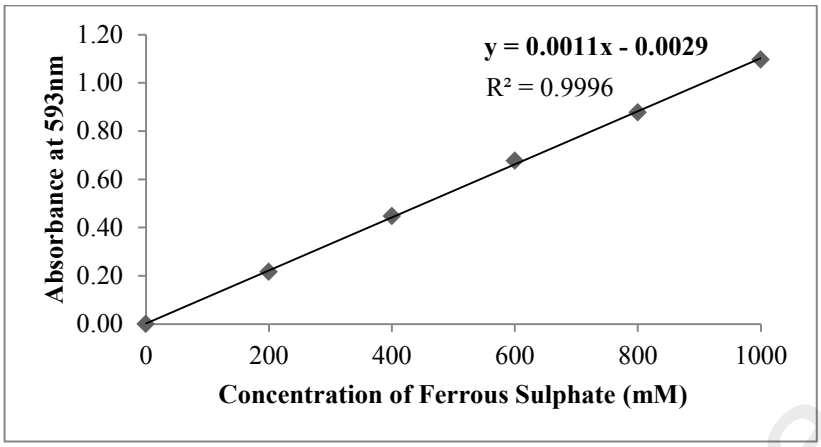


Figure 4.16: Standard curve of ferrous sulphate for evaluation of FRAP assay.

Table 4.13: Antioxidant power of the extracts expressed in mM Fe (II)/g dry mass sample.

Extracts	Reducing power (mM Fe (II)/g dry mass)
Ascorbic acid	1320.82
SC 4% sucrose	720.82
Stem *in vivo*	639.00
LC white light	575.36
Leaf callus	557.18
LC 3% sucrose	557.18
SC 0 h p/period	529.91
Leaf *in vivo*	520.82
Stem callus	484.45
SC 25°C	484.45
SC white light	484.45
LC 30°C	366.27
LC 8 h p/period	320.82
Stem *in vitro*	266.27
Leaf *in vitro*	175.36

*SC: Stem callus; and LC: Leaf callus.

4.7 Identification and estimation of stigmasterol in the extracts of *W. biflora* by using spectrophotometer method and HPLC method.

For callus culture, explants that exhibited intense callus formation and in shortest period were chosen to undergo estimation of stigmasterol content by spectrophotometer and HPLC methods. This is because intense and faster formation of callus is important in mass production of extracts valuable for medicinal purposes. Estimation of stigmasterol content by spectrophotometer method was essential to screen the presence of stigmasterol before the extracts were run in HPLC systems; which is more accurate and expensive procedure.

During analysis of stigmasterol content using spectrophotometer method, all of the extracts showed the presence of stigmasterol but in different concentrations. The extracts were analyzed simultaneously with known concentration of standard stigmasterol to confirm the detection of correct compound. Standard curve of known concentration of stigmasterol obeys the Beer Lambert's law in concentration ranged from 100 to 500 mg/L at 640 nm. The concentration and absorbance of the standard showed a good linearity with correlation coefficient (R^2) of 0.9607 (Fig. 4.17).

The contents of stigmasterol in all callus and explants were analyzed. *In vivo* plant parts showed higher stigmasterol content in both stem (0.078 mg/g) and leaf (0.066 mg/g) parts (Fig. 4.18). The amount of stigmasterol was increased when callus of *W. biflora* was treated by different abiotic stresses especially in different sucrose concentrations. The best sucrose concentration for stem callus was 4% which yielded 0.088 mg/g stigmasterol; whereas 3% sucrose was the optimum sucrose concentration for leaf callus to yield 0.052 mg/g stigmasterol (Figs. 4.19-4.20). Another treatment such as incubation temperature, light colours and photoperiod did not really improve the stigmasterol content. In case of incubation temperature, incubation in 25°C for stem

callus and 30°C for leaf callus gave the optimum stigmasterol content for respective callus (0.052 mg/g and 0.060 mg/g, Figs. 4.21-4.22). White colour light was the best to incubate both stem and leaf callus with the readings of 0.058 mg/g and 0.052 mg/g, respectively (Figs. 4.23-4.24. For stem callus, incubation under total darkness gave significant reading (0.073 mg/g); while leaf callus gave the highest reading (0.052 mg/g) when incubated under 8 h photoperiod condition (Figs. 4.25-4.26).

For each treatment, extracts from callus with greater stigmasterol readings were selected to undergo HPLC analysis. Standard curve of stigmasterol once again built from HPLC analysis and obtained good linearity with R^2 of 0.9878 (Fig. 4.27). The quantification of stigmasterol from each extract were shown in Figure 4.28 and compared with results obtained from spectrophotometer method. The amount of stigmasterol in each samples run through HPLC system showed a slight decrease than amount estimated using spectrophotometer method. Appedix D showed the chromatogram of the standard stigmasterol and all the selected extracts.

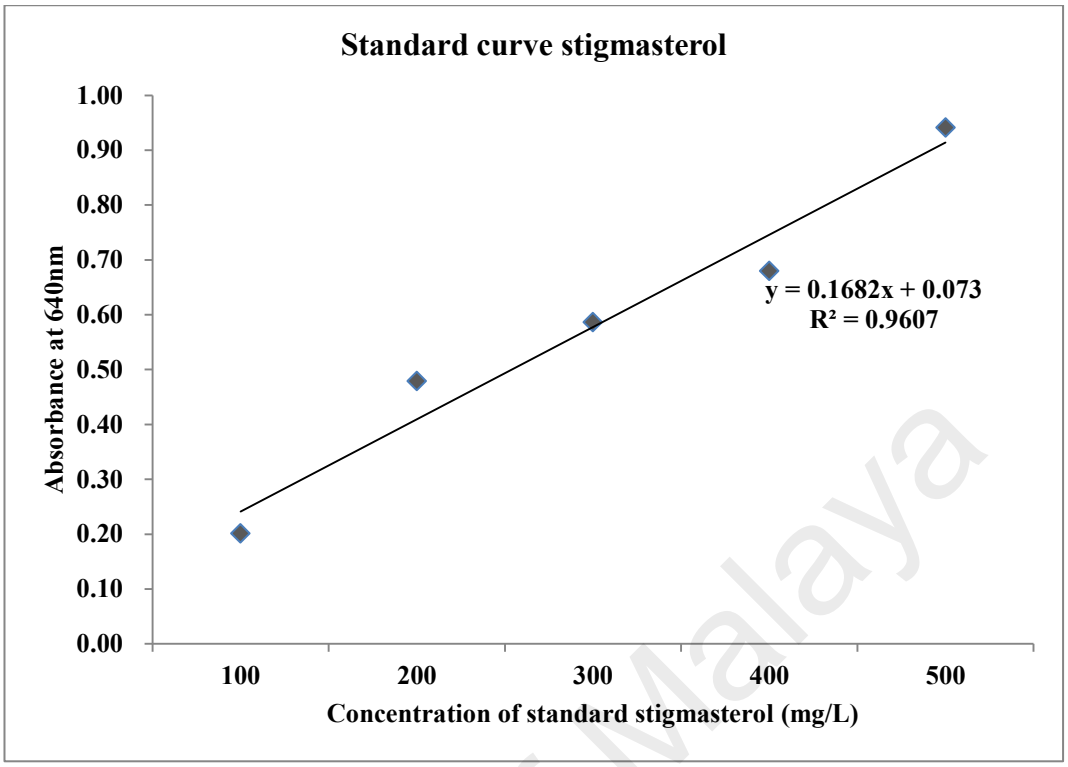


Figure 4.17: Standard curve of standard stigmasterol range from 100 to 500 mg/L (spectrophotometer method).

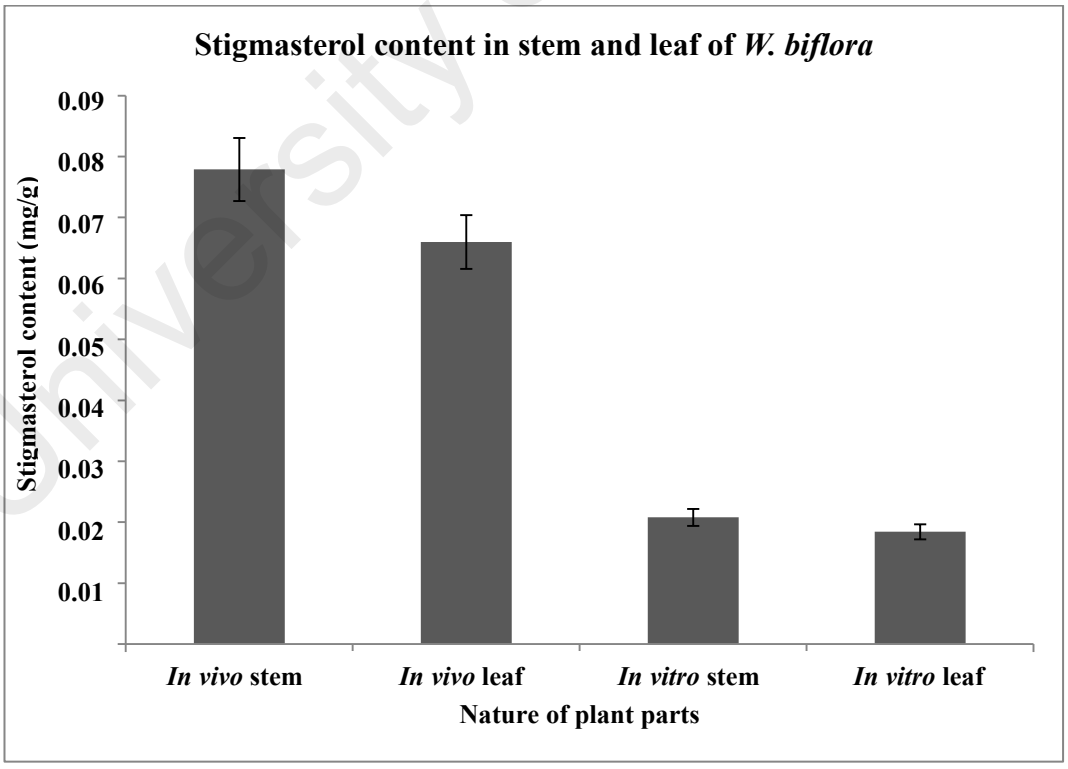


Figure 4.18: Estimation of stigmasterol content in *in vivo* and *in vitro* stem and leaf of *W. biflora* by spectrophotometer method.

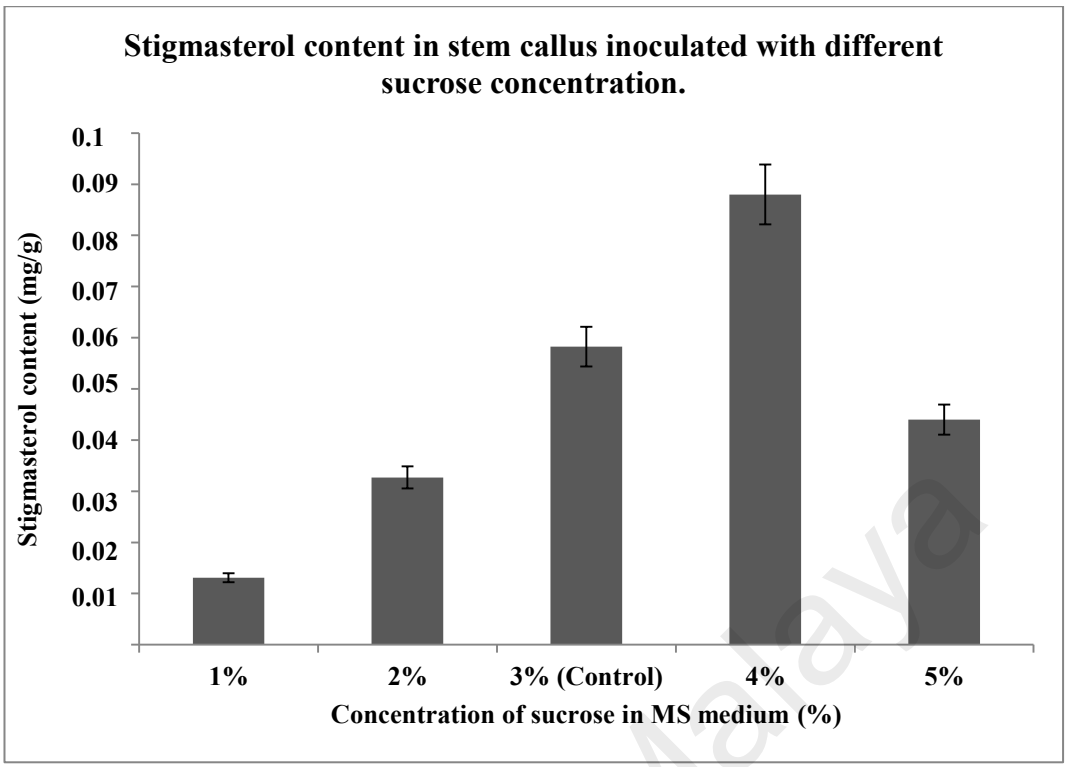


Figure 4.19: Estimation of stigmasterol content in stem callus of *W. biflora* treated in different sucrose concentration by spectrophotometer method.

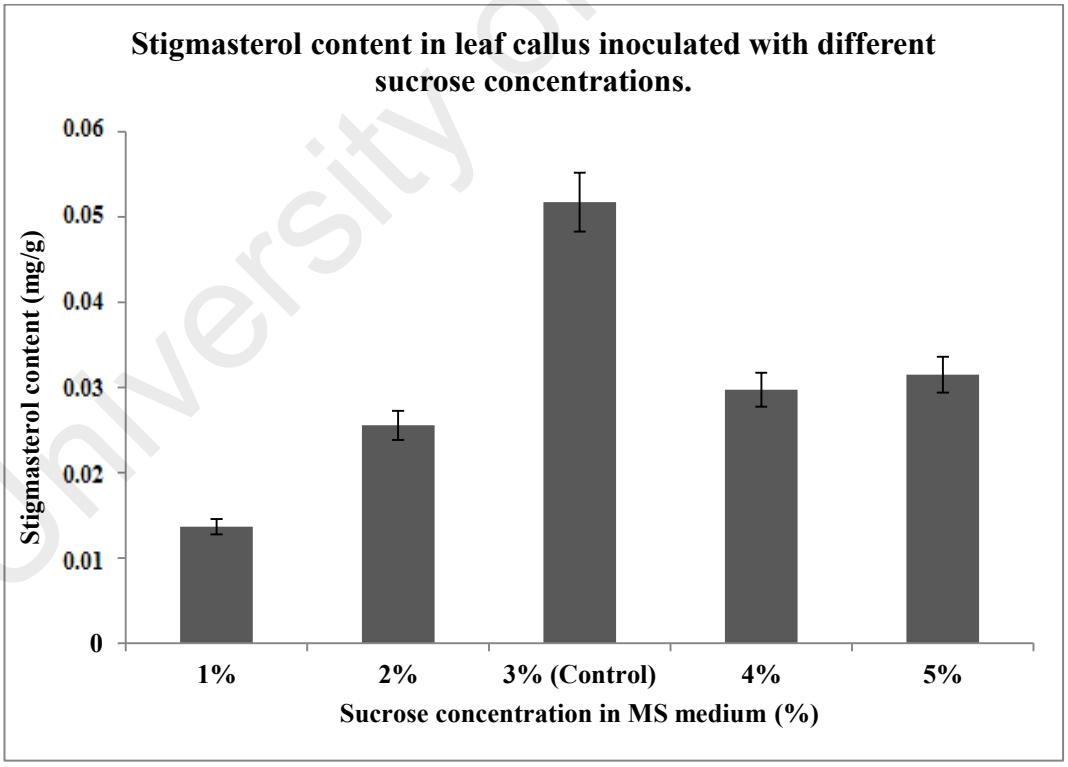


Figure 4.20: Estimation of stigmasterol content in leaf callus of *W. biflora* treated in different sucrose concentration by spectrophotometer method.

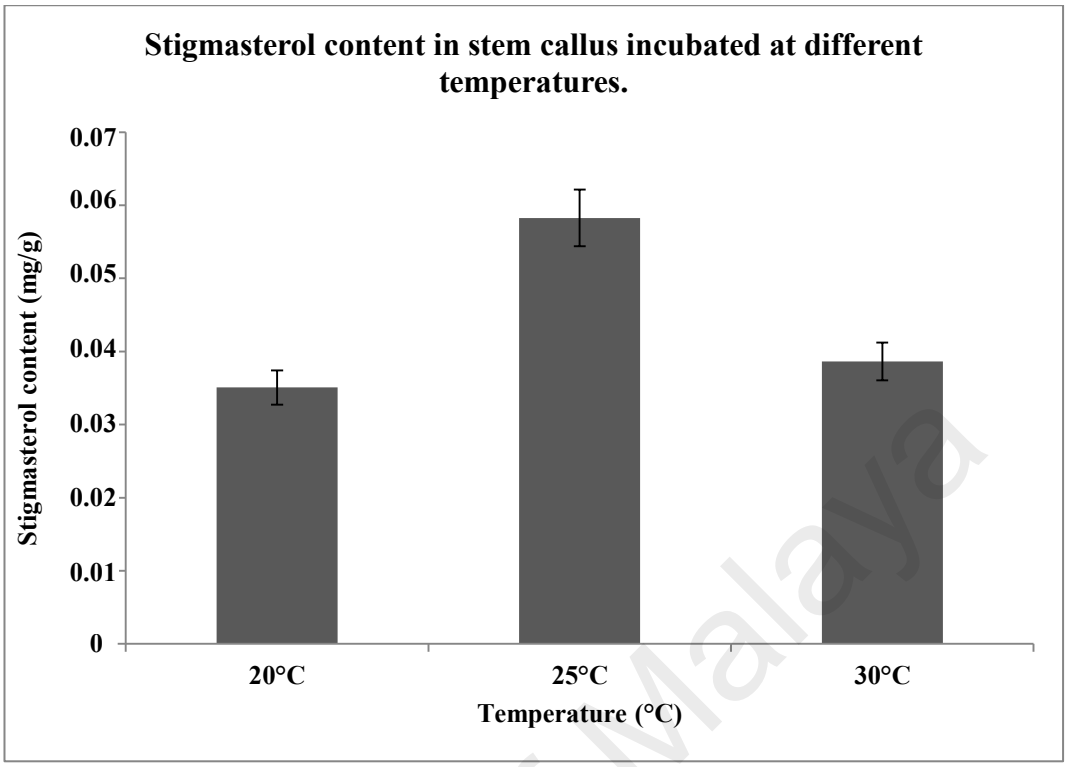


Figure 4.21: Estimation of stigmasterol content in stem callus of *W. biflora* incubated under different temperatures by spectrophotometer method.

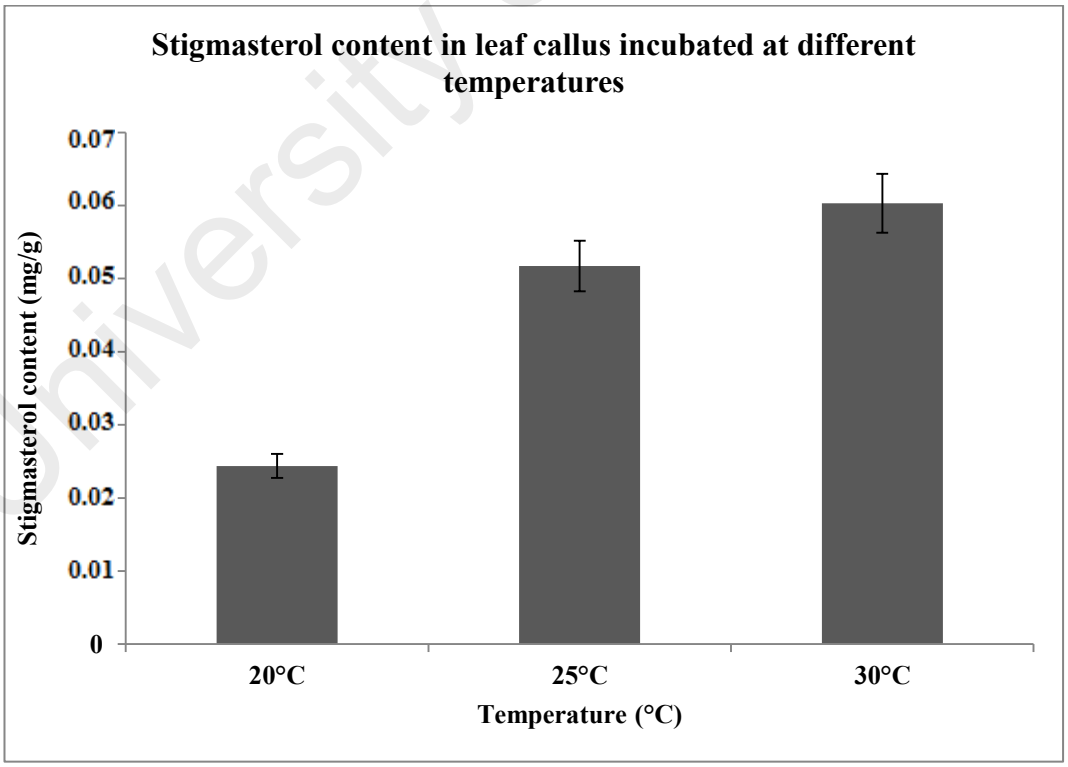


Figure 4.22: Estimation of stigmasterol content in leaf callus of *W. biflora* incubated under different temperatures by spectrophotometer method.

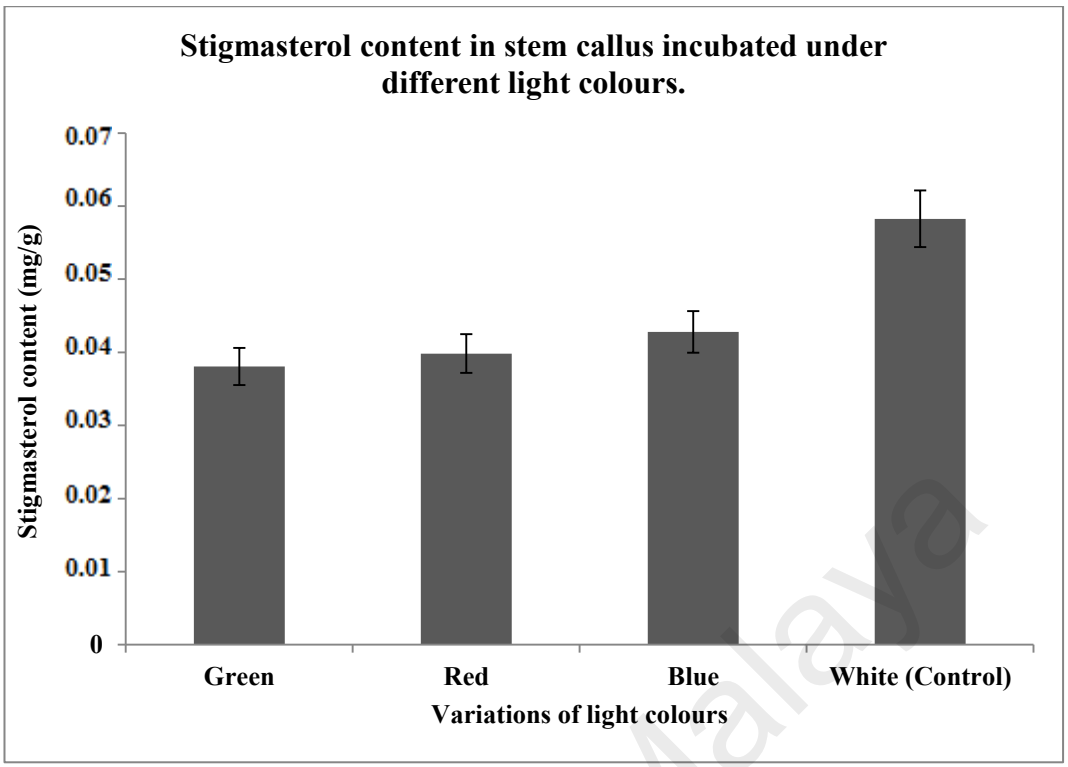


Figure 4.23: Estimation of stigmasterol content in stem callus of *W. biflora* incubated under different light colours by spectrophotometer method.

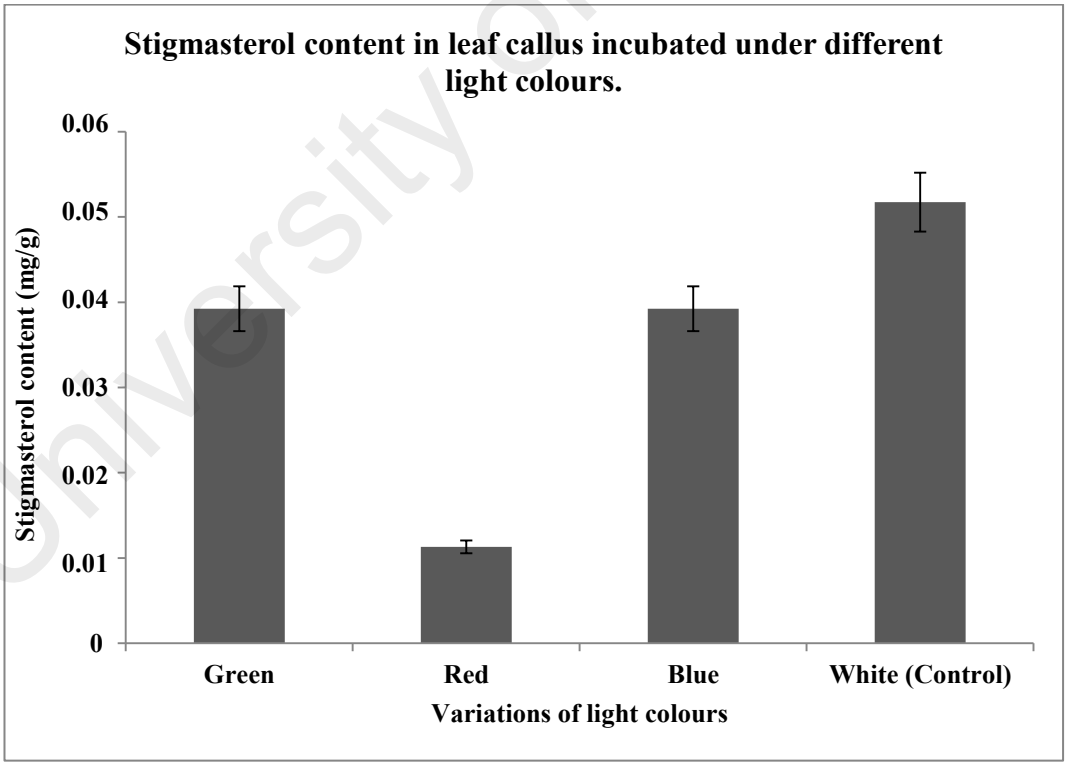


Figure 4.24: Estimation of stigmasterol content in leaf callus of *W. biflora* incubated under different light colours by spectrophotometer method.

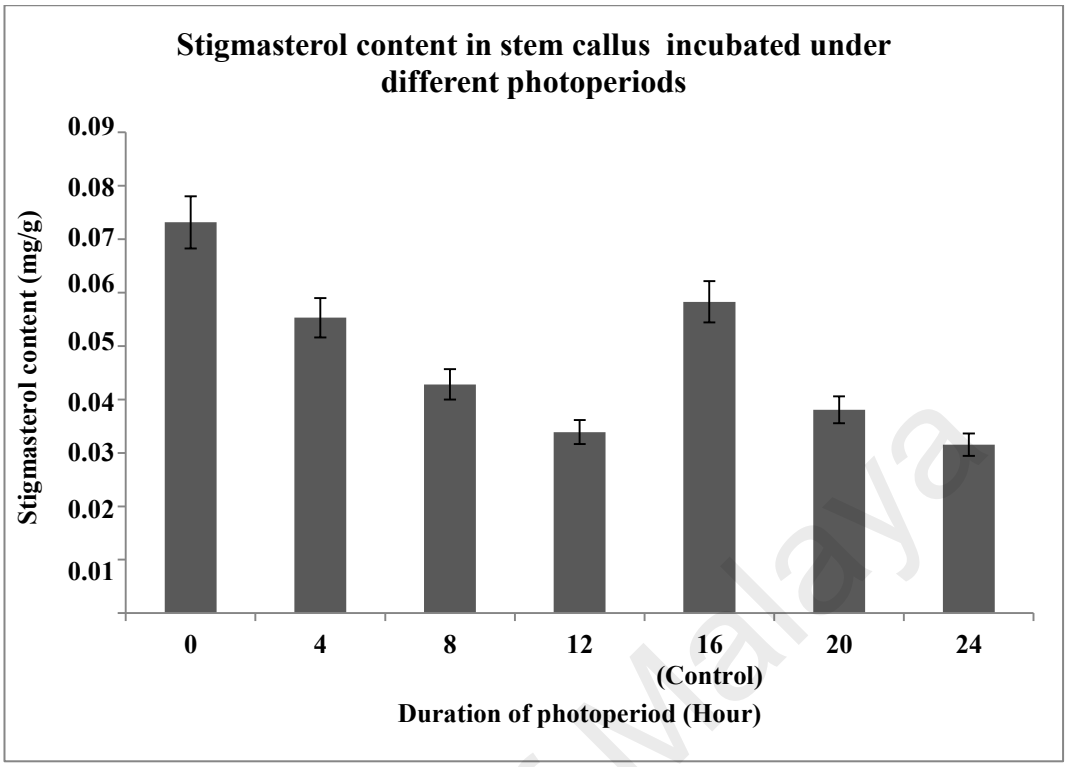


Figure 4.25: Estimation of stigmasterol content in stem callus of *W. biflora* incubated under different duration of photoperiods by spectrophotometer method.

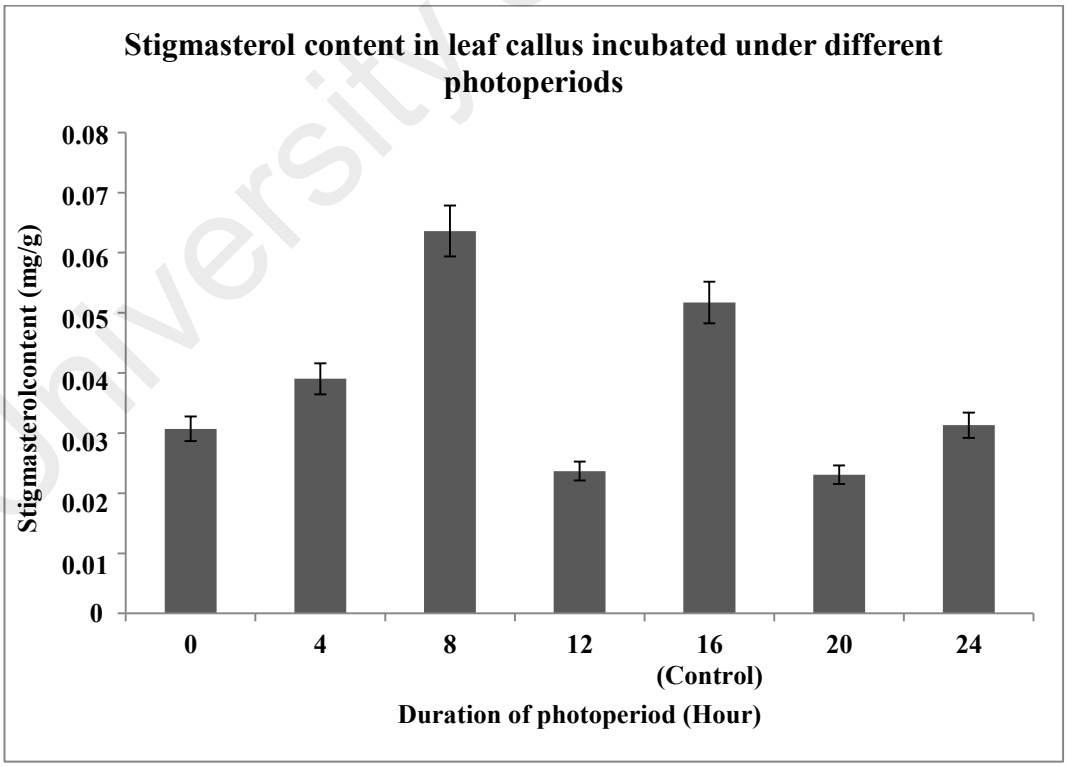


Figure 4.26: Estimation of stigmasterol content in leaf callus of *W. biflora* incubated under different duration of photoperiods by spectrophotometer method.

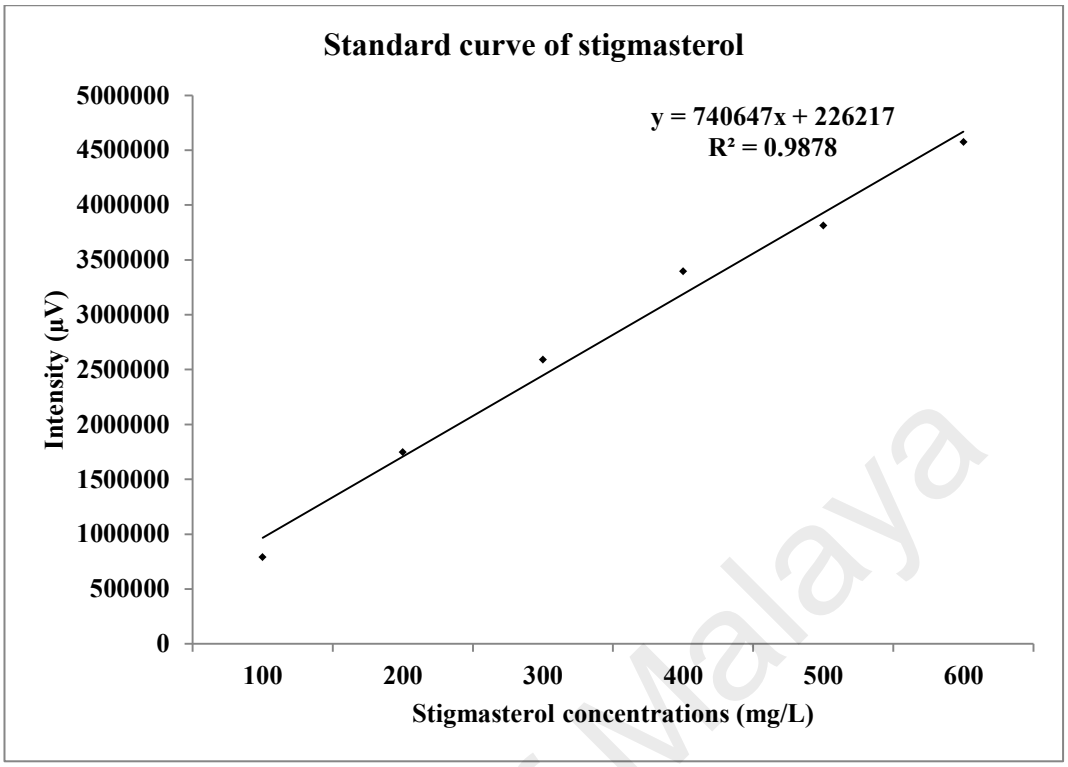


Figure 4.27: Standard curve of standard stigmasterol ranged from 100 to 600 mg/L ran through HPLC system.

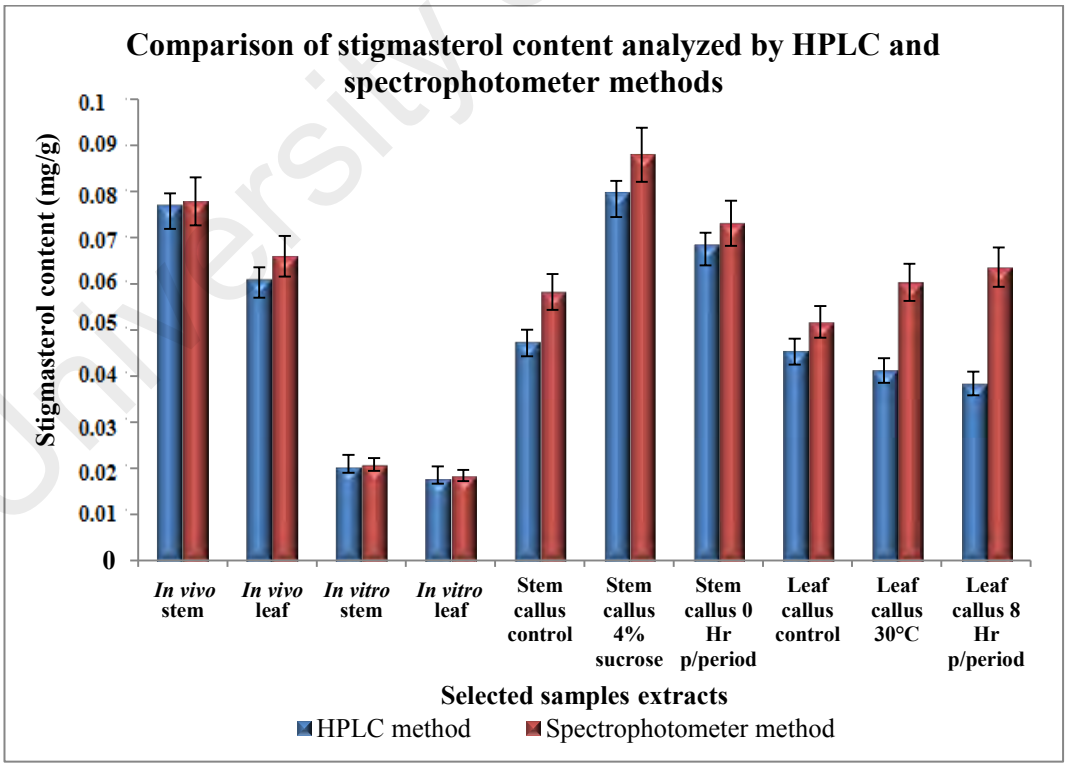


Figure 4.28: Comparison of stigmasterol contents in selected samples (the best samples from spectrophotometer method) analyzed by HPLC and spectrophotometer method.

CHAPTER 5: DISCUSSION

5.1 Optimization of callus induction by using auxins and cytokinins

Callus can be defined as a mass of undifferentiated tissue. In plant tissue culture, regulation of hormone auxins and cytokinins could influence the production of callus. The parts of the explants being used as starting material also could affect the production of callus. On the other hand, manipulation of internal or external factor; or both could also increase or decrease the formation of callus in plant tissues.

In this work, auxins and cytokinins were used to induce the formation of callus from stem and leaf parts of *W. biflora*. The explants were exposed to the treatment of auxins alone or in combination with cytokinins. It was proven in this study that application of auxins and cytokinins in combination (particularly combination of 2.0 mg/L NAA and 2.5 mg/L BAP) resulted in better callus production rather than application of single auxin. This result agreed with most of previous studies, especially from plants within the same family such as *Artemisia vulgaris* L. (Borzabad et al., 2010), *Gerbera jamesonii* (Paduchuri et al., 2010), *Solidago canadensis* L. (Li et al., 2012), *Eclipta alba* (Sharma et al., 2013) and *Leuzea carthamoides* (Zand et al., 2014). Sharma et al. (2013) reported that combination of 1.0 mg/L 2-4,D and 0.5 mg/L BAP produced intense callus formation in leaf explants of *Eclipta alba*. Meanwhile, Zand et al. (2014) found that leaf explants of *Leuzea carthamoides* were best stimulated with combination of 1.0 mg/L 2,4-D and 1.5 mg/L BAP to produce more callus.

There were four types of auxins used in this experiment; which were NAA, IBA, 2,4-D and IAA. Both explants (leaf and stem) were able to produce callus when applied with NAA, 2,4-D and IBA; but in different natures and sizes of callus. Hormone IAA failed to induce callus in both explants. In contrast, Sundram et al. (2012) successfully induced callus in *Curcuma manga* by application of IAA. Application of IAA caused

swelling of the explants (in the present study) and eventually turning black at the edge of the explants. Srivastava (2001) and Tan et al. (2010) stated that hormones IAA and IBA were usually applied for root induction in plant tissue culture method. This is because IBA and IAA are naturally occurring auxins, as compared to 2,4-D and NAA which are chemically synthesized. According to Baskaran et al. (2006), the synthetic auxins are more effective than natural auxins in inducing callus. Hormone IBA however able to produce callus in both explants; but in very small amount and was mixed with formation of roots.

Although derived from the same plant, application of auxins did not give the same results for stem and leaf parts of *W. biflora*. Stem explants were responded more to hormone NAA for callus induction; while 2,4-D was the best hormone for leaf explants. Similarly, Kamruzzaman et al. (2015) reported that leaf callus from *Citrus reticulata* Blanco. was induced by application of 1.0 mg/L 2-4,D, while stem callus from the same plant was induced by combination of 1.0 mg/L 2,4-D, 0.5 mg/L BAP and 0.25 mg/L NAA. The differences may be due to the arrangement of cells from both explants. The cells arrangement of stem explants were more compact than leaf explants. This explanation suggested that NAA could penetrate deeper into harder plant tissues and stimulated the rapid cells proliferation. Even though *W. biflora* is not a woody plant, the stem parts are harder and sturdier than leaf segments (Sureshkumar et al., 2007a).

Combination of auxins with cytokinins accelerated and increased the production of callus even more in both explants. In this experiment, Kinetin and BAP were used as sources of cytokinins. Different types of cytokinins resulted in different callusing frequency and biomass of callus. Combination of auxins with BAP accelerated the production of callus more than combination with Kinetin. The same results were obtained by Xu et al. (2009) where combination of 2-4,D with BAP gave higher

callusing response in *Juncus effusus*. Furthermore, combination of auxins with Kinetin produced rooting callus; even though the rooting percentage was lower than application of IBA alone. In previous studies done by Yang et al. (2008) and Jacinda et al. (2008), they found that more callus were produced when NAA or 2,4-D was combined with BAP. This result showed that regulation of auxins and cytokinins with certain concentration was able to produce more callus. In this experiment, combination of lower concentration of auxins (2.0 mg/L NAA) and higher concentration of cytokinins (2.5 mg/L BAP) resulted in better callus formation. Similarly, Patel and Patel (2013) found that combination of lower concentration of auxins (0.5 mg/L 2-4,D) and higher concentration of cytokinins (3.0 mg/L BAP) produced more callus in leaf explants of *Tecomella undulata*. Angulo-Bejarano and Paredez-Lopez (2011) in contrast reported that similar ratio of auxins (2.26 µM 2-4,D) and cytokinins (2.21 µM BAP) in growth medium will provide better callus response. Another variation was reported by Mahadev et al. (2014) where higher auxins concentration (3.0 mg/L NAA) than cytokinins (0.5 mg/L BAP) resulted in more callus formation in nodal explants of *Solanum viarum*. This variety of results showed that the effects of auxins and cytokinins ratios were different in different plants (Zhao et al., 2008).

In the current study, stem explants showed better results than leaf explants in terms of callusing frequency and the biomass of callus. Stem explants were easily and faster to form callus than leaf explants. This result was supported by Yaacob et al. (2013) which showed stem explants were better starting materials than leaf explants for *Justicia betonica*. Another study by Kumlay and Ercisli (2015) showed that stem explants of *Solanum tuberosum* were more responsive than its leaf explants in terms of callus formation. This is because the nature of stem tissues is more rigid than leaf tissues. Leaf explants (tissues) tend to curl inwards a few days after inoculation which caused the leaf tissues unable to absorb sufficient nutrients for cell growth. The stem explants however

able to remain sticky onto the medium which allowed the full nutrients absorption from all parts of stem tissues (Yaacob et al., 2013).

5.2 Optimization of callus induction under abiotic stress conditions

The other factor tested for better callus induction was manipulation of abiotic stress conditions, which also represented the manipulation of internal or external factors during explants incubation. In this experiment, only external factors were manipulated such as sucrose concentration, incubation temperature, exposure to different light colours during incubation and the photoperiod during incubation. The manipulation of abiotic stress conditions was not only increased the callusing frequency, but also able to regulate the production of secondary metabolites in callus.

Sucrose is the most common carbon source used in tissue culture application due to its feasibility to be obtained and could be easily absorbed by plant cells. Normal concentration of sucrose used is 3% as according to Wilson et al. (1994) for optimum carbon supply to plant cells growth during *in vitro* incubation period. Lower concentration of sucrose will retard the cells growth, while higher concentration would inhibit the nutrient uptake by lowering the water potential of the medium (Shim et al., 2003). Results from this experiment showed that both explants favoured moderate concentration of sucrose (stem explants: 4%; leaf explants: 3%) for optimum callus growth as shown in Table 4.2. Previous study by Pande and Khetmalas (2012) found that 3% of sucrose resulted in higher callus response in leaf explants of *Stevia rebaudiana*. Another study by Saikia et al. (2012) reported more callus was produced in leaf explants of *Aquilaria malaccensis* when 4% of sucrose was added into the MS medium.

On the other hand, different incubation temperatures will alter the cells growth differently. Endress (1994) suggested that lower temperature (22-27°C) was more favourable for callus induction; whereas temperature above 30°C and below 21°C will diminish cells growth rapidly. Most of growth chambers for *in vitro* incubation were set at 25±2°C. Lower temperature could also lower the presence of contaminants. The lowest temperature being tested in this study was 20°C, whereby the callus was able to form but at a slower rate and smaller in size (Fig. 4.3). This is because this temperature was too cold that the plant cells slowly proliferate and the production of callus was almost halted. Similar study by Sengar et al. (2011) reported that incubation at 20°C decreased the growth rate of two varieties of sugarcane callus. The highest temperature tested was 35°C which kill all the explants after two weeks of incubation (Table 4.2). Kokotkiewicz et al. (2014) in agreement reported that 35°C was extremely hot for tissue culture incubation and could kill the plant cells after longer exposure. For stem explants of *W. biflora*, 25°C incubation temperature gave the optimum results, while 30°C was better for the leaf explants. Previous study by Kumar et al. (2012) agreed with these results where they reported stem callus of *Heliotropium indicum* grew best under 25°C when compared with 20, 30 and 32°C. On the other hand, Arif et al. (2014) reported that incubation of leaf callus under 27°C resulted in optimum growth rate for *Dianthus caryophyllus*.

In nature, light is essential for plant to optimally adapt its growth and development to the ambient environment (Cheikhrouhou et al. 2015). The formation of different colour lights strongly related to their wavelength regimes. Visible light (white light) is the main light with the widest range of wavelength (400 to 710 nm). The dispersion of visible light will produce distinct blue light (400-495 nm), green light (495-570 nm), yellow light (570-590 nm) and red light (590-710 nm). The colours of lights involved in this experiment were white, blue, green and red. Among all these light colours, white light

gave the best callus formation and biomass for both stem and leaf callus as shown in Table 4.3. The worst light colour for callus induction was red light. Ahmed et al. (2012) similarly reported red light caused slow growth on callus of *Gymnema sylvestre*; and decreased its biomass. This is because red light has lower energy and chlorophyll a, which is crucial for photosynthesis, could not absorb enough energy for photosynthesis (due to absent of blue light) and subsequently inhibited the nutrient uptake in the plant cells (Muneer et al. 2014). Kokotkiewicz et al. (2014) on the other hand claimed that treatment with different light regimes did not significantly influence the growth of *Cyclopia subternata* callus. White light is however the most commonly light used in tissue culture application and successfully produce callus in most cases. For instance, Cybularz-Urban et al. (2015) reported that leaf callus of *Cattleya* grew best when incubated in white light among other light colours tested. Since white light possessed all range of wavelength, including red and far-red wavelength, the photoequilibrium in callus cells was established immediately (Zoratti et al. 2014). This condition allowed the interconversion of inactive phytochrome (P_r) and active phytochrome (P_{fr}) and served as on and off switch to regulate better growth of callus.

Regulation of photoperiod in plant growth enable the control of plant developmental process such as flowering induction, bud dormancy, stem elongation, leaf growth, formation of storage organ as well as axillary branching (Thomas, 2006). On the other hand, photoperiodic conditions could also affect the biosynthesis of secondary metabolites (Zoratti et al., 2014). Zoratti et al. (2014) reported that anthocyanins content in Chinese bayberry is increased by 0.5% in the presence of light. In current experiment, different explants showed different callogenesis responds towards light exposure (Table 4.3). Stem callus seems to favour total darkness condition (24 h darkness) while leaf callus favoured short period of light exposure (8 h of light/16 h of darkness). Afshari et al. (2011) and Yaacob et al. (2014a) similarly reported that 24 h of darkness was

beneficial for production of callus in *Brassica napus* and *Citrus assamensis*; while Ahmad et al. (2014) suggested that reversed photoperiod (8 h light/16 h darkness) improved callus formation and piperine production in *Piper nigrum*. In dark condition, the secretion of phenolic compound was decreased; thus extending the shelf life of the explants (Tan et al., 2010). This condition (total darkness) allowed the explants to proliferate actively under dark condition and increased callus biomass.

5.3 Growth curve analysis of *W. biflora* callus

The current study aimed to determine the most suitable growth time point for callus harvesting with the highest stigmasterol content. The growth of callus was measured by their fresh and dry weights. The pattern of the growth curve was obtained and showed an initial lag phase, followed by a period of rapid growth (exponential) and finally decreasing to complete decline phase (Fig. 4.8-4.27).

Limited reports were found on production of stigmasterol in callus of any plants. However, Flores-Sanchez et al. (2002) reported that overall sterols synthesis was higher during exponential phase of *Uncaria tomentosa* cells suspension cultures. Similarly, Ponis et al. (2006) reported that stigmasterol was produced during the exponential phase of microalgae growth, while most of other secondary metabolites were produced during the stationary or decline phase (Miguel-Chaves et al., 2003; Abbasi et al., 2007). Exponential phase is a phase where the cells were rapidly proliferated and consumed a lot of energy. In this period, secondary metabolites such as flavonoids and triterpenes are actively produced (Tan et al, 2010) to support the actively growing cells. Sterols synthesis was actively occurred during the exponential phase of cellular growth in order to provide enough sterols for new membrane of the cells and to support cellular stability (Hartmann, 1998).

5.4 Indirect and direct regeneration of *W. biflora*

Many researchers had agreed that cytokinins are the essentials plant growth regulators which are responsible in plant cell division, induction of adventitious shoots and lateral buds formation, as well as in cell cycle control (van-Staden et al., 2008; Gatica-Arias et al., 2010; Quintero-jimenez et al., 2010). Therefore, different concentrations of cytokinins, particularly BAP were used to induce multiple shoots from callus of *W. biflora* as shown in Fig. 4.28. Optimum shoots regeneration was observed on MS medium with 3.0 mg/L BAP; and shoots formation decreased at higher concentrations of BAP. Collado et al. (2013) supported the fact that higher concentrations of BAP decreased the formation of shoots from callus. Results from Collado et al. (2013) reported that formation of shoots from callus of *Phaseoulus vulgaris* was decreased with application of 4.5 mg/L BAP and above.

Lower concentration of BAP (1.0 mg/L) was needed to optimize shoots formation from nodal segments of *W. biflora*. Nodal segments (*in vivo* parts) were naturally ready to produce shoots rather than callus (*in vitro* grown) because the internodes served as shooting or branching points for the explants. Therefore, little external stimulation was needed to induce the shoots formation. Cirak et al. (2007) agreed that internodal explants were more responsive towards shoots formation and lower concentrations of BAP (2.0 mg/L) needed for optimum shoots formation in *Hypericum bupleuroides.*

In previous studies, better shoots regeneration was obtained when cytokinins were combined with auxins such as in *Ariocarpus kotschoubeyanus* (Moebius-Goldammer et al., 2003), *Coffea arabica* (Zoriniants et al., 2003), *Eleusine indica* (Yemets et al., 2003), *Cardispermum halicacabum* (Thomas & Maseena, 2006) and *Opuntia ficus-indica* (Angulo-Bejarano and Paredez-Lopez, 2011). However, in the present study, combinations of BAP with auxins resulted in lower shoots regeneration as compared to

application of single BAP. Therefore, single application of BAP was the best method to induce shoots regeneration from callus and nodal segments of *W. biflora* (Table 4.4).

IBA was commonly supplemented in the growth medium for root induction. This hormone had been widely used to induce roots in many plants such as *Artemisia judaica* (Liu et al., 2003), *Bita orellana* (Neto et al., 2003) and *Dioscorea zingiberensis* (Chen et al., 2003). In the current study, low concentrations of IBA needed for root induction from callus explants (2.0 mg/L) and nodal explants (1.0 mg/L; Table 4.5). These results were supported by those obtained by Ling et al. (2013) in *Labisia pumila*. They found that higher root formation obtained when *in vitro* stem and leaf explants of *L. pumila* were cultured in MS media supplemented with 1.0 mg/L and 3.0 mg/L BAP, respectively. Thomas and Maseena (2006) reported that hormone IBA was more effective for root induction in *Cardiospermum halicacabum* as compared to NAA and IAA. Even so, the effects may be varied for different species as *Rosa hybrida* (Ibrahim & Debergh, 2001) and *Hypericum bupleuroides* (Cirak et al., 2007) preferred hormone-free medium for roots induction, while the explants of *Ziziphus jujuba* were best rooted with application of 1.5 mg/L IAA (Zhou and Liu, 2009).

Prior to acclimatization, the regenerated plantlets were allowed to grow longer or taller; to produce more leaves and roots and to extend the stems. Yaacob et al. (2014b) reported that plantlets which had more leaves before acclimatization able to maintain the growth and produced even more leaves during acclimatization. Even though *in vitro* leaves lacked of photosynthetic ability, but the leaves can be recovered by formation of new leaves after acclimatization which had higher photosynthetic activity than *in vitro* leaves (Seelye et al., 2003). Also, taller plantlets were more capable to survive acclimatization period as compared to shorter plantlets. Mohammed & Vidaver (1990) and Yaacob et al. (2014b) supported this result when taller plantlets of *Psudotsuga*

menziesii and *Agapanthus praecox* survived better when acclimatized *ex vitro*. This showed that the plantlets should grow in sufficient height and size before acclimatization for better survival rates.

5.5 Antimicrobial and antioxidant activities of *W. biflora* extracts

In order to explore the medicinal value of *W. biflora*, antimicrobial and antioxidant assays were carried out to test the extracts for potential antimicrobial and antioxidant activities. Biswas et al. (2013) had been reported the antimicrobial and antioxidant activities of ethanol extracts of *W. biflora* leaves against *Klebsiella pneumonias* and *Candida albicans*. The ethanol extracts of *W. biflora* leaves therefore have very high potential to be used as wound healing agent due to its higher antimicrobial and antioxidant activity. In the current study, methanolic extracts of *W. biflora* callus, leaves and stems were used for the assays. Antimicrobial assay was carried out by disc diffusion method; where the extracts showed a clear zone of inhibitions towards tested microbes should it has any antimicrobial effects. Antioxidant ability of the extracts was confirmed by four assays; DPPH radical scavenging, H_2O_2 radical scavenging, Reducing Power Assay and Ferric Reducing Antioxidant Power assay.

It was expected that leaves extracts have more potential as antimicrobial and antioxidant agent. However, in both bioactivity assays in this study, extracts from stems of *W. biflora* showed better results than extracts from its leaves (Table 4.9). This result was not in agreement with Biswas et al. (2013) where they evaluated the ethanolic leaves extract of *W. biflora* for antimicrobial and wound healing property. They reported that the ethanol extracts from leaves of *W. biflora* inhibited the growth of *Klebsiella pneumoniae* and *Candida albicans* as well as possessed the ability to heal the excision wound model in Wistar albino rats. In another study, Govindappa et al. (2011) reported that leaf and flower extracts of *W. trilobata* were more potent in showing

antimicrobial and antioxidant ability. Previous study by Senthilkumar et al. (2008) reported that ethanol extracts from leaves of *W. chinensis* possessed antihyperglycaemic activity and prevents oxidative stress associated with diabetes mellitus in alloxan-induced diabetic rat. Meanwhile, aqueous extracts from leaves of *W. chinensis* was validated to show antimicrobial activity against *Streptococcus mutans*, *Lactobacillus casei* and *Staphylococcus aureus*.

On the other hand, Manjamalai et al. (2012) and Dai et al. (2013) extracted the essential oils of *W. chinensis* and *W. prostrata* to be tested for anticancer and antimicrobial agent. The essential oils from *W. chinensis* had the ability to suppress the metastatic potential of B16F-10 cells in lungs of C57BL/6 mice as reported by Manjamalai et al. (2012). Meanwhile, Dai et al. (2013) reported the antimicrobial potential of *W. prostrata* essential oils especially against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, etc. These variations from previous results give the idea that the whole parts of *Wedelia* spp. could serve as potential medicinal agents. Current findings which pointed stem extracts as the potential candidates further strengthen the usage of the whole parts of *W. biflora* to be extracted for medicinal purpose; without wasting any of the plant parts.

In this study, proper stimulation such as manipulation of sucrose concentration in MS media and manipulation of photoperiod during incubation could increase the antimicrobial and antioxidant potential in the stem extracts. Higher concentrations of sucrose (4%) increased the production of stigmasterol in stem callus culture of *W. biflora* more than in intact stems. Stigmasterol is the compound believed to play an important role in antimicrobial and antioxidant properties in *W. biflora*, and higher antimicrobial and antioxidant activities were exhibited by *W. biflora* stem callus inoculated with 4% sucrose in the MS medium. Similarly, Qian et al. (2009) found that

application of 4% sucrose B5-medium increased the growth rate of *Coleus blumei* suspension culture as well as its rosmarinic acid production, which is responsible in antibacterial and antioxidant activities. Incubation of *W. biflora* stem callus in total darkness (0 h photoperiod) had also increased the bioactivities; but the readings were lower than intact stems and stem callus with 4% sucrose (Table 4.9-4.13). This result agreed with Lopez-Laredo et al. (2009) which reported that antioxidant activity of *Tecoma stans* callus decreased when cultured in extended darkness.

5.6 Estimation of stigmasterol in *W. biflora* extracts

Stigmasterol is one of the phytosterol which is rich in medicinal properties such as wound healing (Verma et al., 2008), antimicrobial and antioxidant (Sureshkumar et al., 2007b; Biswas et al., 2013), analgesic (Sureshkumar et al. 2007a) etc. Stigmasterol can be obtained from extracts of naturally grown *W. biflora*. However, the mass extraction of the compound will eventually decreased the amount of the naturally grown plants and inhibited its continuous production. Therefore, *in vitro* approach was taken in the current study to provide continuous supply of the materials while keeping or even increasing the production of stigmasterol.

In this study, *in vivo* parts of *W. biflora* (naturally grown stem and leaf) exhibited higher amount of stigmasterol as compared to *in vitro* parts. This phenomenon was highly expected as *in vitro* grown plants are lacked of growth metabolism. However, *in vitro* grown plants may be manipulated easily to increase the stigmasterol content, especially via callus culture which could provide a lot more cell lines. Recently, Castro et al. (2016) reported higher content of total phenol and flavonoids in callus derived from leaf of *Byrsonima verbascifolia* as compared to its wild-grown leaf. Production of bioactive compounds becoming more feasible by application of callus culture because a number of explants can be obtained from single plant; and subsequently able to sustain its wild population. The callus of *W. biflora* in the current study were stimulated by

different conditions of abiotic stress such as temperature, light colours, sucrose concentration, and photoperiod in order to increase the production of stigmasterol.

From all these treatments, manipulation of sucrose concentration showed a very promising result, which surpassed the production of stigmasterol from *in vivo* parts; particularly when the stem callus treated with 4% sucrose. Praveen and Murthy (2012) and Doma et al. (2012) also found that 4% sucrose concentration could increase the production of withanolides in *Whitania somnifera*. The latter observed the accumulation of withaferin A in hairy roots culture of the species incubated in medium fortified with 2%, 3% and 4% of sucrose with the highest accumulation at 4% of sucrose and no accumulation of withaferin A observed at 6% of sucrose. Excessive application of sucrose could be harmful to the growth of the callus as shown in current study. In agreement with Yin et al. (2013), they reported that application of 5% and 6% of sucrose concentration in MS medium decreased the biomass of *Pseudostellaria heterophylla* callus as well as its saponins and polysaccharides content when compared to application of 4% sucrose where they recorded the highest biomass and metabolites production.

The second best treatment was photoperiod. Zoratti et al. (2014) stated that regulation of photoperiod could affect the production of secondary metabolites in plants due to the interconnection between light signaling pathway and circadian clocks in the regulation of the photoreceptor genes (Lopez et al., 2012). In this study, total darkness (0 h photoperiod) increased the production of stigmasterol in stem callus, but still lower than *in vivo* stems. Artanti and McFarlane (1996) reported that dark condition had increased the production of asparagine in *Coronilla rostrata* callus. In contrast, many previous studies had proven that light treatment had been positively increased the flavonoids contents in various fruits such as Chinese bayberry, tomato and grapes (Niu et al., 2010; Lovdal et al., 2010; Koyama et al., 2012; Azuma et al., 2012).

Other than the photoperiod, the exposure of the explants in different light colours could also affect the production of secondary metabolites. There were four different light colours investigated in this study. Among all the light colours, white light resulted in higher production of stigmasterol followed by blue light, red light and green light. This result supported by He et al. (2005) where they found that white light increased the production of total isoflavones in hairy root cultures of *Pueraria phaseoloides* by 1.15 times and 1.19 times as compared to blue light and dark condition, respectively. On the other hand, Reis et al. (2015) found that different light colours affected different metabolites production; such as red and white lights increased the production of betacyanin while blue light increased the production of flavonoids in *Alternanthera* spp.

Incubation temperature also plays an important role in callus growth rate as well as in secondary metabolites production. In the current study, maintenance of the callus culture under optimum temperature (25°C) resulted in the highest biomass and stigmasterol accumulation. In contrast with previous study, Zhang et al. (1997) reported an increased as much as 13-fold and 4.6-fold of anthocyanins in suspension culture of strawberry incubated at 15°C as compared to incubation at 35°C and 25°C, respectively. Meanwhile, Chan et al. (2010) also found that culture of *Melastoma malabathricum* grew better and had higher anthocyanins accumulation when incubated at 20°C than those incubated at 26°C and 29°C. In this study, lower temperature (20°C) reduced the callus growth rate and stigmasterol content significantly while higher temperature (30°C) killed the explants.

CHAPTER 6: CONCLUSION

The present study proved that stem explants of *W. biflora* are a good starting material rather than leaf explants for efficient callus induction. Combinations of auxins and cytokinins provided mass production of callus in both stem and leaf explants. Best percentage of callusing and biomass production were achieved with combinations of NAA and BAP for stem explants and 2,4-D and BAP for leaf explants. The formation of callus was further optimized by stimulation of abiotic stress conditions. For stem callus, 4% of sucrose concentration, 25°C incubation temperature under white colour light and incubated in total darkness improved the growth of callus. Growth of leaf callus could be optimized by application of 3% sucrose, incubated at 30°C under white colour light and exposed to 8 h light/16 h darkness.

Indirect regeneration from previously obtained callus was successfully achieved by addition of 3.0 mg/L BAP (for shoot regeneration) and 2.0 mg/L IBA (for root regeneration). Direct regeneration of *W. biflora* was optimized by addition of 1.0 mg/L BAP for shooting and 1.0 mg/L IBA in ½ MS medium for rooting. Combination of BAP and auxins for shooting purpose did not increase the multishoot production. The acclimatization of in vitro grown *W. biflora* was achieved in combination of red soil and black soil in 1:1 ration.

All extracts including *in vivo* stem and leaf, *in vitro* stem and leaf, callus under normal conditions and callus under abiotic stress conditions were screened for antimicrobial and antioxidant activities. It was observed that stem callus treated with 4% sucrose possessed superior antimicrobial and antioxidant activities followed by *in vivo* stem, *in vivo* leaf, and stem callus incubated in total darkness. The screening of stigmasterol content from the selected extracts also showed higher stigmasterol content in stem callus with 4% sucrose as compared to *in vivo* stem parts. Antimicrobial and antioxidant studies of these methanolic extracts suggested the efficiency of the callus

(especially stem callus) to produce secondary metabolites comparable to intact plants when stimulated with proper stress condition; in this study it was 4% sucrose concentration. The present callus induction protocol can be recommended for large scale production of active constituents from *W. biflora*, especially stigmasterol.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

1. Siti Norayu Idris, Bakrudeen Ali Ahmed Abdul and Rosna Mat Taha, (2014). Optimization of callus induction, antimicrobial and antioxidant properties of *wedelia biflora* – a potential medicinal plant. Monash Science Symposium 2014, Kuala Lumpur. Poster presented on 16-17 June 2014.

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Optimization of callus induction, antimicrobial and antioxidant properties of *Wedelia biflora* – A potential medicinal plant.

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INTRODUCTION

- *Wedelia biflora* is a member of Asteraceae family and is highly distributed in Middle East Asia.
- It is a type of medicinal herbs with pharmacological potential eg: antimicrobial, antioxidant, antidiabetic, etc.
- It is easily grown through stem cuttings and is a creeping herb.

OBJECTIVES

- To optimize callus induction from stem and leaf explants of *W. biflora* and
- To evaluate and compare antimicrobial and antioxidant properties of *W. biflora* callus extracts with *in vivo*- and *in vitro*-grown plant extracts.

METHODOLOGY

Leaf and stem explants

↓

Inoculated in MS medium supplemented with: Auxin alone (NAA, IAA, 2,4-D, and IBA); Auxin and cytokinin (BAP and Kinetin)

↓

The best callus selected based on nature and biomass

↓

Dried callus *in vivo*- and *in vitro*-grown plant extracted in methanol

↓

Antimicrobial test: *Escherichia coli, Bacillus subtilis, Aspergillus niger and Candida albicans.*

↓

Antioxidant test: DPPH assay

CONCLUSION

- Callus from stem and leaf explants of *W. biflora* could be induced by inoculation of the explants in MS media supplemented with combinations of 2.0 mg/L NAA + 2.5 mg/L BAP and 1.0 mg/L 2,4-D + 3.5 mg/L BAP, respectively.
- Callus extracts of *W. biflora* have potential in antimicrobial and antioxidant activity.
- The analysis of compound and quantification of potential phytoconstituents from this plant could be investigated further for various medicinal purposes.

ACKNOWLEDGMENT

Authors would like to thank University of Malaya for providing the financial support (PRPUM grant, GC040-2013).

RESULTS

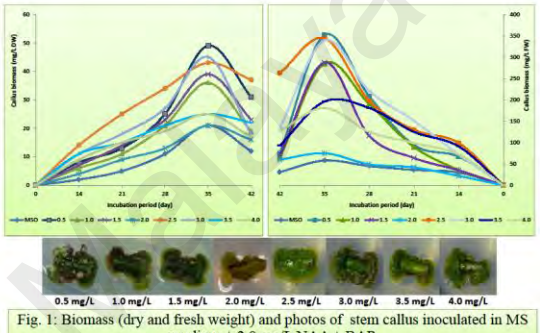


Fig. 1: Biomass (dry and fresh weight) and photos of stem callus inoculated in MS medium + 2.0 mg/L NAA + BAP

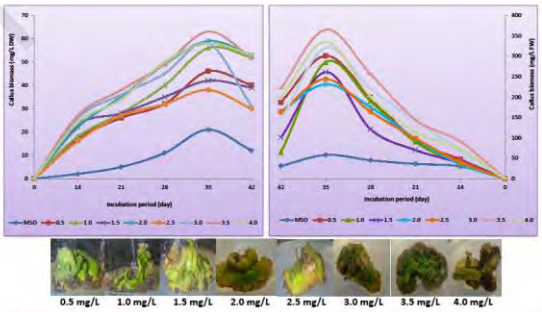
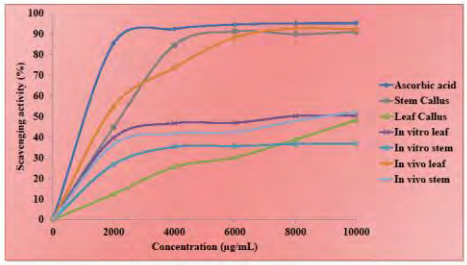


Fig. 2: Biomass (dry and fresh weight) and photos of leaf callus inoculated in MS medium + 1.0 mg/L 2,4-D + BAP

Extracts/ Microbes	Stem Callus	Leaf Callus	*In vivo* stem	*In vivo* Leaf	*In vitro* Stem	*In vitro* Leaf	Amp.	Fluco.
Bacillus subtilis	10	9	12	10	11	9	33	-
Escherichia coli	8	9	10	11	9	9	33	-
Aspergillus niger	11	7	9	8	8	7	-	30
Candida albicans	9	8	14	10	13	10	-	30

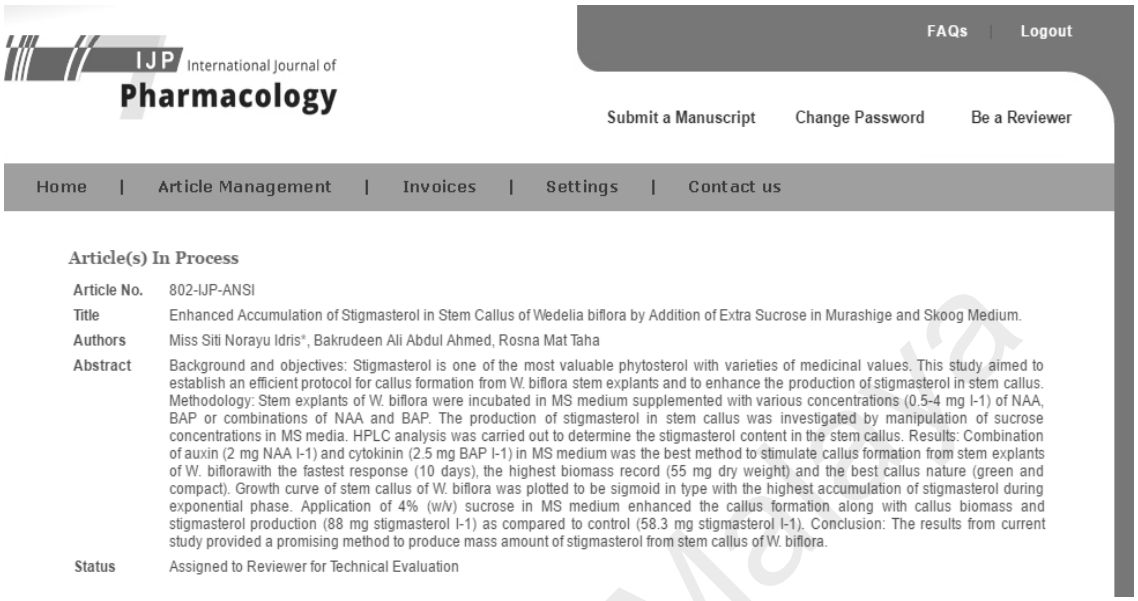
Fig. 3: Zone of inhibitions (in mm) for extracts treated on selected microbes.



Extracts	Ascorbic acid	Stem callus	Leaf callus	*In vitro* stem	*In vitro* leaf	*In vivo* stem	*In vivo* leaf
IC_{50} value (µg/mL)	1630	2530	6104	7009	4860	5060	2500

Fig. 4: Scavenging activity (%) and IC_{50} values (µg/mL) of extracts from DPPH assay.

2. Siti Norayu Idris, Bakrudeen Ali Ahmed Abdul and Rosna Mat Taha, (2016). Enhanced accumulation of stigmasterol in stem callus of *Wedelia biflora* by addition of extra sucrose in Murashige and Skoog medium. International Journal of Pharmacology, under review, Q4.



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Article(s) In Process

Article No.	802-IJP-ANSI
Title	Enhanced Accumulation of Stigmasterol in Stem Callus of Wedelia biflora by Addition of Extra Sucrose in Murashige and Skoog Medium.
Authors	Miss Siti Norayu Idris*, Bakrudeen Ali Abdul Ahmed, Rosna Mat Taha
Abstract	Background and objectives: Stigmasterol is one of the most valuable phytosterol with varieties of medicinal values. This study aimed to establish an efficient protocol for callus formation from W. biflora stem explants and to enhance the production of stigmasterol in stem callus. Methodology: Stem explants of W. biflora were incubated in MS medium supplemented with various concentrations (0.5-4 mg l-1) of NAA, BAP or combinations of NAA and BAP. The production of stigmasterol in stem callus was investigated by manipulation of sucrose concentrations in MS media. HPLC analysis was carried out to determine the stigmasterol content in the stem callus. Results: Combination of auxin (2 mg NAA l-1) and cytokinin (2.5 mg BAP l-1) in MS medium was the best method to stimulate callus formation from stem explants of W. biflorawith the fastest response (10 days), the highest biomass record (55 mg dry weight) and the best callus nature (green and compact). Growth curve of stem callus of W. biflora was plotted to be sigmoid in type with the highest accumulation of stigmasterol during exponential phase. Application of 4% (w/v) sucrose in MS medium enhanced the callus formation along with callus biomass and stigmasterol production (88 mg stigmasterol l-1) as compared to control (58.3 mg stigmasterol l-1). Conclusion: The results from current study provided a promising method to produce mass amount of stigmasterol from stem callus of W. biflora.
Status	Assigned to Reviewer for Technical Evaluation