EVALUATION OF THE EFFECT OF JASMONIC ACID ELICITATION ON COMPOSITION OF PIGMENTS IN GREEN CALLUS OF NEEM (*Azadirachta indica*)

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FACULTY OF SCIENCE
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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
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EVALUATION OF THE EFFECT OF JASMONIC ACID ELICITATION ON COMPOSITION OF PIGMENTS IN GREEN CALLUS OF NEEM (*Azadirachta indica*)

ABSTRACT

This project was carried out with the aim of determining the effects of jasmonic acid (JA) elicitation on the bioactive pigments biosynthesis and the antioxidant activities in green callus of *Azadirachta indica*. Plant tissue culture technique was employed to induce the formation of green callus from leaf explants of *A. indica* on media using 0.6 mg/L thidiazuron (CM) and three different concentrations of jasmonic acid (2, 4 and 6 mg/L) for 4 and 8 weeks of incubation time. The methanolic extracts from the green callus were used for determination of total anthocyanin content (TAC), total chlorophyll content (TCh), total carotenoid content (TC), total phenolic content (TPC), and total flavonoid content (TFC) through colorimetric and HPLC analysis. Phytochemical analysis were done using standard established phytochemical qualitative analysis. The usage of thidiazuron (TDZ) with JA did not exert any significant effect on callus fresh weight and growth index (GI). The highest amount of yield was from CM and 2 mg/L jasmonic acid (2JA) extracts for 4-week-old and 8-week-old samples respectively. Phytochemical screening revealed the presence of similar constituents in both 4-week-old and 8-week-old green callus extracts such as alkaloids, flavonoids, phenols, tannins, and terpenoids. The highest value for TAC, TCh, TC, TPC, and TFC (0.31 ± 0.00 mg/g DW, 1.00 ± 0.03 mg/g DW, 0.13 ± 0.01 mg/g DW, 3.96 ± 0.02 g GAE/g DW, and 0.55 ± 0.03 g QE/g DW respectively) of 4-week-old samples were from callus cultured on media supplemented with 6 mg/L jasmonic acid (6JA) extract. Meanwhile, the highest value for TAC, TCh, TC TPC, and TFC (0.32 ± 0.01 mg/g DW, 0.65 ± 0.00 mg/g DW, 0.10 ± 0.01 mg/g DW, 3.40 ± 0.05 g GAE/g DW, and 0.52 ± 0.01 g QE/g DW respectively) of 8-week-old samples were from callus cultured on media supplemented with 4 mg/L jasmonic acid

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(4JA) extract. The inhibitory concentration (IC_{50}) value for 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) of all samples were calculated and the lowest IC_{50} values were found to be 8.29 ± 0.10 mg/mL (6JA) for 4-week-old and 7.73 ± 0.03 mg/mL (4JA) for 8-week-old samples, respectively. The highest Ferric Reducing Antioxidant Power (FRAP) values obtained in this study were 90.60 ± 1.55 g/g (6JA), and 74.59 ± 3.91 g/g (4JA) respectively for 4- and 8-week-old samples. Overall, the findings suggest that elicitation with JA significantly improved the content of bioactive pigments in the sample, where 6 mg/L and 4 mg/L had been identified as the optimum JA concentration for 4 and 8 weeks of incubation time, respectively. Moreover, Pearson's correlation analysis revealed a significant correlation between TAC, TCh, TC, TPC, and TFC with ABTS and FRAP assays.

Keywords: jasmonic acid, *Azadirachta indica*, plant tissue culture, bioactive pigments, antioxidants activities

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PENILAIAN KESAN ELISITASI ASID JASMONIK KE ATAS KOMPOSISI PIGMEN-PIGMEN DI DALAM KALUS HIJAU POKOK SEMAMBU (*Azadirachta indica*)

ABSTRAK

Projek ini dijalankan dengan tujuan untuk menentukan kesan elisitasi asid jasmonik (JA) terhadap penghasilan pigmen bioaktif dan aktiviti antioksidan kalus hijau *Azadirachta indica*. Dalam kajian ini, teknik kultur tisu tumbuhan digunakan untuk menginduksi penghasilan kalus hijau daripada daun *A. indica* dalam media menggunakan 0.6 mg/L thidiazuron (CM) dan tiga kepekatan asid jasmonik (2, 4 and 6 mg/L) selama 4 dan 8 minggu tempoh inkubasi. Ekstrak metanolik kalus hijau digunakan untuk penentuan jumlah kandungan antosianin (TAC), jumlah kandungan klorofil (TCh), jumlah kandungan karotenoid (TC), jumlah kandungan fenolik (TFC), dan jumlah kandungan flavonoid (TFC) melalui analisis kolorimetrik dan HPLC. Analisis fitokimia juga telah dijalankan menggunakan prosedur-prosedur standard yang telah diterima pakai. Penggunaan thidiazuron (TDZ) bersama-sama JA tidak menyebabkan apa-apa kesan signifikan terhadap berat basah dan indeks pertumbuhan (GI). Jumlah hasil tertinggi adalah daripada ekstrak CM dan 2 mg/L asid jasmonik (2JA) untuk sampel 4 minggu dan 8 minggu mengikut turutan. Ujian fitokimia menunjukkan kehadiran konstituen yang sama dalam ekstrak kalus hijau berumur 4 dan 8 minggu seperti alkaloid, flavonoid, fenol, tannin, dan terpenoid. Nilai tertinggi untuk TAC, TCh, TC, TPC, dan TFC (0.31 ± 0.00 mg/g DW, 1.00 ± 0.03 mg/g DW, 0.13 ± 0.01 mg/g DW, 3.96 ± 0.02 g GAE/g DW, dan 0.55 ± 0.03 g QE/g DW mengikut turutan) bagi sampel-sampel berumur 4 minggu adalah dari ekstrak kalus yang dikultur di atas media yang ditambah dengan 6 mg/L asid jasmonik (6JA). Manakala, nilai tertinggi untuk TAC, TCh, TC, TPC, and TFC (0.32 ± 0.01 mg/g DW, 0.65 ± 0.00 mg/g DW, 0.10 ± 0.01 mg/g DW, 3.40 ± 0.05 g GAE/g DW, and 0.52 ± 0.01 g QE/g DW mengikut turutan) bagi sampel-sampel berumur 8 minggu

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adalah dari ekstrak kalus yang dikultur di atas media yang ditambah dengan 4 mg/L asid jasmonik (4JA). Nilai kepekatan perencat (IC_{50}) untuk 2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) bagi kesemua sampel telah dikira dan nilai IC_{50} terendah ialah 8.29 ± 0.10 mg/mL (6JA) bagi sampel berumur 4 minggu dan 7.73 ± 0.03 mg/mL (4JA) bagi sampel berumur 8 minggu. Nilai tertinggi bagi analisis '*Ferric Reducing Antioxidant Power*' FRAP ialah 90.60 ± 1.55 g/g (6JA) bagi sampel berumur 4 minggu dan 74.59 ± 3.91 g/g (4JA) bagi sampel berumur 8 minggu. Secara keseluruhan, hasil kajian mendapati 6 mg/L and 4 mg/L asid jasmonik ialah kepekatan optimum bagi tempoh 4 dan 8 minggu inkubasi. Selain itu, analisis korelasi Pearson menunjukkan korelasi signifikan antara TAC, TCh, TC, TPC dan TFC dengan ujian ABTS dan FRAP.

Kata kunci: asid jasmonik, *Azadirachta indica*, kultur tisu tumbuhan, pigmen bioaktif, aktiviti antioksidan

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LIST OF SYMBOLS AND ABBREVIATIONS

TDZ : 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea

ABTS : 2, 2-azino-bis-3-ethylbenzotiazoline-6-sulfonic acid

DPPH : 2,2-diphenyl-1-picrylhydrazyl

2,4-D : 2,4-dichlorophenoxyacetic acid

TPTZ : 2,4,6-tri(2-pyridyl)-s- triazine

BAP : 6-benzylaminopurine

NAA : α-naphthaleneacetic acid

α : alpha

$AlCl_3.6H_2O$: aluminium chloride

β : beta

CO_2 : carbon dioxide

Ca : chlorophyll a

Cb : chlorophyll b

Cu : copper

℃ : degree Celsius

ddH_2O : double distilled water

Fe^{3+} : ferric

Fe^{2+} : ferrous

Fe : ferrum

HCl : hydrochloric acid

$FeSO_4$: iron(II) sulfate

$<$: less than

$\leq$: less than or equal to

Mg : magnesium

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$MgCO_3$	:	magnesium carbonate
MeOH	:	methanol
>	:	more than
2iP	:	N^6-(2-isopentyl) adenine
NO^{3-}	:	nitrate
N	:	nitrogen
%	:	percentage
K	:	potassium
$K_2S_2O_8$	:	potassium persulfate
$NaC_2H_3O_2.3H_2O$	:	sodium acetate
Na_2CO_3	:	sodium carbonate
NaOH	:	sodium hydroxide
C(x+c)	:	total carotenoid (xanthophyll and carotene)
Ca + Cb	:	total chlorophyll a and b
Zn	:	zinc
BA	:	benzyladenine
BM	:	basal media
CEC	:	capillary electrochromatography
cm	:	centimeter
CM	:	callus induction media
DF	:	dilution factor
DMRT	:	Duncan's multiple range test
ET	:	electron transfer
FCR	:	Folin–Ciocalteu reagent
FRAP	:	ferric reducing power
g	:	gram

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g GAE/g DW	:	gram of gallic acid equivalents/gram of dry weight
g QE/g DW	:	gram of quercetin equivalents/g of dry weight
GC	:	gas chromatography
GI	:	growth index
HAT	:	hydrogen atom transfer
HPLC	:	high performance liquid chromatography
IAA	:	indole-3-acetic acid
IBA	:	indole-3-butyric acid
JA	:	jasmonic acid
JSM	:	jasmonic acid-stress media
kg	:	kilogram
LC-MS	:	liquid chromatography – mass spectrometry
µg/g	:	microgram per gram
µg/mL	:	microgram per milliliter
µL	:	microliter
mg	:	milligram
mg/g DW	:	milligram per gram of dry weight
mg/L	:	milligram per liter
mg/mL	:	milligram per milliliter
mm	:	millimeter
mM	:	millimolar
MAPKs	:	mitogen activated protein kinases
MW	:	molar weight
MS	:	Murashige and Skoog
MSO	:	Murashige and Skoog media without plant growth regulators
NMR	:	nuclear magnetic resonance

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ANOVA	:	one-way analysis of variance
g^{-1}	:	per gram
min^{-1}	:	per minute
PGR	:	plant growth regulators
RNS	:	reactive nitrogen species
ROS	:	reactive oxygen species
SE	:	standard error
SPSS	:	Statistical Package for the Social Sciences
TF		transcription factor
TAC	:	total anthocyanins content
TCh	:	total chlorophyll content
TC	:	total carotenoid content
TFC	:	total flavonoid content
TPC	:	total phenolic content
UV	:	ultraviolet
UV-B	:	ultraviolet-B
v/v	:	volume per volume

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Plants are known to be one of the essential food sources for all animals including human beings to continue living. Fundamental life processes in plants are supported by the production of primary metabolites. These primary metabolites include carbohydrates, lipids, and amino acids. In addition, plants are also able to produce secondary metabolites which have been proven to have significant roles in their interaction with environmental stresses and pathogen attacks (Yang et al., 2018).

In the industry, plant secondary metabolites are used in the production of drugs since they possess various pharmacological properties and have been identified to be safe to be utilized for medicinal purposes (Seca & Pinto, 2018). *Azadirachta indica* or neem plant used in this study is well known for its pharmaceutical values (Alzohairy, 2016; Saleh Al-Hashemi & Hossain, 2016). Several studies have shown that secondary metabolites isolated from neem plants have the potential to be an anti-bacterial (Quelemes et al., 2015; Sarmiento et al., 2011), anti-cancer (Arumugam et al., 2014; Paul et al., 2011), as well as anti-diabetic agents (Patil et al., 2013; Dholi et al., 2011). The usage of plant secondary metabolites in drug manufacturing in spite of current dependence on synthetic chemical drugs elevates the importance of plants in preventing and treating diseases.

However, it is difficult to ensure a continuous supply of secondary metabolites from plant sources because the growth of plants in nature is highly influenced by exogenous factors such as environmental and climate changes (Gahukar, 2014). Moreover, most of the compounds produced through secondary metabolism of plants are often present in a very small amount therefore, insufficient for testing a wide range of biological activities (Guerriero et al., 2018). Therefore, biotechnology approach is taken by introducing plant

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tissue culture as an alternative to help in producing and extracting valuable secondary metabolites with more reliable and simpler techniques, compared to extraction from complex whole plants (Hussain et al., 2012; Karuppusamy, 2009). Besides, better and greater production of plant secondary metabolites *in vitro* can be achieved by the addition of elicitors in the plant growth media (Ramakrishna & Ravishankar, 2011). Elicitors are compounds that trigger secondary metabolites formation in plants which can be biotic or abiotic. Examples of biotic elicitors are bacterial, fungal, polysaccharides, and yeast extract whereas abiotic elicitors can be grouped into three which are chemical, physical, and hormonal (Patel & Modi, 2018). Jasmonic acid (JA), a class of plant growth regulator, is one type of hormonal elicitors and has been commonly used as an abiotic elicitor in production of secondary metabolites from higher plants by *in vitro* tissue and cell cultures techniques (Guerriero et al., 2018; Golovatskaya & Karnachuk, 2008; Kovač & Ravnikar, 1998, 1994; Gundlach et al., 1992).

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CHAPTER 2

LITERATURE REVIEW

2.1 Origin and Taxonomy of *Azadirachta indica*

Based on a recent study, the exact origin of neem tree or scientifically known as *Azadirachta indica*, was reported to be in Upper Myanmar before it can be widely and commonly found in India since many years ago (Sujarwo et al., 2016). It is also found in other countries such as Bangladesh, Thailand, Nepal and Pakistan and was famous for its traditional usage especially in medicine and as bio-pesticides (Hossain et al., 2013; Debashri & Tamal, 2012). Therefore, several names has been attributed to *A. indica* due to its usefulness including "Divine Tree", "Life giving tree", "Nature's Drugstore", "Village Pharmacy" and "Panacea for all diseases" (Patel et al., 2016).

Table 2.1: Taxonomic classification of *Azadirachta indica* (References Kumar, Shankar, Subhapriya, & Nandhini (2018)).

The taxonomy of *A. indica*	
Kingdom	Plantae
Order	Rutales
Suborder	Rutinae
Family	Meliaceae
Subfamily	Melioideae
Tribe	Melieae
Genus	*Azadirachta*
Species	*Indica*

A. indica is classified in the family of Meliaceae (Table 2.1) or also called mahogany, in which, trees in this family are generally grown in the tropical and sub-tropical area and known for their ambrosial wood (Yadav et al., 2015). In one study, comparison of 44,495 genes of protein sequences against proteome of 23 sequenced plant species revealed that 23,125 genes (52%) of *A. indica* genome were categorized into 18,327 families (Figure 2.1) with 4,320 of them are multi-gene families (Kuravadi et al., 2015). Currently, there

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are two types of *Azadirachta* species: *Azadirachta indica* and *Azadirachta excelsa* which are classified and documented with several chemical and biological activities (David et al., 2017; Dewi et al., 2017; Gumilar et al., 2017; Rahmawathi et al., 2017; Koul et al., 1990).

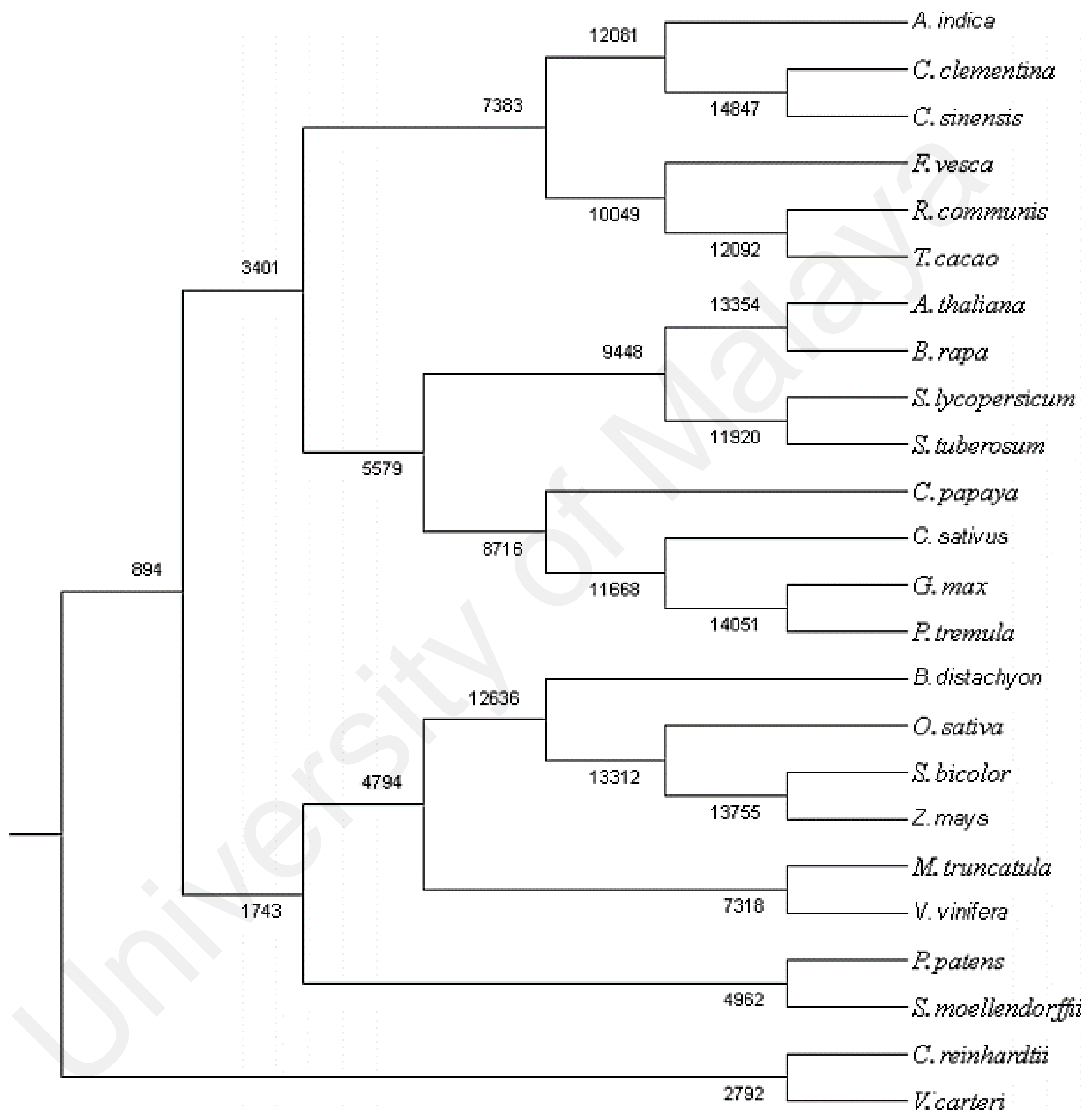

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Figure 2.1: Cluster of co-orthologous groups detected based on proteome similarity within the selected plant species (References Kuravadi et al. (2015)).

2.2 Morphological Description of *A. indica*

A. indica (Figure 2.2 [A]) is a medium to large, deep-rooted, and evergreen tree that can reach a height of 15 to 30 m tall, with a round and large crown of 10 to 20 m in diameter; branches spread and trunk branchless for up to 7.5 m and up to 90 cm in diameter respectively; bark characterized by moderate thickness, small and scattered but deeply fissure and flaked in old trees and dark grey outside and reddish inside, with colorless, sticky fetid sap (Ogbuewu et al., 2011; Orwa et al., 2009).

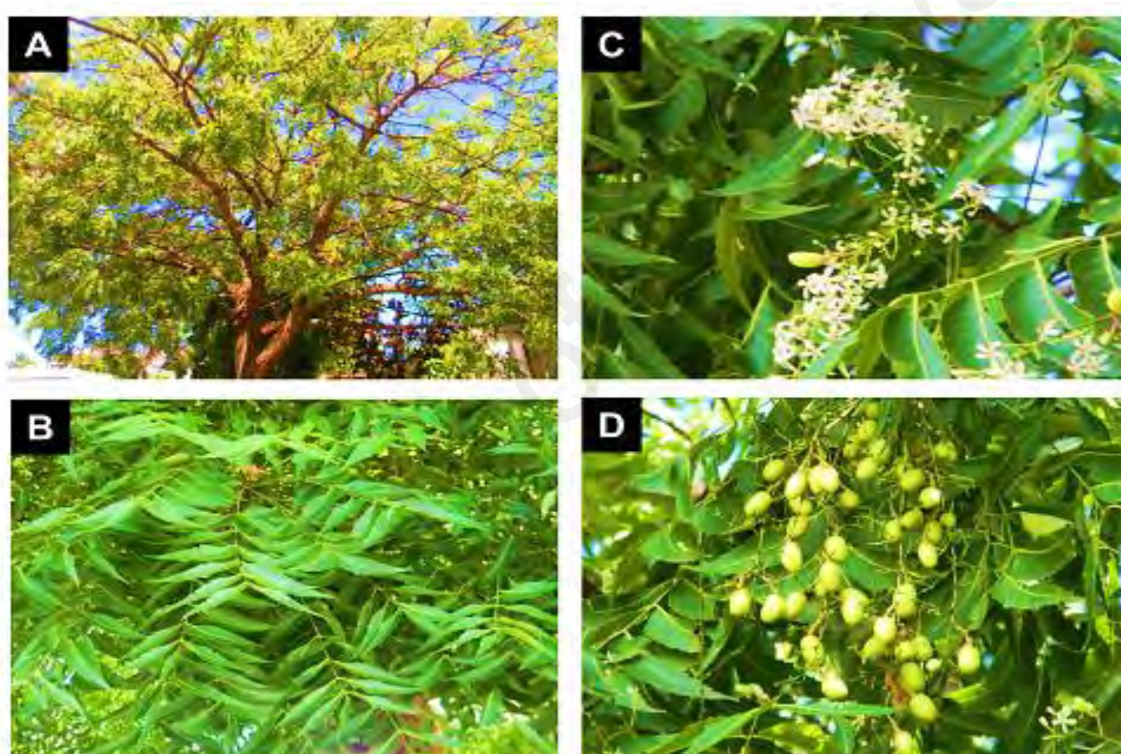

Figure 2.2: *A. indica* and its various parts; [A] the whole tree, [B] leaves, [C] flowers and [D] fruits (References Patel et al. (2016)).

Leaves of *A. indica* (Figure 2.2 [B]) are alternated and crowded near the end of branches; shape is simply pinnate with 20 to 40 cm long and of light green color with 2 pairs of glands at the base and or else glabrous; has petiole about 2 to 7 cm long and axial structure channeled above; leaflets are about 8 to 19 cm with very short petiole that alternate proximally and more or less distally (Orwa et al., 2009).

A. indica flowers (Figure 2.2 [C]) have an axillary mode of development and arrangement with many-flowered thyrsus that reach up to 30 cm long with minute and caduceus bracts; flowers are bisexual or can be male on same tree, actinomorphic, small and pentamerous; color of the flowers can be white or pale yellow with a slightly sweet scent. *A. indica* calyx lobes imbricate, sizably elliptic and thin and puberulous inside and petals free, imbricate, spathulate, spreading, ciliolate inside (Orwa et al., 2009).

The fruit (Figure 2.2 [D]) can be one- or two-seeded drupe, ovate, 1 to 2 cm long with greenish, greenish-yellow to yellow or purple color when ripe; thin exocarp, a pulpy mesocarp and cartilogenous endocarp; fruit's seed can be ovoid or spherical in shape with pointed apex and a thin testa that is composed of a shell and a kernel (sometimes 2 or 3 kernels) and each is about half of the seed's weight (Orwa et al., 2009).

2.3 Importance of *A. indica*

A. indica is well-acknowledged for its various beneficial properties such as antimicrobial, anti-inflammatory, anti-diabetic, antioxidant, antifeedant, and anticancer. Those properties are attributed to *A. indica* biologically active phytochemicals which can be extracted from almost all parts of the plant (Saleem et al., 2018; Alzohairy, 2016; Naik et al., 2014; Akter et al., 2013; Hossain et al., 2013; Gunadharini et al., 2011). Previous study had reported that methanolic extracts of *A. indica* were shown to exhibit zone of inhibition against Gram-positive as well as Gram-negative bacteria thus, proving its potential as antibacterial agent (Panda et al., 2016). In another study, extract of *A. indica* leaves inhibited the formation of *Pseudomonas aeruginosa* which proved the role of *A. indica* as antimicrobial agent (Harjai et al., 2013) whereas in a different study, *A. indica* oil extracts exhibited role as an antimicrobial agent for dental plaque when inhibition zones were seen on agar plate (Elavarasu et al., 2012).

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Other than as a health-promoting agent, antimicrobial properties of *A. indica* extract could help the textile industry by inhibiting microbial activity to produce an eco-friendly and non-toxic fabrics (Joshi et al., 2010). Tetranorterpenoid compounds for example, nimbolide, nimbin, deactylnimbin, mahmoodin, salannin, epoxy-azadiradione, deactylgedunin, gendunin, and including azadirachtin are among valuable phytochemical constituents of *A. indica* and believed to be responsible for the antiproliferative activity and cytotoxic effects of *A. indica* in inhibiting cancer activities (Santos et al., 2018; Nagini, 2014).

A. indica also has a significant value in timber industry due to its hardness and resistance towards termites, borers and fungi, hence, it is suitable to be used for making doors, windows, agricultural implements, carts, in ship and boat building, and in furniture (Council, 2002). As in repellent, *A. indica* was reported to affect more than 200 insect species, as well as some mites, nematodes, fungi, bacteria and viruses. It is widely planted in farms and plantation fields to discourage diseases and pests (M. Hussain et al., 2011; Council, 2002). As a biopesticide, *A. indica* significantly increase the yield of rice (*Oryza sativa*) when compared to conventional pesticide (Kamarulzaman et al., 2018). Other than that, the effect of toxicity of *A. indica* biopesticide was proven in a study involving *Drosophila melanogaster* when the fecundity and fertility of the insect was adversely affected after being subjected to Neem Azal; a commercial formulation of *A. indica* biopesticide (Oulhaci et al., 2018). In addition, field trials done by Khattak, Rashid, & Abdullah (2009) showed that at all tested concentrations, neem oil and seed water extracts reduced melon fruit fly (*Bactrocera cucurbitae* Coq.) infestation, where less number of pupae was observed from the fruits that were randomly collected in the treated plots in comparison to the control. The effectiveness of neem derivatives from this study was dependent on the treatment dose and nonetheless, at any concentration, gave no effect towards adult flies emergence.

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Azadirachtin (Figure 2.3) is one of the active components of *Azadirachta* species and remarkably known for its role in preventing insects attack because of its antifeedant, anti-infective, and antimicrobial properties (Archana et al., 2017; Chaudhary et al., 2017). It can be found in all parts of the tree but concentrated in mature seeds of *A. indica* (Krishnan et al., 2011) and was one of the first *A. indica* active compounds isolated and grouped into tetranorterpenoid (also known as limonoid) with molecular formula $C_{35}H_{44}O_{16}$ (Archana et al., 2017; Butterworth & Morgan, 1968).

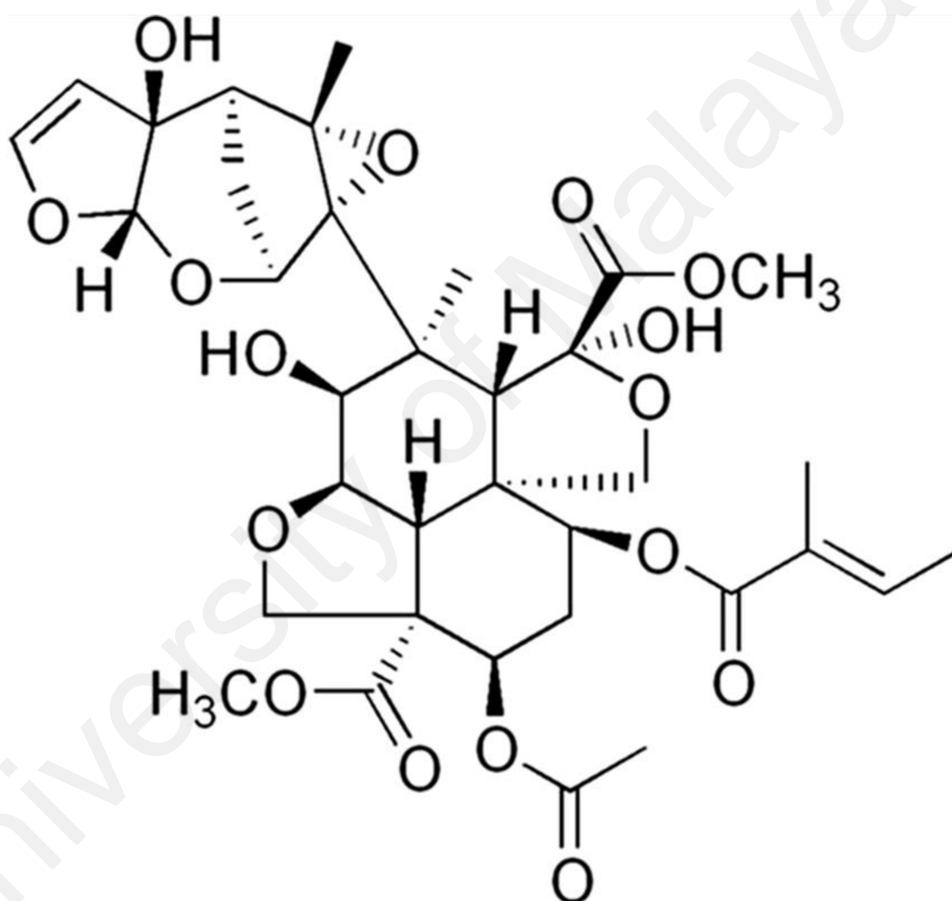

Figure 2.3: The azadirachtin molecule structure (References Baligar et al. (2014)).

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Commercially, there are about hundred *A. indica* formulations available in the market such as Azatin, Bio-Neem, Neemies, as well as Neemguard. The effectiveness of azadirachtin activity as biopesticide increases by formulating the compound with *A. indica* oil medium and other natural products of *A. indica* instead of using the pure compound solely (Sundari et al., 2016). Azadirachtin acts by exerting physiological effect in insect's midgut, and obstructing the growth and molting process of insects in addition

to inhibiting stimulation of insects feeding (Chaudhary et al., 2017). A study showed that azadirachtin caused apoptosis in midgut of *Spodoptera litura* larval which affected its nutrients intake and digestion, thus inhibited the process of *S. litura* to become a mature insect (Shu et al., 2018). Meanwhile, the field study of soybean plants revealed that, azadirachtin (AzaMax™) at concentration of 50 and 100 mg/L was effective and efficient in controlling velvetbean caterpillar (*Anticarsia gemmatalis*) reproduction and attack in soybean plants and exerted no adverse effect on the plant's major agent in biological control which was parasitoid *Trichogramma pretiosum*. Therefore, the product containing azadirachtin also can be used with other biological controlling agent as such *T. pretiosum* to prevent pest attack in the field (Almeida et al., 2010).

Besides, *A. indica* is also useful for wasteland recovery, therefore by growing and cultivating this species, soil fertility and water holding capacity can be improved, and acidic soils can be neutralized because it tolerates saline and alkaline soils with pH of up to 9.8 and soluble salt content up to 0.45 % in the subsoil (Yadav & Singh, 1970). Moreover, *A. indica* can be utilized to help improve soil fertility by using it as fertilizer, manure, soil conditioner and fumigant to eliminate soil denitrifying bacteria without harming the environment and the plant itself (Mondal & Chakraborty, 2016).

2.4 Application of Plant Tissue Culture Technology in Natural Compounds Biosynthesis

Plant tissue culture can be described as a system where totipotent cells maintains undifferentiated under specific conditions (Sussex, 2008; White, 1939) and the technique has been developed over years since it was first introduced through experiments of single cells culture in 1902 by a German scientist named Haberlandt (Espinosa-Leal et al., 2018; Haberlandt, 2003). The development of plant tissue culture technique application (Figure 2.4) in producing natural compounds or secondary metabolites originated from plant is

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driven by commercial significance of these compounds in various fields such as pharmaceutical, food, agricultural, cosmetic, and textile industries (Hussain et al., 2012).

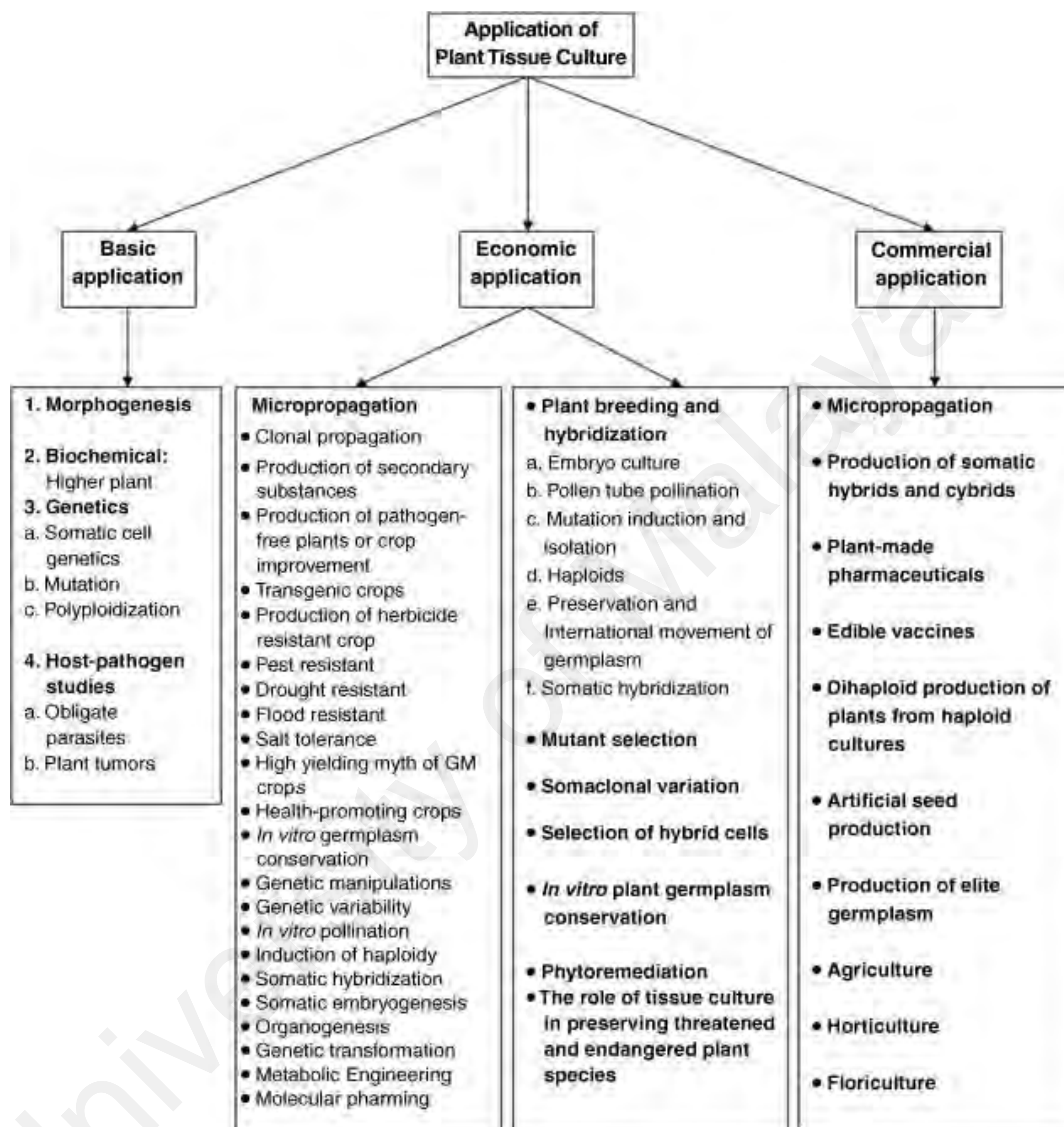

Figure 2.4: Plant tissue culture applications in the basic and applied research for plants improvement (References Bhatia (2015b)).

Basically, plant tissue culture technique starts with induction of callus (mass of cells) stage, where freshly cut explant, such as leaves or seeds, is transferred onto growth hormones supplemented nutrient medium (Espinosa-Leal et al., 2018). Callus (Figure 2.5) is formed as a result of unorganized cell division for wound healing process at the cut or wounded area and it can either be maintained undifferentiated yet still capable of growing

unlimitedly in new nutrient medium or further differentiated to form adventitious roots, shoots, as well as embryo depending on provided particular conditions (Neumann et al., 2009; White, 1939). To ensure the success of callus formation, it is very important that this technique is being executed under sterile and aseptic conditions (Ikenganyia et al., 2017).

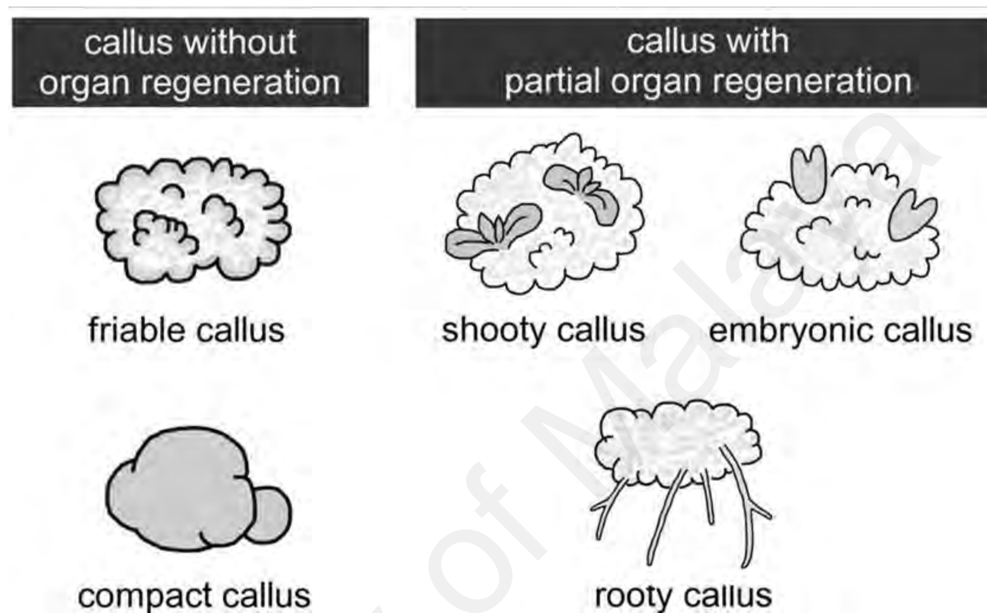

Figure 2.5: Illustration of callus with different observable tissues characteristics (References Ikeuchi, Sugimoto, & Iwase (2013)).

In isolation and characterization of plant natural bioactive compounds analysis, the quality of the starting material is very crucial therefore, cultivating plants in fields to get the starting material for this purpose is not practical due to time restriction, management issues and environmental factors (Patel, 2013). *Panax ginseng*, for example, is a slow-growing plant in which its roots take about 6 years before they can be harvested (Bonfill et al., 2002). Besides, the composition of starting material planted in fields is influenced by external factors which barely can be controlled such as soil composition, endophytic organisms, altitude, climate, processing, and storage conditions, which later will affect the evaluation of targeted natural bioactive compounds (Atanasov et al., 2015).

Plants cultivated through plant tissue culture have comparatively short growth cycle and their growth is independent of geographical, seasonal, and, environmental differences, enabling the continuous production of many genetically identical plants with favorable characteristics as well as uniform quality and yield in a short amount of time (Akin-Idowu et al., 2009). Besides, a disease-free plant material can be produced through plant tissue culture technology, which will omit the usage of pesticide and herbicide in plantation (García-Gonzáles et al., 2010). Examples of plant pathogenic organism elimination techniques are explants disinfection and meristem cultures which can be done by chemical or physical methods (Smith, 2012).

2.5 Types and Importance of Plants Secondary Metabolites

The two different groups of plant metabolites are classified into primary and secondary metabolites. All basic processes carried out by plants such as photosynthesis, respiration, growth and development make use of primary metabolites (Gandhi et al., 2015). Meanwhile, plants secondary metabolites are generally known to be involved in plant defense, interaction between plants and symbiotic microorganisms and being attractors of pollinators as well as seed dispersers (Yang et al., 2018). Figure 2.6 shows various type of stresses that influence physiological processes in plants in which later also affect the production of plant secondary metabolites (Savvas & Gruda, 2018). Environmental components that may induce physiological changes of plants include external conditions such as temperature, light, humidity, as well as other types of geo-climatic and seasonal changes (Berini et al., 2018; Zykin et al., 2018; Chetri et al., 2013; Ramakrishna & Ravishankar, 2011; Morison & Lawlor, 1999).

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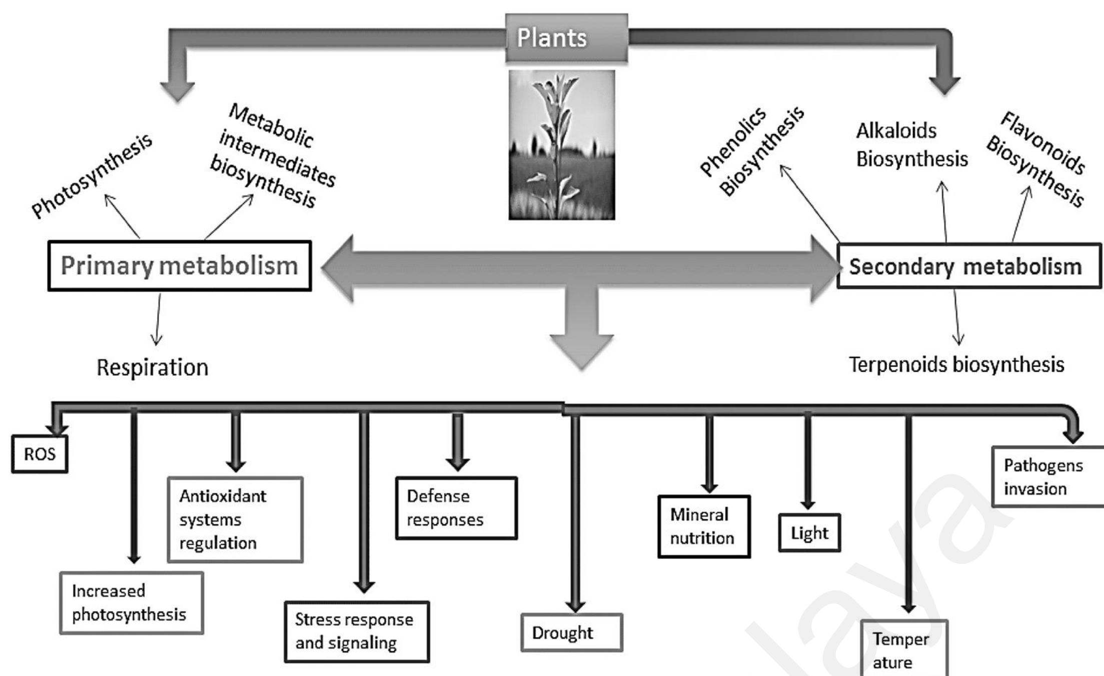

Figure 2.6: Two types of metabolisms in plants and different types of stresses that may trigger plants' physiological processes and influencing their metabolites production (References Isah (2019)).

The classification of plants secondary metabolites can be made based on their chemical structures which divided them into three main groups namely terpenoids, alkaloids and phenolics (Hussein & El-Anssary, 2018). Terpenoids are the largest class of secondary metabolites which comprise of 40,000 different compounds that have therapeutic effects on different kind of diseases (Misawa, 2011) (Figure 2.7). In one study, a diterpenoid produced by *Taxus brevifolia* and other *Taxus*-species named paclitaxel (taxol) was reported to have a great potential as an anticancer agent (Lenka et al., 2012). Classification of terpenoids is basically made depending on the number and structural organization of the five carbon isoprene units involved in their synthesis which includes C5 hemiterpenoids, C10 monoterpenoids, C15 sesquiterpenoids, C20 diterpenoids, C25 sesterterpenoids, C30 triterpenoids, C40 tetraterpenoids, and C>40 polyterpenoids (Abdallah & Quax, 2017).

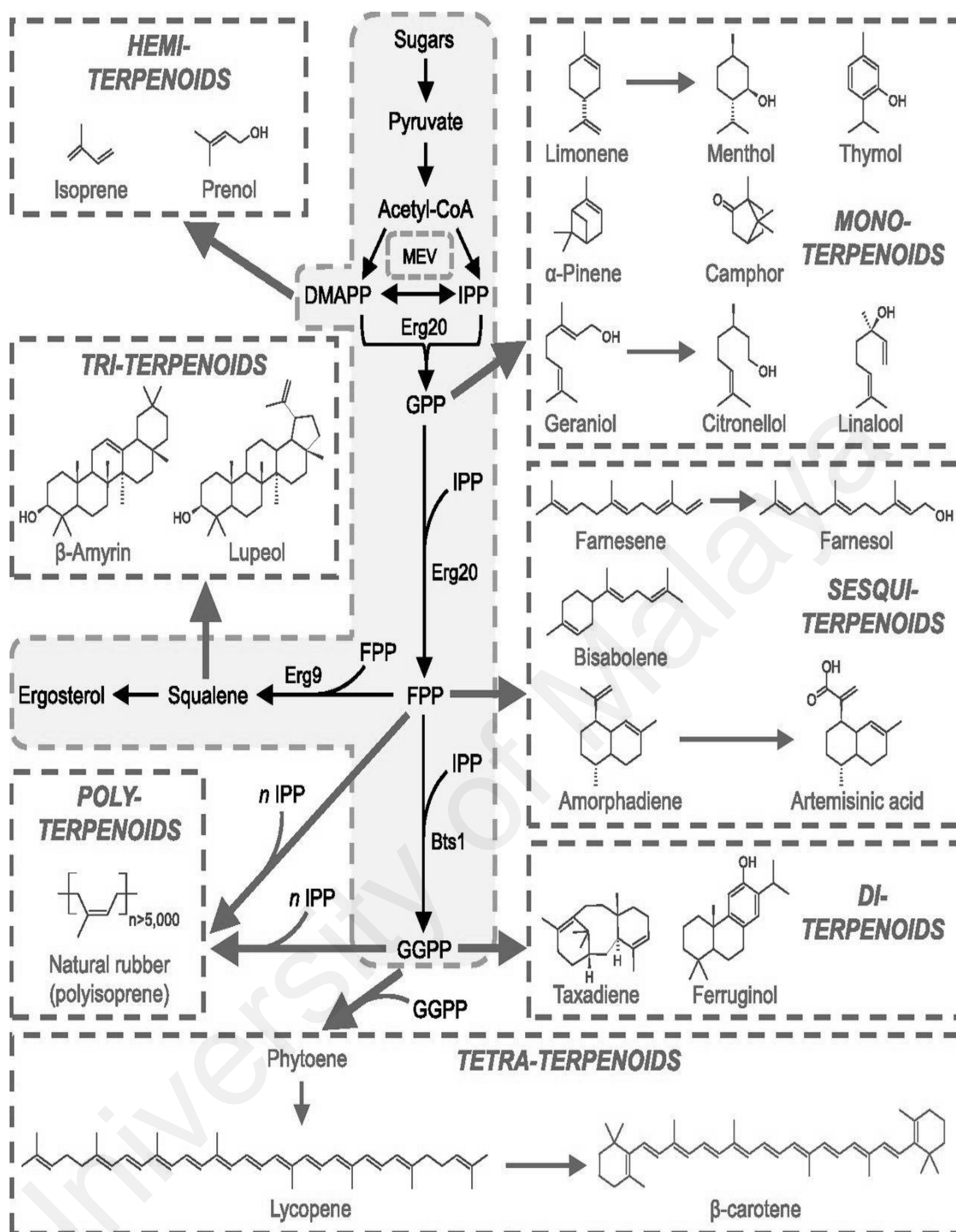

Figure 2.7: Biosynthetic pathway and structure of different groups of terpenoids (References Pyne, Narcross, & Martin (2019)).

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The next largest class of plant secondary metabolites known as alkaloids consists of approximately 12,000 low-molecular weight compounds (Ziegler & Facchini, 2008). The presence of a basic nitrogen atom at any position in the molecule becomes the basis in categorizing these compounds by excluding nitrogen in an amide or peptide bond (Ncube

& Van Staden, 2015) (Figure 2.8). Ecologically, alkaloids act as anti-feedants and toxins to pests by interfering with their nervous system in which they interact directly with molecular targets in the system (Kennedy & Wightman, 2011).

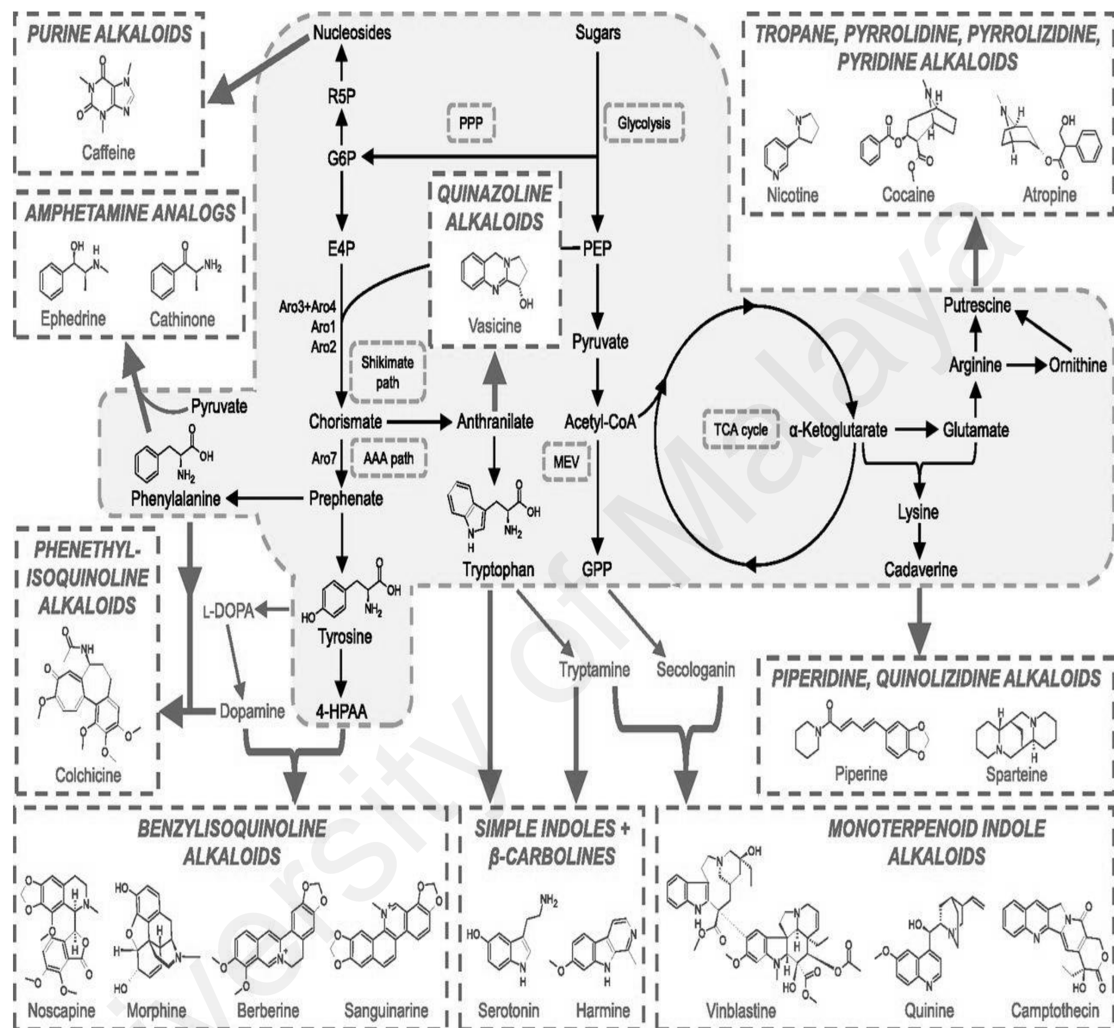

Figure 2.8: Biosynthetic pathway and structure of different groups of alkaloids (References Pyne et al. (2019)).

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The third largest class of secondary metabolites encompasses of phenolic compounds which is biosynthesized from precursors provided by glycolytic and pentose phosphate pathways to the shikimate pathway (Caretto et al., 2015). Figure 2.9 shows different classes of phenolic compounds which are flavonoids, stilbenes, coumarins, and other phenols and the number of aromatic ring possessed by the compounds which becomes the main principal of their classification (Dai & Mumper, 2010). In higher plants, these phenolics compounds are believed to be involved in defense reaction against different types of stresses (Nakabayashi & Saito, 2015; Suzuki et al., 2014; Atkinson & Urwin, 2012; Ahuja et al., 2010). Structurally, phenolics have at least one aromatic ring with one or more hydroxyl groups which may further be esterified, methylated, etherified or glycosylated (Fresco et al., 2006). In addition to that, grouping of phenolic compounds are also influenced by several other factors such as the nature and complexity of the basic carbonaceous skeleton, the degree of skeletal modification and the link between the base unit and other molecules, including primary and secondary metabolites (Ewané et al., 2012).

These metabolites are diverse and some of them accumulate specifically depending on taxonomically related species but usually have similar roles (Table 2.2) in plants interaction with environment and immune mechanism against microbial pathogens for plant defense (Delgoda & Murray, 2017). Besides, numerous researches have been done to study the potential of these metabolites to be used as raw materials or active ingredients particularly in foods, cosmetics and medicine due to their astounding biological activities (Grof, 2018; Korkina et al., 2018; Seca & Pinto, 2018; Clerici & Carvalho-Silva, 2011).

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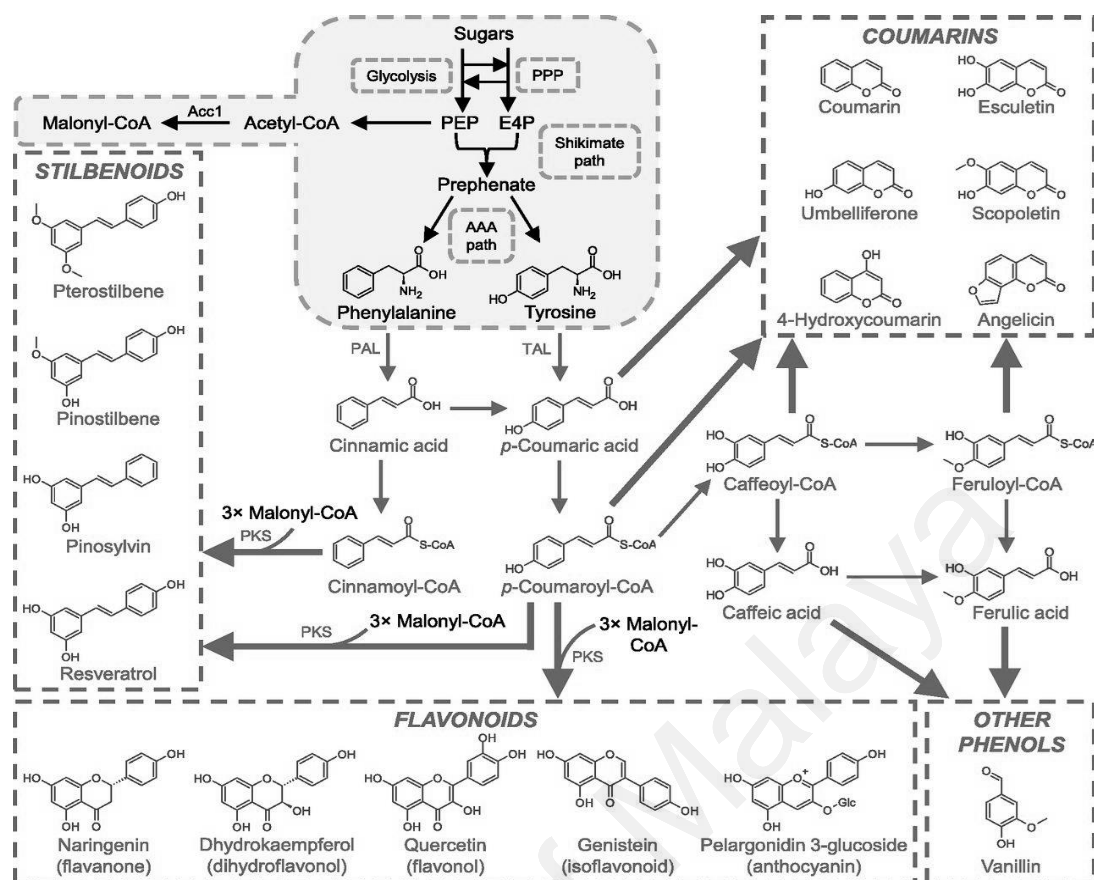

Figure 2.9: Biosynthetic pathway and structure of different groups of phenolics (References Pyne et al. (2019)).

Table 2.2: Different types of secondary metabolites produced in specific plants and their roles.

Plant species	Secondary metabolites	Key roles	Reference(s)
Allium sativum (garlic)	Allicin	Provides protection against bacteria such as *Bacillus* spp., *Streptococcus* spp., *Vibrio cholerae* and *Salmonella typhimurium*.	Borlinghaus et al., 2014; Small et al., 1947; Cavallito & Bailey, 1944
Solanum lycopersicum (tomato)	Sorbitol	Provides protection against abiotic stresses like salinity of soil, drought and osmotic stresses.	Almaghamsi et al., 2020; Tari et al., 2010

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Table 2.2, continued.

Plant species	Secondary metabolites	Key roles	Reference(s)
Antirrhinum (snapdragon flowers)	Methyl benzoate	A scent compound or volatile ester known to be attractants for pollinators (bumblebees).	Horiuchi et al., 2007; Negre et al., 2003; Pichersky & Gershenzon, 2002; Dudareva et al., 2000

2.6 Factors Influencing *in vitro* Plant Regeneration and Biosynthesis of Secondary Metabolites

For an efficient *in vitro* production of secondary metabolites, research was done by taking into account different factors influencing the response of culture in *in vitro* plant regeneration which include type and age of explants (Sánchez-Ramos et al., 2018; Adhikari et al., 2017), utilization of plant growth hormones (Twaij et al., 2020; Phillips & Garda, 2019; Osman et al., 2016; Hill & Schaller, 2013; Gaspar et al., 1996), elicitors supplementation (Darwish & Ahmed, 2020; N. Singh & Kumaria, 2020; Lian et al., 2019; Munim Twaij et al., 2019) and others. Artificial synthesis of secondary metabolites is difficult and not currently possible because of chemical and structural complexity of the compounds thus, optimization of factors contributing to high-quality natural compounds production with improved yield like secondary metabolites using *in vitro* technique is crucial (Bhatia, 2015b).

2.6.1 Selection of Elite Cell Lines for an Efficient Production System

In tissue culture, explant is the living tissue obtained from plant for *in vitro* culture purpose (Ali et al., 2007). Research done using tobacco as a model system showed that any part of the plant can be a totipotent explant, capable of proliferating into a callus and

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further develops to grow and regenerate various type of organs at different rates but in some cases, may not grow at all (Song et al., 2018; G. Ali et al., 2007).

In vitro cultures, particularly callus culture, have been chosen and used in many studies relating to plant secondary metabolites production because in comparison to the normal growth plants, they are more flexible and reliable (Adhikari et al., 2017; Dias et al., 2016; M. Ali & Abbasi, 2014). Therefore, to ensure the growth of healthy callus culture or plant regeneration for secondary metabolites production purpose, it is important to select for an explant or starting material that has a high potential of regenerating and not easily contaminated.

Potential of different organs and explants regeneration depends on which stage the cells are in the cell cycle, the availability or ability to transport endogenous growth regulators like auxins and cytokinins and the metabolic capabilities of the cells (Scofield et al., 2014). In a research study, meristematic ends of plants such as shoot tip, auxiliary bud tip and root tip are frequently used as the tissue explants because the cells in these types of tissue are capable of dividing at high rates and generating or accumulating important plant growth regulators (Machida et al., 2013). Besides, a study using banana plant showed that, explant material chosen for tissue culture also later determined the ploidy level (haploid or diploid) of the plantlets and if the explants used are ill-suited for tissue culture, high chances that microbial contamination will occur (Suman et al., 2012).

Problems may arise during culture establishment processs when the explants used are contaminated with endophytic microbes and to eliminate endophytic microbial contamination is quite troublesome (C. R. Singh, 2018). To eliminate of endophytic microbes, fungicides or antibiotics such as bavistin and chloramphenicol, can be added to the medium during growth (Bhatia & Sharma, 2015). In comparison to endophytic microorganisms, epiphytic microbes are easier to be removed from explants by gentle

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rinsing and surface sterilization as most of them do not form tight association with plant tissue (Tiwari et al., 2012). The association of epiphytic microbes with the explants can be observed through visual inspection and seen as a mosaic, de-colorization or localized necrosis (Oo et al., 2018). It is vital to eliminate these microbes because overgrow of these microbial contamination on the tissue culture medium will later affect the growth of plant tissue.

One way to obtain uncontaminated explants is by taking seedlings that are grown from surface sterilized seeds aseptically. Harsh surface sterilizing agents such as hypochlorite are used to sterilize seeds and these agents can barely penetrate the hard surface of the seed, making the sterilization of seeds conditions stricter than vegetative tissues (Bhatia, 2015a). Consequently, selecting elite cell lines with proper sterilization techniques in *in vitro* culture will reduce the risk of sample contamination thus, elevate the chance of callus establishment and secondary metabolites production efficiency.

2.6.2 Types of Plant Growth Hormones

The determination of developmental pathway of plant cells and tissues in culture medium is highly influenced by growth hormones such as auxins and cytokinins. For example, high concentration of auxins and cytokinins are known to promote formation of root and shoot respectively and a balanced amount of both will cause the formation of callus (García-Gonzáles et al., 2010). Synthetic auxins such as α-Naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) are among commonly used plant growth hormones that induce adventitious root formation in high concentrations relative to cytokinins. Meanwhile, 4-hydroxy-3-methyl-trans-2-butenylaminopurine (Zeatin), 6-furfurylaminopurine (Kinetin), 6-benzylaminopurine (BAP), N^6-(2-isopentenyl) adenine (2iP), and 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron or TDZ) are examples of growth hormone cytokinins used for callus induction and shoot formation (Bhatia, 2015a).

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A perusal of literature showed that other than functioning solely as cytokinin, TDZ has shown to exert both auxin and cytokinin-like effects and may induce or enhance biological activities in cells, even though chemically, TDZ is totally different from auxin and cytokinin (Guo et al., 2011). A recent study using different concentration of plant growth hormones treatments including TDZ, purine, picloram, and 2,4-D, reported that TDZ application had successfully induced maximum callus formation of four different species of *Garcinia* compared to other plant growth hormones used (Suwanseree et al., 2019). TDZ also has been used as elicitor to enhance the production of secondary metabolites where one study reported that the addition of 0.2 mM TDZ at day 10 resulted in better capsaicin accumulation by 181.48% in *Capsicum annuum* L. cell suspensions (Islek et al., 2016).

Moreover, a study using different combinations of plant growth hormones had succeeded in devising an efficient protocol for development of callus and regeneration system for *Allium sativum* L. In the study, addition of 0.5 mg/L of 2,4-D and 0.5 mg/L of Kinetin in MS medium were shown to be optimum for calli induction meanwhile, 0.5 mg/L of benzyladenine (BA) alone or 1.0 mg/L of BA together with 0.5 mg/L Kinetin in MS medium promoted optimum shoots regeneration and 2.0 mg/L of IAA and 0.5 mg/L of NAA in MS medium induced optimum rooting (Khan et al., 2017). At concentration of 0.5 mg/L of BA along with 0.5 mg/L TDZ, direct shoot organogenesis of *Lysionotus serratus* was shown to be effectively induced. Meanwhile, shoot proliferation was observed to be most effective in MS medium supplemented with 0.5 mg/L BA alone or combined with 0.1 mg/L NAA (Li et al., 2013). Besides, *Artemisia abrotanum* L. was successfully regenerated *in vitro* through somatic organogenesis where callus and shoot formed in MS medium containing 4.44 μM BA and 0.54 μM or 0.81 μM NAA and subsequently rooted in MS media supplemented with 0.49 μM IBA, 0.54 μM NAA, or without hormones (Bolyard, 2018).

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In one study, other than inducing callus formation, supplementation of plant growth hormones in MS medium was shown to enhance secondary metabolites production such as phenols, flavonoids, phthalide, and 3-butylidenephthalide in *Cnidiumofficinale* (Adil et al., 2018). Another study done using *Salvia leriifolia* Benth. cell suspension cultures showed that, 5 mg/L BAP and 5 mg/L NAA supplemented in 30 mL of liquid MS medium enhanced the production of phenolic acids, caffeic acid, salvianolic acid B, as well as rosmarinic acid at different stage of the cultivation cycle (Modarres et al., 2018).

2.6.3 Types of Biotic and Abiotic Elicitors

One of plant secondary metabolites' major roles is to survive biotic and abiotic stresses exerted upon plants (Zaynab et al., 2018). Based on this principle, some strategies for *in vitro* production of these metabolites have been developed to increase the yield which include treatment with different types of elicitors such as biotic and abiotic stresses (Thakur et al., 2019). The elicitation by biotic and abiotic elicitors *in vitro* or *in vivo*, invoked signals that will highly induce the formation of plant defense phytoalexins (Figure 2.10) and has been shown to be a feasible way to trigger the biosynthesis of plant secondary metabolites (Shakya et al., 2019).

Classification features of elicitors are made based on their origin and nature. Endogenous and exogenous elicitors are two types of elicitor that formed inside and outside of plant cells respectively. Exogenous elicitors originate from outside of the cell may induce a reaction through endogenous mediators in the cell meanwhile, endogenous elicitors formed through secondary metabolism are induced by a signal that is either of biotic or abiotic nature in cell (Goel et al., 2011). Biotic and abiotic elicitors are the two groups of elicitors classified based on their nature. Abiotic elicitors such as heavy metal ions, temperature, ultraviolet light and fungicides work via endogenously formed biotic elicitors (Biswas et al., 2016). Fungal homogenates and bacterial fractions are common biotic elicitors used to stimulate secondary metabolites production (Chandra & Chandra,

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2011). In one study, fungal elicitation of *Catharanthus roseus* callus was found to improve the biomass yield and increase the biosynthesis of vincristine and vinblastine via somatic embryogenesis (Tonk et al., 2016).

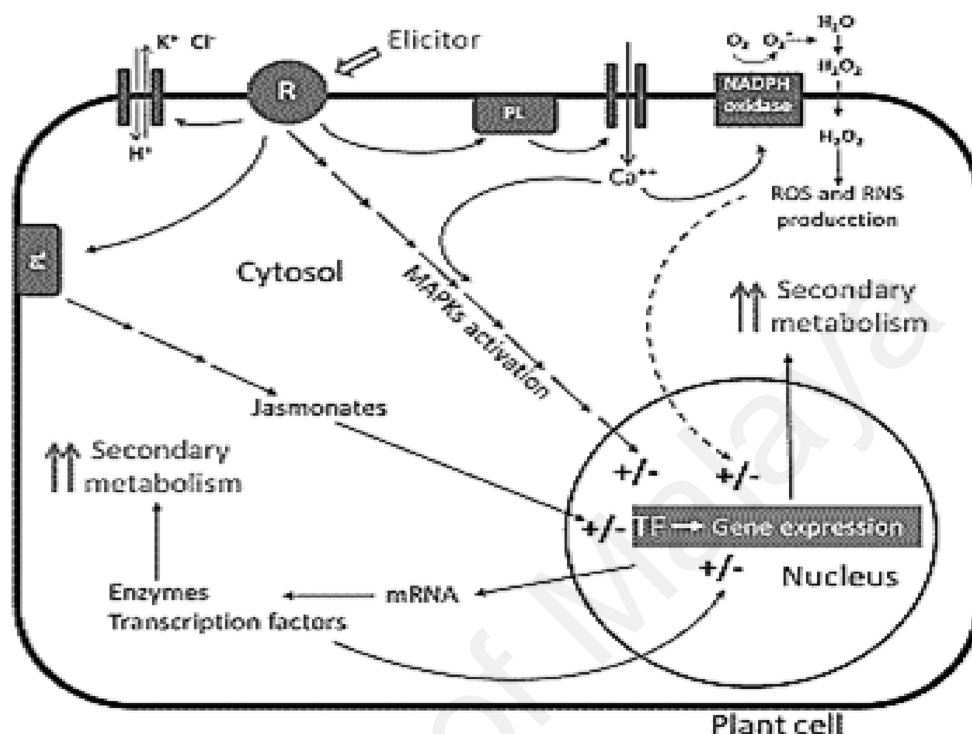

Figure 2.10: A diagram depicting molecular mechanisms of elicitation in plant cell (References Ramirez-Estrada et al. (2016)).

Jasmonic acid is one of the common elicitors used in experimental studies of secondary metabolites biosynthesis. Other than inducing the production of secondary metabolites, jasmonic acid plays a crucial role in protecting cells from the toxic effects of abiotic stresses and causes diverse responses resulted from jasmonic acid signaling through derepression of transcription factors (M. S. Ali & Baek, 2020; Gomi, 2020; Wang et al., 2020). A study reported by Złotek et al. (2016) showed that elicitation with 1 µM and 100 µM of jasmonic acid did improved the overall yield of basil essential oil and enhanced the biosynthesis of several chemical composition of the oil content which were linalool, eugenol, limonene and methyl eugenol. In one recent study, elicitation of *Hypericum aviculariifolium* and *H. pruinatum* with jasmonic acid was shown to increase the production of several secondary metabolites including hypericin, pseudohypericin,

hyperforin, adhyperforin, chlorogenic acid, neochlorogenic acid, hyperoside, isoquercitrin, quercitrin, quercetin, avicularin, rutin, (+)-catechin, and (-)-epicatechin in the plants (Cirak et al., 2020).

Factors that contribute to optimum elicitor employment include the concentration of elicitor and culture growth stage (Narayani & Srivastava, 2017). One study had reported that, in comparison to non-transgenic cells, the growth of *sts*-expressing transgenic *Vitis vinifera* cells was affected by addition of cyclodextrins and methyl jasmonate at the same concentration and the production of *trans*-resveratrol was enhanced (Chu et al., 2017). Other than that, *Puerarie tuberose* cell suspension culture was found to be best elicited with yeast extract at late stationary phase to improve the production of isoflavonoid (Goyal & Ramawat, 2008). It is important to apply elicitor the best way to make sure an efficient elicitor uptake by cultures in enhancing production of secondary metabolites.

Besides, an elicitor may exert non-specific effects which were seen in a study where four different types of fig leaves cultivars namely Deym, Sabz, Siah, and Shah were subjected to drought stress. Results revealed that, in all cultivars, α-tocopherol did increase in amount whereas concentration of ascorbic acid decreased in response to water deprivation (Gholami et al., 2012). Also, the elicitation using chitosan and salicylic acid in *Fagonia indica* callus cultures increased the amount of phenolic (16.9 µgGAE/mg) and flavonoid (2.2 µgQE/mg) content of the samples in comparison with the control which was TDZ induced callus of *F. indica* (Khan et al., 2019).

The effect of elicitation with biotic and abiotic stress on accumulation of secondary metabolites is also influenced by sample age or elicitor contact duration factor. A study of accumulation of ascorbic acid in tomato cell culture showed that, the amount of ascorbic acid determined in the samples harvested at 15 days interval until day 60 were statistically different from each other in which the oldest sample was shown to give the highest amount of ascorbic acid value when compared to 0, 15, 30, and 45 days samples

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(Minutolo et al., 2020). However, the sample of oldest age or longer time of incubation with elicitor does not necessarily always give the highest amount of metabolites accumulation as study done by Alhaithloul et al. (2020) demonstrated that the amount of secondary metabolites (phenols, flavonoids, and saponins) of *Mentha piperita* and *Catharanthus roseus* after 14 days of stress exposure was significantly lower in comparison to respective samples that were exposed to the same stress for 7 days. Meanwhile, a study done by Hashemi & Naghavi (2016) revealed that, exposure of *Papaver orientale* hairy roots to methyl jasmonate for 24 h did enhanced the accumulation of codeine compared to the control and higher exposure time decreased the amount of codeine of the cultures.

2.6.4 Light Source

Light plays a significant role in controlling *in vitro* biosynthesis of various types of bioactive metabolites and there are several key factors related to light radiation which include intensity, photoperiod (duration), and quality (Zoratti et al., 2014; Carvalho et al., 2010). The influence of light exposure in production of secondary metabolites was supported by a positive correlation between the growth-lighting condition and the content of flavonoids and chlorogenic acid (Alqahtani et al., 2015). Besides, several secondary metabolites such as flavonoids, chlorogenic acid, asiaticoside and madecassoside were shown to increase in amount when the plants were exposed to full day sunlight in comparison to plants grown under 50% shade condition (H. et al., 2012).

Besides, the growth and development of plants were shown to be affected by photoperiod which then also influenced the regulation of secondary metabolites production in plants (B. Yang et al., 2013; Jaakola & Hohtola, 2010). In one study, flavonoids and phenolic acids content in leaves of *Ipomoea batatas* was shown to increase greatly after being exposed to light irradiation for 16 h long period (Carvalho et al., 2010).

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Another study comparing metabolites composition of *Vaccinium myrtillus* in Northern and Southern regions of Finland revealed that, in southern clones, anthocyanin and its derivatives were at the highest amount during a 24-h light period and chlorogenic acid level was enhanced compared to a 12-h light period (Uleberg et al., 2012).

In one study using callus culture and UV irradiation, it has been shown that light quality also have an influence on synthesis of plants secondary metabolites (Ku et al., 2005). Moreover, Ramani and Jayabaskaran (2008) reported that, UV-B light did enhance the production of catharanthine and vindoline in suspension cultures of *Catharanthus roseus*. In addition, comparative study of UV irradiation effect on rutin, catechin and quercetin concentration in *Fagopyrum esculentum* and *F. tatricum* revealed that, exposure to enhanced UV irradiation could increase the concentration of quercetin specifically in *F. esculentum* (Regvar et al., 2012).

2.7 Problem Statement

Despite its distinct advantages, neem plants grown in nature has been reported to be infected by fungi and afflicted with various diseases. The infection often occurs at its early growth stages such as seedling stage and causes destruction to the growth of the plant as well as its metabolites production (Bhanumathi & Rai, 2007). It gets harder when most of plant metabolites produced through secondary metabolism can only be found in small amounts within the cells (Guerriero et al., 2018). Therefore, biological studies, specifically biotechnology through plant tissue culture is anticipated to maximize the production plant secondary metabolites through elicitor-induced stress response. It is of utmost importance so that these metabolites production can be of benefit to various fields to aid human lives.

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2.8 Significance of Study

This study utilizes plant tissue culture technique to improve the cultivation of *A. indica in vitro* and employs JA in the growth medium as an abiotic elicitor to trigger and boost the production of secondary metabolites in green callus of *A. indica*. These secondary metabolites are expected to accumulate and their production is enhanced in response to stress.

2.9 Objectives of Study

The objectives of the present study are as follow:

1. To evaluate the effect of jasmonic acid elicitation on the total amount of plant secondary metabolites (anthocyanins, chlorophylls, carotenoids, phenolics, flavonoids, chlorophylls and carotenoids,) in the elicited and non-elicited green callus of *A. indica.*
2. To analyze the effect of jasmonic acid elicitation on antioxidant potential (ABTS and FRAP assays) between elicited and non-elicited green callus of *A. indica.*
3. To analyze the effect of culture period on the stability of secondary metabolites in *A. indica*.

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CHAPTER 3

METHODOLOGY

3.1 Methodology Flowchart

Figure 3.1 shows the methodology outline for this research project starting from induction of *A. indica* green callus until the final statistical data analysis.

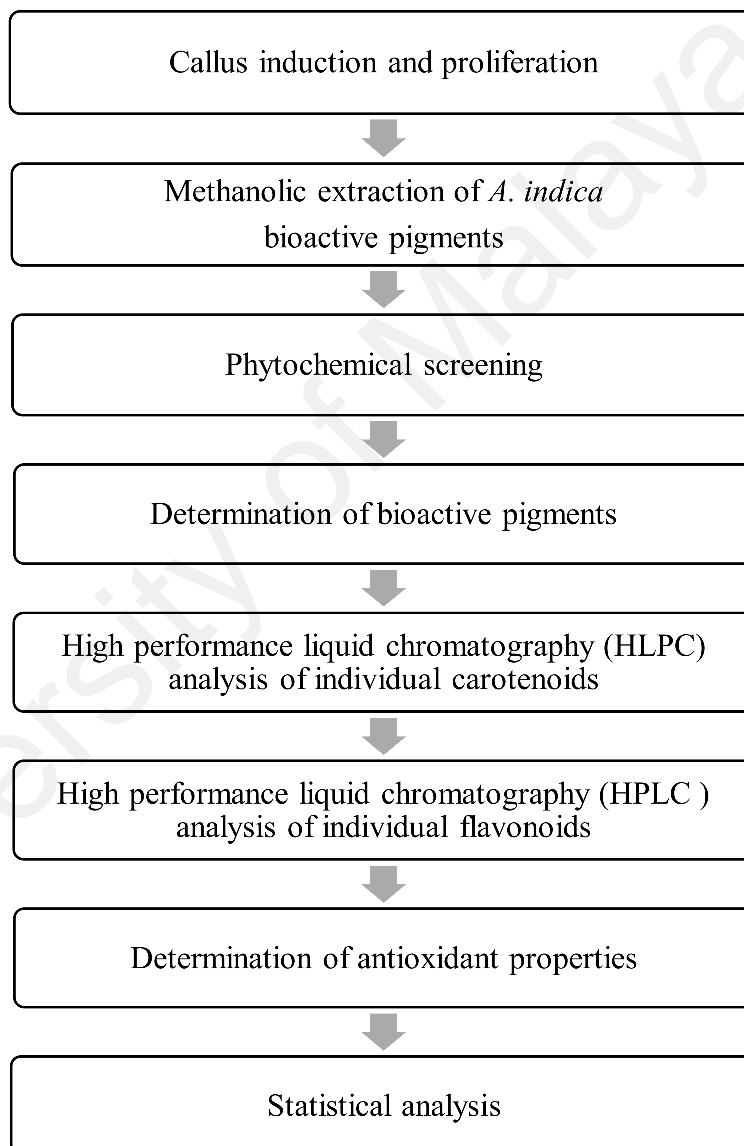

Figure 3.1: Methodology employed in this study includes methanolic extraction of *A. indica*, quantitative bioactive pigments, antioxidant, and statistical analysis.

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3.2 Callus Induction and Proliferation

Formation of callus was induced by cultivating young expanded *A. indica* leaves which were cut into small pieces on callus induction (CM) and MSO media. CM media consisted of 4.4 g of Murashige & Skoog (MS) media, 30 g/L of sucrose and 2 g/L of gelrite and supplemented with TDZ at concentration of 0.6 mg/L while MSO media was similar to CM media but devoid of any plant growth regulators. The pH of both media, CM and MSO, were adjusted to 5.8 using 0.1 M NaOH or 0.1 M HCl (Ashokhan et al., 2018). The callus cultures were maintained in the culture room at 25 ± 2ºC under a white fluorescent light. After 8 weeks of incubation, successfully developed green calli were subcultured onto JA-stress media (JSM), composed of CM added with different concentrations (0, 2, 4, and 6 mg/L) of jasmonic acid (Sigma chemical company St. Louis, Mo 63178 USA). The cultures were maintained in the culture room for another 4 weeks as well as 8 weeks before being harvested for subsequent analysis. Fresh weight of green callus developed on JSM media was recorded and growth index (GI) of each sample for each treatment was calculated to determine the growth efficiency of *A. indica* green callus (Bhatia, 2015a). The growth index (GI) was calculated using the formula described below (Sahraroo et al., 2014):

$$GI = \frac{W_1 - W_0}{W_0} \tag{3.1}$$

Where, $W_0 =$ weight of callus before treatment

$W_1 =$ final weight of callus after culture period

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3.3 Extraction of Bioactive Pigments from Green Callus of *A. indica*

Green calli were harvested from CM and JSM media and subjected to freeze drying process using Labconco freeze dryer (Labconco Corporation, MO 64132 United States) at -50 °C. About 2 g of freeze-dried green callus was ground on a chilled mortar and pestle together with trace amounts of $MgCO_3$ and sand to enhance pigment extraction process. Homogenized samples were then soaked in 60 mL of absolute methanol and incubated in the dark for 48 h at 4 °C.

After 48 h, the homogenates were filtered using filter paper before being centrifuged at 8000 rpm for 10 min, at 4 °C (Universal 32 R centrifuge Hettich Zentrifugen, D-78532 Germany). The clear colored supernatants were transferred into new tubes. A portion of these supernatants were used instantly for subsequent pigment analysis to measure the total chlorophyll, carotenoid and anthocyanin contents meanwhile, the remaining portion of the supernatants were concentrated under reduced pressure at 60 °C using rotary evaporator (Rotavapor R-3 BÜCHI Labortechnik, 9230 Switzerland) to remove excess solvent. The resultant dried extracts were weighed and re-dissolved in absolute methanol to a concentration of 20 mg/mL and used for subsequent total phenolics, total flavonoids, phytochemical and antioxidants analysis (Ashokhan et al., 2018).

3.4 Phytochemical Screening

Standard methods were used to screen the presence of different types of bioactive pigments such as alkaloids, flavonoids, phenols, tannins, and terpenoids (Solihah, 2012).

3.5 Determination of Bioactive Pigments

3.5.1 Total Anthocyanin Content

The pH differential method was used to determine the amount of monomeric anthocyanin pigment (cyanidin-3-glucoside) *of A. indica* green callus (Giusti & Wrolstad, 2001). The pH of each extract was adjusted to pH 1 and pH 4.5 and the absorbance was

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taken in triplicate at 510 nm and 700 nm using a Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA). The following formula was used to calculate the concentrations of monomeric anthocyanin pigments in green callus of *A. indica*:

$$Anthocyanin\,pigment\ content\ (mg/L) = \frac{(A \times MW \times DF \times 1000)}{(\varepsilon \times 1)} \tag{3.2}$$

$$Where, A = (Abs_{510} - Abs_{700})pH\ 1 - (Abs_{510} - Abs_{700})pH\ 4.5$$

$$MW(cyanidin - 3 - glucoside) = 449$$

$$DF = dilution\,factor \qquad \varepsilon = 26{,}900$$

3.5.2 Total Chlorophyll and Carotenoid Content

Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA) was used for photometric determination of chlorophylls (a and b) as well as carotenoids and the absorbance for every wavelength involved was taken in triplicate. The chlorophylls and carotenoids content was calculated as follows (Wellburn, 1994):

$$C_a(\mu g/mL) = 15.65\,A_{666} - 7.34\,A_{653} \tag{3.3}$$

$$C_b(\mu g/mL) = 27.05\,A_{653} - 11.21\,A_{666} \tag{3.4}$$

$$C_{(x+c)}(\mu g/mL) = \frac{(1000\,A_{470} - 2.86\,C_a - 129.2\,C_b)}{221} \tag{3.5}$$

3.5.3 Total Phenolic Content

Quantification of phenolic content was done using method described by Yusof et al. (2018), but with several changes. About 20 µL of 20 mg/mL sample extract was mixed with 150 µL of diluted Folin-Ciocalteu reagent (FCR) and incubated for 10 min at room temperature. Prior to the mixture incubation, FCR was diluted 10-fold with deionized

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water. Then, 150 µL of 2% Na_2CO_3 was added to the mixture and incubated for another 45 min in the dark. Blank was prepared using 70% methanol and the absorbance was taken at 765 nm in triplicate using Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA). A standard curve was prepared using gallic acid as the standard with six different concentrations (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 mg/mL). TPC of the samples was expressed as mg of gallic acid equivalents/g dry weight (g GAE/g DW) of callus.

3.5.4 Total Flavonoid Content

Total flavonoid content (TFC) in *A.indica* green callus was evaluated based on aluminium chloride colorimetric method (Yusof et al., 2018) with minor modifications. A total of 30 µL of 20 mg/mL sample extract was mixed together with 90 µL of 70% methanol, 6 µL of 10% aluminium chloride hexahydrate, 6 µL of 1 M sodium acetate, and 168 µL of distilled water. The mixture was incubated for 40 min and the absorbance was taken at 415 nm in triplicate using Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA). A quercetin standard curve of concentration ranging from 0.15 to 0.40 mg/mL was made to calculate TFC of the samples and the results were expressed as quercetin equivalents in g per gram of dry weight (g QE/g DW) of callus.

3.6 High Performance Liquid Chromatography (HLPC) Analysis of Individual Carotenoids

3.6.1 Extraction of Carotenoids for HPLC Analysis

HPLC-based quantification of individual carotenoids was done according to the method described by Othman (2009). For every sample, 1 g of freeze-dried green callus was mixed with 1 g of calcium carbonate and then rehydrated in 1 mL of distilled water. The tissue was soaked in 5 mL of acetone:methanol at ratio 7:3 overnight at room temperature. The next day, the mixture was vortexed and centrifuged at 13500 g for 2 min

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(Universal 32 R centrifuge Hettich Zentrifugen, D-78532 Germany). The supernatant was collected and transferred into foil-covered 50 mL graduated polypropylene centrifuge tubes. This procedure was repeated after adding in 5 mL of acetone:methanol of ratio 7:3 without addition of calcium carbonate until the sample became colourless. To remove residual fine particulates, centrifugation of combined supernatants was repeated at 13500 g for 5 min. Extraction of the carotenoids was done by adding 1:1 ratio of diethyl ether:distilled water to the sample mixture. After that, the solution was allowed to separate and the upper layer containing carotenoids was collected. By using diethyl ether alone, this procedure was repeated and the combined upper phase was then dried using a vacuum concentrator (Thermo Scientific, Waltham, MA, USA) to remove adhering solvents. The vials were then immediately capped, sealed with parafilm and stored at -80 °C until subsequent analysis.

3.6.2 Quantification of Individual Carotenoids by HPLC

Quantification of individual carotenoids content was analyzed using HPLC (Agilent 1200 series model) as described by Othman (2009). Separation was attained using ZORBAX SB-C18 end capped column (5 µm, 4.6 x 250 mm) (Waters, Milford, MA, USA). The eluents used for mobile phase were; (A) 9:1 v/v acetonitrile:water, and (B) ethyl acetate. The gradient program used was 0-40% solvent B (0-20 min), 40-60% Solvent B (20-25 min), 60-100% Solvent B (25-25.1 min), 100% Solvent B (25.1-35 min), and 100-0% Solvent B (35-35.1 min) and the flow rate was 1.0 mL/min. Prior to next sample injection, the column was allowed to re-equilibrate in 100% Solvent A for 10 min. The temperature of the column and injection volume were 20°C and 10 µL respectively. Carotenoid peaks were detected between wavelengths ranging from 350 to 550 nm. The extracts were screen for eight types of carotenoid , namely α-carotene, β-carotene, lycopene, lutein, neoxanthin, violaxanthin, zeaxanthin and β-cryptoxanthin.

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Individual carotenoids concentration were calculated as microgram per 1 g dry weight (μg/g DW).

3.7 High Performance Liquid Chromatography (HLPC) Analysis of Individual Flavonoids

3.7.1 Extraction of Flavonoids for HPLC Analysis

The extraction of flavonoids for HPLC analysis was done based on the method described by Seo et al. (2016) with minor modifications. The freeze-dried *A. indica* green callus of each treatment (2 g) was extracted with 20 mL of 80% ethanol at room temperature. After three days, the sample was filtrated. Ethanol was evaporated and freeze-dried to concentrate the extracts and then stored at -80 °C. Crude extracts were redissolved in ethanol and filtered using a 0.2 μM membrane.

3.7.2 Quantification of Individual Flavonoids by HPLC

Quantification of individual flavonoids content was performed on Agilent Technologies 1100 series model HPLC system (Waters, Milford, MA, USA) by following the method described by Seo et al. (2016). The column used was Agilent ZORBAX SB-C18 (5 μm, 4.6 x 250 mm) with mobile phase consisting of 100% acetonitrile. Sample was injected at a flow rate of 0.8 mL/min. The extracts were screened for six types flavonoids, namely rutin, kaempherol, kaempherol 3-glucoside, flavone, myricetin, and quercetin. Individual flavonoids concentration were calculated as microgram per 1 g dry weight (μg/g DW).

3.8 Determination of Antioxidant Properties

3.8.1 ABTS Free Radical Scavenging Activity Assay

Decolorization assay was used to determine the scavenging activity of ABTS free radical (Rajurkar & Hande, 2011). Initially, the solution of ABTS radical cation ($ABTS^{\cdot+}$)

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was prepared by mixing 10 mL of 2.45 mM potassium persulfate and 10 mL of 7 mM ABTS solution. Then, the mixture was incubated for 12 hours in the dark at room temperature. After that, double distilled water (ddH_20) was used to dilute the mixture until the absorbance produced is 0.70 ± 0.2 at 734 nm. Diluted $ABTS^{\cdot+}$ solution of 200 µL in volume was mixed with 20 µL of sample extract of six different concentrations and incubated for 30 minutes at room temperature. Absorbance was taken in triplicates at 734 nm.

$$\boldsymbol{ABTS^{\cdot+} scavenging\ activity}\ (\%) = \frac{\boldsymbol{A_0 - A_1}}{\boldsymbol{A_0}} \times \boldsymbol{100} \tag{3.6}$$

$$Where, A_0 = absorbance\ of\ ABTS^{\cdot+} and\ methanol$$

$$A_1 = absorbance\ of\ ABTS^{\cdot+}\ and\ sample\ extract\ or\ standard$$

3.8.2 Ferric Reducing Antioxidant Power (FRAP) Assay

FRAP assay of *A. indica* green callus extract was done following the standard protocol with slight changes (Benzie & Strain, 1999). FRAP reagent was prepared fresh during the assay by mixing 300 mM of acetate buffer (pH 3.6), 10 mM 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) solution and 20 mM iron (III) chloride hexahydrate ($FeCl_3.6H_2O$) in the ratio of 10:1:1 respectively. Then, FRAP reagent was incubated at 37 °C in the water bath for 30 minutes. 10 µL of each sample extract was mixed with 300 µL of FRAP reagent and vortexed gently. To prepare the blank, sample extract was substituted with methanol. The mixture was incubated for another 30 minutes at room temperature. Absorbance was taken in triplicate at a wavelength of 593 nm. Ferrous sulfate of known concentration was used to construct a standard curve thus, antioxidant value will be shown as ferrous equivalent Fe (II) in gram per gram of dry weight of callus.

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3.9 Statistical Analysis

The experiments were conducted in a completely randomized block design with 15 replicates of three cultures for each treatment. Results obtained were presented as mean values ± standard error (SE) of triplicate measurements. The significance of the difference between means was determined using one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) with significant difference level at $p < 0.05$ was then used to analyze the differences between the treatments. Meanwhile, correlations among antioxidant potential exhibited by the green callus extracts and bioactive pigments present in the callus were determined by Pearson correlation analysis using IBM SPSS Statistics version 24.

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CHAPTER 4

RESULTS

4.1 Induction and Development of *A. indica* Green Callus

4.1.1 Production of Green Callus of *A. indica in vitro*

Callus induction on CM media which contained 0.6 mg/L of TDZ started after 7 days of incubation and leaf explants were observed to further develop into green compact calli. The growth and morphology of the callus were observed for eight weeks (Figure 4.1). In contrast, the development of callus on MSO media was slower and there was no complete green callus formation noted from the leaf explants after eight weeks therefore, it could not be used for further analysis (Figure 4.2).

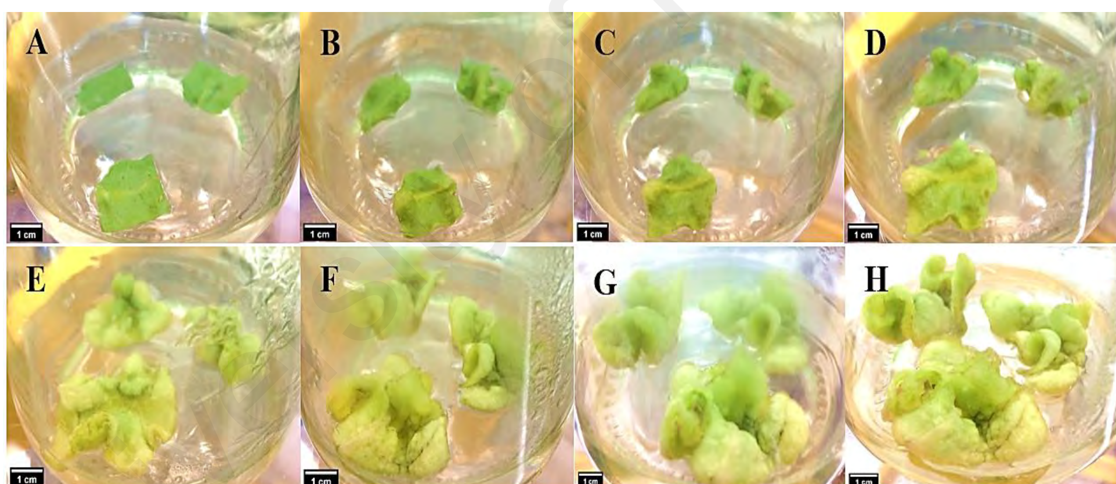

Figure 4.1: Callus induction and development observed on MS medium supplemented with 0.6 mg/L of TDZ (CM media) for 8 weeks; [A] week 1, [B] week 2, [C] week 3, [D] week 4, [E] week 5, [F] week 6, [G] week 7, and [H] week 8.

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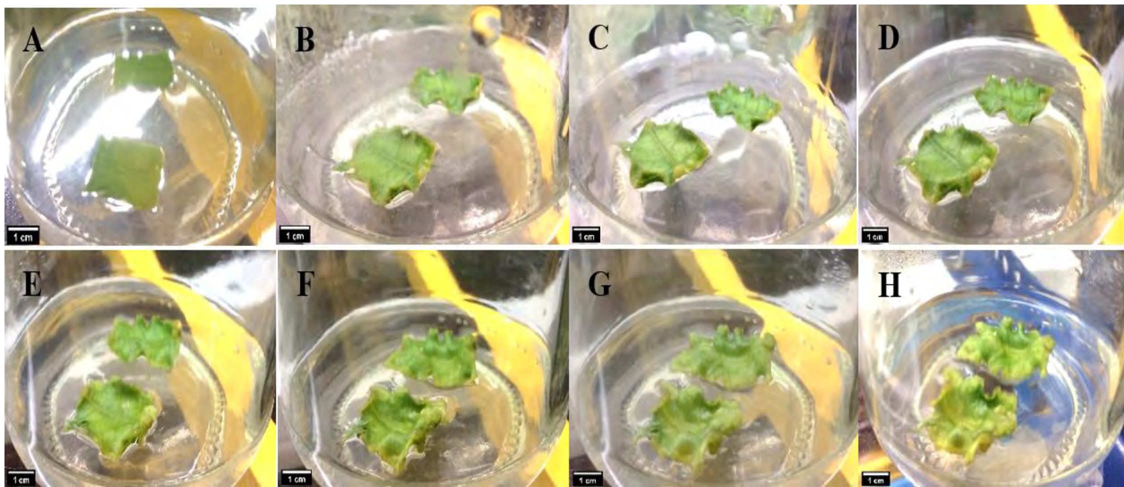

Figure 4.2: Callus induction and development observed on MS medium without PGR (MSO media) for 8 weeks; [A] week 1, [B] week 2, [C] week 3, [D] week 4, [E] week 5, [F] week 6, [G] week 7, and [H] week 8.

4.1.2 Fresh Weight and Growth Index (GI)

The effects of sample ages and addition of different concentrations of JA into CM media were evaluated to better understand their influence on callus performance. Callus growth was monitored by measuring the callus fresh weight and growth index (GI), as shown in Table 4.1. The results between different treatments are generally not significant to each another. However, treatment with 2 mg/L JA was the most effective in enhancing callus regeneration capacity after 4 weeks of culture, with callus fresh weight and growth index (GI) of 1.90 ± 0.18 g and 0.65 ± 0.02 respectively. Meanwhile, CM media produced the highest callus fresh weight (2.30 ± 0.35 g) and growth index (0.74 ± 0.02) after 8 weeks of culture.

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Table 4.1: Effect of TDZ and JA concentrations on callus fresh weight and growth index.

Sample ID	Treatment	Sample age			
		4-week-old		8-week-old	
		Fresh weight (g)	GI	Fresh weight (g)	GI
CM	0.6 mg/L TDZ	1.39 ± 0.21[ab]	0.61 ± 0.03[b]	2.30 ± 0.35[a]	0.74 ± 0.02[a]
2JA	0.6 mg/L TDZ + 2 mg/L JA	1.90 ± 0.18[b]	0.65 ± 0.02[b]	1.63 ± 0.24[a]	0.70 ± 0.03[ab]
4JA	0.6 mg/L TDZ + 4 mg/L JA	1.39 ± 0.14[ab]	0.53 ± 0.03[a]	2.03 ± 0.27[a]	0.71± 0.02[ab]
6JA	0.6 mg/L TDZ + 6 mg/L JA	1.23 ± 0.19[a]	0.50 ± 0.03[a]	1.98 ± 0.43[a]	0.66 ± 0.03[a]

Data expressed as mean values ± standard error (SE) of 15 replicates, each replicate consisted of 4 callus cultures. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *GI*: growth index.

4.1.3 Yield of Extract

A portion of the methanolic extracts were concentrated under reduced pressure. The yield of extracts gained from the concentration process were presented in Table 4.2. Concentrated extracts were then dissolved in methanol to a final concentration of 20 mg/mL and used for total phenolic, flavonoid, antioxidant, and phytochemical analysis.

Table 4.2: Yield of extracts of *A. indica* green callus.

Sample ID	Treatment	Samples age	
		4-week-old	8-week-old
		Yield of extract (g)	
CM	0.6 mg/L TDZ	0.41	0.42
2JA	0.6 mg/L TDZ + 2 mg/L JA	0.38	0.48
4JA	0.6 mg/L TDZ + 4 mg/L JA	0.36	0.34
6JA	0.6 mg/L TDZ + 6 mg/L JA	0.37	0.38

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid

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4.2 Phytochemical Screening

Methanolic extracts of *A. indica* green callus were subjected to qualitative screening of phytochemical constituents by following the standard protocols described by Solihah (2012). Results from the screening revealed the presence of alkaloids, flavonoids, phenols, tannins, and terpenoids in the methanolic extracts of *A. indica* green callus (Table 4.3).

Table 4.3: Qualitative screening of phytochemical constituents in green callus of *A. indica* methanolic extract.

Chemical constituents	Sample age	Treatment			
		CM	2JA	4JA	6JA
Alkaloids	4-week-old	+	+	+	+
	8-week-old	+	+	+	+
Flavonoids	4-week-old	+	+	+	+
	8-week-old	+	+	+	+
Phenols	4-week-old	+	+	+	+
	8-week-old	+	+	+	+
Tannins	4-week-old	+	+	+	+
	8-week-old	+	+	+	+
Terpenoids	4-week-old	+	+	+	+
	8-week-old	+	+	+	+

+ Present

**CM*: callus induction media composed of 0.6 mg/L TDZ; *JA*: jasmonic acid

4.3 Determination of Pigment Contents

4.3.1 Determination of Total Anthocyanin, Total Phenolic and Total Flavonoid Contents

The TAC, TPC and TFC of the 4-week-old and 8-week-old green callus were determined. TAC was expressed as mg per g dry weight of sample, while TPC was expressed as g Gallic Acid (GAE) per g dry weight of the sample, and TFC was expressed as g Quercetin (QE) per g dry weight of the sample. An increase in anthocyanins, phenolics, and flavonoids accumulation was noticed after elicitation with JA (for all concentrations) but the maximum accumulation was observed at two different time

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intervals; 4 weeks and 8 weeks. Data analysis revealed that *A. indica* green callus cultured on CM added with 6 mg/L JA (6JA) and 4 mg/L JA (4JA) resulted in the highest amounts of TAC, TPC, and TFC after 4 and 8 weeks of culture. In general, the TAC, TPC and TFC of methanolic extracts of 4-week-old *A. indica* green callus increased significantly with the increase in JA concentration (Table 4.4). Green callus extract of 4-week-old 6JA sample showed the highest TAC, TPC, and TFC with compounds content of 0.31 ± 0.00 mg/g DW, 3.96 ± 0.02 g GAE/g DW, and 0.55 ± 0.03 g QE/g DW respectively, followed by 4JA, 2JA, and CM.

Table 4.4: Effect of different concentration of jasmonic acid on total anthocyanin, phenolic, and flavonoid contents of 4-week-old *A. indica* green callus.

Sample ID	Treatment	Total anthocyanins content (mg/gDW)	Total phenolics content (g GAE/g DW)	Total flavonoids content (g QE/gDW)
CM	0.6 mg/L TDZ	0.14 ± 0.00^{a}	2.88 ± 0.10^{a}	0.29 ± 0.00^{a}
2JA	0.6 mg/L TDZ + 2 mg/L JA	0.23 ± 0.02^{b}	3.32 ± 0.00^{b}	0.37 ± 0.01^{b}
4JA	0.6 mg/L TDZ + 4 mg/L JA	0.25 ± 0.01^{b}	3.62 ± 0.01^{c}	0.43 ± 0.01^{c}
6JA	0.6 mg/L TDZ + 6 mg/L JA	0.31 ± 0.00^{c}	3.96 ± 0.02^{d}	0.55 ± 0.03^{d}

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid

However, the TAC, TPC, and TFC of the extracts of 8-week-old samples were found to decrease in CM media supplemented with high concentration of JA (6JA) (Table 4.5). Interestingly, green callus extract from 8-week-old 4JA samples showed significantly highest values for TAC, TPC, and TFC which were 0.32 ± 0.01 mg/g DW, 3.40 ± 0.05 g GAE/g DW, and 0.52 ± 0.01 g QE/g DW respectively, followed by 6JA, 2JA, and CM.

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Table 4.5: Effect of different concentration of jasmonic acid on total anthocyanin, phenolic, and flavonoid contents of 8-week-old *A. indica* green callus.

Sample ID	Treatment	Total anthocyanins content (mg/g DW)	Total phenolics content (g GAE/g DW)	Total flavonoids content (g QE/g DW)
CM	0.6 mg/L TDZ	0.08 ± 0.01[a]	1.76 ± 0.01[a]	0.18 ± 0.04[a]
2JA	0.6 mg/L TDZ + 2 mg/L JA	0.16 ± 0.01[b]	2.47 ± 0.02[b]	0.31 ± 0.01[b]
4JA	0.6 mg/L TDZ + 4 mg/L JA	0.32 ± 0.01[d]	3.40 ± 0.05[d]	0.52 ± 0.01[c]
6JA	0.6 mg/L TDZ + 6 mg/L JA	0.21 ± 0.00[c]	3.23 ± 0.07[c]	0.44 ± 0.02[c]

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).
**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid

4.3.2 Analysis of Chlorophylls and Carotenoids Content

The total amount of photosynthetic pigments such as chlorophylls and carotenoids were measured and expressed as mg per g dry weight (mg/g DW) of callus. Based on Table 4.6, photosynthetic pigments in 4-week-old *A. indica* green callus increased as the concentration of JA increased. 6JA showed the highest amount of total chlorophylls (1.00 ± 0.03 mg/g DW) and carotenoids (0.13 ± 0.01 mg/g DW) contents for 4-week-old samples when compared to control. Meanwhile, the amount of photosynthetic pigments contained in 8-week-old samples were generally lower as compared to 4-week-old samples (Table 4.7). 4JA green callus extract also contained the highest amount of total carotenoids (0.10 ± 0.01 mg/g DW) compared to the control, followed by 6JA (0.09 ± 0.00 mg/g DW), 2JA (0.08 ± 0.00 mg/g DW), and CM (0.07 ± 0.00 mg/g DW). Generally, the amount of chlorophyll a is higher in all extracts compared to chlorophyll b for both 4-week-old and 8-week-old samples.

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Table 4.6: Effect of different concentration of jasmonic acid in JSM on total chlorophyll and carotenoid contents of 4-week-old *A. indica* green callus.

Sample ID	Treatment	Pigments content (mg/g DW)			
		Ca	Cb	Ca + Cb	C (x+c)
CM	0.6 mg/L TDZ	0.50 ± 0.01^{a}	0.33 ± 0.01^{a}	0.82 ± 0.02^{a}	0.11 ± 0.00^{a}
2JA	0.6 mg/L TDZ + 2 mg/L JA	0.52 ± 0.00^{ab}	0.33 ± 0.01^{a}	0.85 ± 0.01^{bc}	0.12 ± 0.01^{ab}
4JA	0.6 mg/L TDZ + 4 mg/L JA	0.56 ± 0.00^{b}	0.34 ± 0.01^{a}	0.90 ± 0.01^{b}	0.12 ± 0.00^{ab}
6JA	0.6 mg/L TDZ + 6 mg/L JA	0.61 ± 0.02^{c}	0.39 ± 0.02^{b}	1.00 ± 0.03^{c}	0.13 ± 0.01^{c}

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT);

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *Ca*: chlorophyll a; *Cb*: chlorophyll b*;* *Ca* + *Cb*: total chlorophyll a and b*;* *C(x+c)*: total carotenoid (xanthophyll and carotene).

Table 4.7: Effect of different concentration of jasmonic acid in JSM on total chlorophyll and carotenoid contents of 8-week-old *A. indica* green callus.

Sample ID	Treatment	Pigments content (mg/g DW)			
		Ca	Cb	Ca + Cb	C (x+c)
CM	0.6 mg/L TDZ	0.33 ± 0.00^{a}	0.25 ± 0.00^{ab}	0.59 ± 0.00^{a}	0.07 ± 0.00^{a}
2JA	0.6 mg/L TDZ + 2 mg/L JA	0.36 ± 0.00^{b}	0.25 ± 0.01^{ab}	0.61 ± 0.01^{b}	0.08 ± 0.00^{ab}
4JA	0.6 mg/L TDZ + 4 mg/L JA	0.40 ± 0.00^{d}	0.26 ± 0.00^{c}	0.65 ± 0.00^{c}	0.10 ± 0.01^{b}
6JA	0.6 mg/L TDZ + 6 mg/L JA	0.38 ± 0.01^{c}	0.24 ± 0.01^{a}	0.61 ± 0.01^{b}	0.09 ± 0.00^{b}

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT);

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *Ca*: chlorophyll a; *Cb*: chlorophyll b*;* *Ca* + *Cb*: total chlorophyll a and b*;* *C(x+c)*: total carotenoid (xanthophyll and carotene).

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Table 4.8 and Table 4.9 presented below show the ratios of pigments content in methanolic extracts of *A. indica*. Weight ratio of chlorophyll a to chlorophyll b (Ca/Cb ratio) for 4-week-old extracts of CM, 2JA, 4JA, and 6JA were 1.52, 1.58, 1.66, and 1.56 respectively. As for CM, 2JA, 4JA, and 6JA of 8-week-old extracts, the Ca/Cb ratios were 1.32, 1.43, 1.54, and 1.58 respectively. Other than that, weight ratio of total chlorophylls to carotenoids (Ca + Cb/C(x+c) ratio) was determined to demonstrate the sample's greenness. Generally, greenness of samples were about the same, as can be observed based on Ca + Cb/C(x+c) ratios obtained in this study. The ratios for CM, 2JA, 4JA, and 6JA of 4-week-old samples were 7.19, 7.14, 7.34, and 7.68, respectively. Meanwhile, Ca + Cb/C(x+c) ratios for CM, 2JA, 4JA, and 6JA of 8-week-old samples were 8.19, 7.62, 6.96, and 6.80, respectively.

Table 4.8: Ratio of pigments content of 4-week-old *A. indica* green callus extracts.

Sample ID	**Treatment**	**Pigments content (mg/g DW)**	
		Ca/Cb ratio	**Ca + Cb/C(x+c) ratio**
CM	0.6 mg/L TDZ	1.52	7.19
2JA	0.6 mg/L TDZ + 2 mg/L JA	1.58	7.14
4JA	0.6 mg/L TDZ + 4 mg/L JA	1.66	7.34
6JA	0.6 mg/L TDZ + 6 mg/L JA	1.56	7.68

Data expressed as mean values ± standard error (SE) of three replicates.

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *Ca*: chlorophyll a; *Cb*: chlorophyll b; *Ca + Cb*: total chlorophyll a and b; *C(x+c)*: total carotenoid (xanthophyll and carotene).

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Table 4.9: Ratio of pigments content of 8-week-old *A. indica* green callus extracts.

Sample ID	Treatment	Pigments content (mg/g DW)	
		Ca/Cb ratio	Ca + Cb/C(x+c) ratio
CM	0.6 mg/L TDZ	1.32	8.19
2JA	0.6 mg/L TDZ + 2 mg/L JA	1.43	7.62
4JA	0.6 mg/L TDZ + 4 mg/L JA	1.54	6.96
6JA	0.6 mg/L TDZ + 6 mg/L JA	1.58	6.80

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT);
**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *Ca*: chlorophyll a; *Cb*: chlorophyll b; *Ca + Cb*: total chlorophyll a and b; *C(x+c)*: total carotenoid (xanthophyll and carotene).

4.3.3 Analysis of Individual Carotenoids through HPLC

Previous data analysis revealed that 6JA and 4JA samples resulted in the highest amount of total carotenoid content in 4-week-old and 8-week-old green callus, respectively. Thus, the samples were subjected to a HPLC analysis to determine and compare the presence of individual carotenoids in the samples compared to the control (CM). The extracts were screened for the presence of eight common carotenoids such as α-carotene, β-carotene, lycopene, lutein, neoxanthin, violaxanthin, zeaxanthin and β-cryptoxanthin. Data analysis revealed the presence of four types of carotenoid in the samples, including β-carotene, lutein, neoxanthin, and violaxanthin. For 4-week-old callus extracts, CM was found to contain neoxanthin (0.93 ± 0.02 µg/g DW), violaxanthin (9.02 ± 0.61 µg/g DW), lutein (263.24 ± 4.96 µg/g DW), and β-carotene (2.59 ± 0.04 µg/g DW), however 6JA callus was found to contain only lutein (431.81 ± 1.60 µg/g DW) (Table 4.10). As depicted in Table 4.11, 8-week-old CM callus also showed the presence of only lutein (478.97 ± 4.59 µg/g DW), meanwhile 4JA callus extracts showed the absence of all eight carotenoids. The HPLC chromatograms also revealed the presence of

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unresolved peaks for 4-week-old 6JA callus extracts and 8-week-old CM and 4JA callus extracts (Figure 4.3 and 4.4).

Table 4.10: Distribution and amount of individual carotenoids present in 4-week-old green callus of *A. indica.*

Sample ID	Treatment	Individual carotenoid (µg/g DW) Neoxanthin	Violaxanthin	Lutein	*β*-carotene
CM	0.6 mg/L TDZ	0.93 ± 0.02	9.02 ± 0.61	263.24 ± 4.96[a]	2.59 ± 0.04
6JA	0.6 mg/L TDZ + 6 mg/L JA	ND	ND	431.81 ± 1.60[b]	ND

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Independent samples t-test.

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *ND*: not detected; *U*: unknown.

Table 4.11: Distribution and amount of individual carotenoids present in 8-week-old green callus of *A. indica.*

Sample ID	Treatment	Individual carotenoid (µg/g DW) Neoxanthin	Violaxanthin	Lutein	*β*-carotene
CM	0.6 mg/L TDZ	ND	ND	478.97 ± 4.59	ND
4JA	0.6 mg/L TDZ + 4 mg/L JA	ND	ND	ND	ND

Data expressed as mean values ± standard error (SE) of three replicates.

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *ND*: not detected; *U*: unknown.

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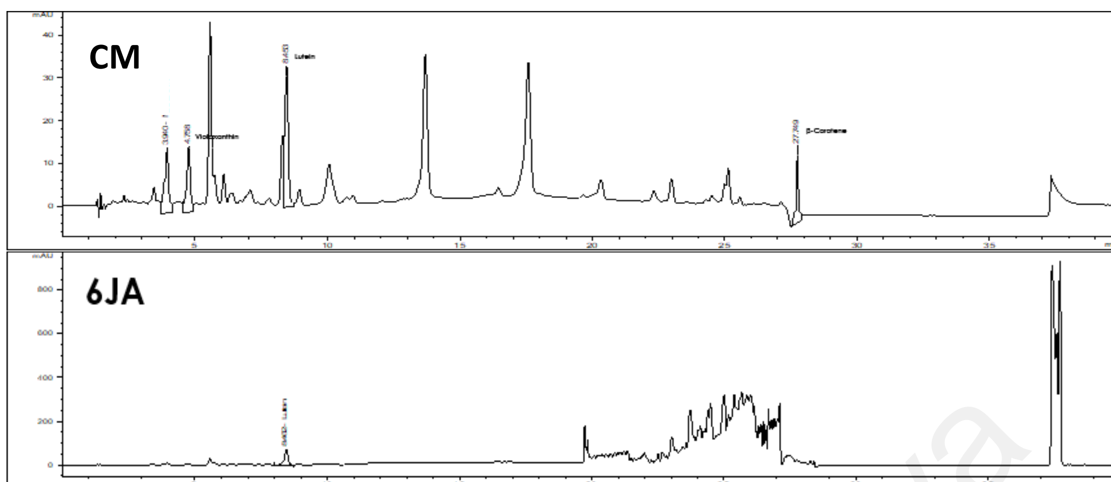

Figure 4.3: HPLC chromatograms showing the presence of carotenoid in green callus extracts of 4-week-old CM and 6JA samples.

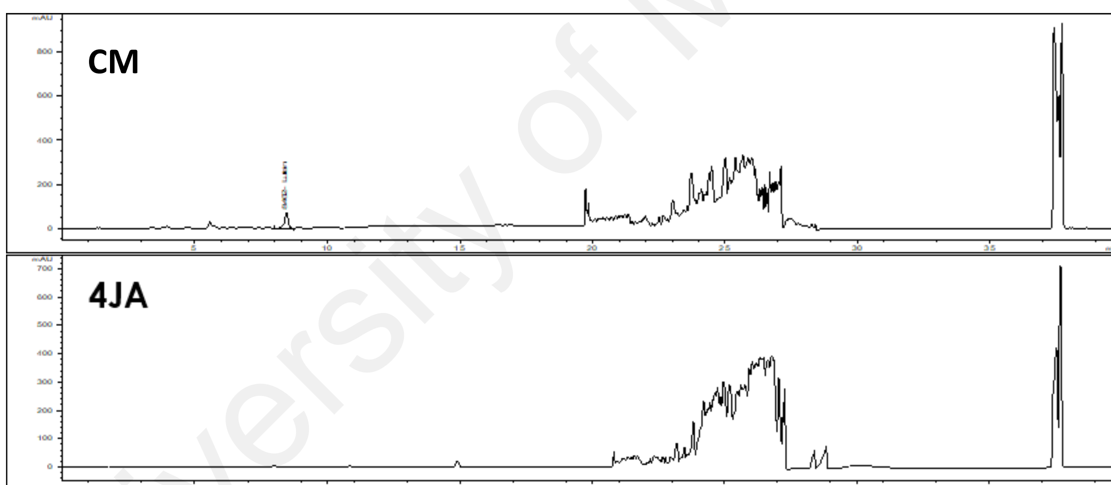

Figure 4.4: HPLC chromatograms showing the presence of carotenoid in green callus extracts of 8-week-old CM and 4JA samples.

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4.3.4 Analysis of Individual Flavonoids through HPLC

Previous data analysis revealed that 6JA and 4JA samples resulted in the highest amount of TFC in 4-week-old and 8-week-old green callus, respectively. Thus, the samples were subjected to a HPLC analysis to determine and compare the presence of individual flavonoids in the samples compared to the control (CM). Flavonoids detected include rutin, kaempherol, flavone, and myricetin (Table 4.12 and Table 4.13).

According to the results, green callus extract of 4-week-old CM sample exhibited the highest amount of rutin (218.08 ± 18.08 µg/g DW) and flavone (5.30 ± 0.00 µg/g DW). On the other hand, 4-week-old 6JA extract showed significantly higher amount of kaempherol (14.45 ± 0.43 µg/g DW) and myricetin (45.32 ± 0.01 µg/g DW). For 8-week-old sample extracts, the amount of rutin (224.73 ± 1.94 µg/g DW) and kaempherol (4.20 ± 0.12 µg/g DW) were significantly higher in 4JA compared to CM. Interestingly, the amount of flavone and myricetin for 8-week-old CM and 4JA green callus extracts are similar, despite the fact that 4JA extract was elicited with JA. Figure 4.5 and 4.6 represent HPLC chromatograms showing the peak area of detected compounds.

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Table 4.12: Distribution and amount of individual flavonoids present in 4-week-old green callus of *A. indica*.

Sample ID	Treatment	Individual flavonoid (µg/g DW)			
		Rutin	Kaempherol	Flavone	Myricetin
CM	0.6 mg/L TDZ	218.08 ± 18.08[a]	6.45 ± 0.09[a]	5.30 ± 0.00[a]	44.82 ± 0.01[a]
6JA	0.6 mg/L TDZ + 6 mg/L JA	110.58 ± 1.66[b]	14.45 ± 0.43[b]	5.28 ± 0.00[a]	45.32 ± 0.01[b]

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Independent samples t-test.

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid

Table 4.13: Distribution and amount of individual flavonoids present in 8-week-old green callus of *A. indica*.

Sample ID	Treatment	Individual flavonoid (µg/g DW)			
		Rutin	Kaempherol	Flavone	Myricetin
CM	0.6 mg/L TDZ	216.61 ± 2.33[a]	2.81 ± 0.18[a]	5.25 ± 0.01[a]	44.57 ± 0.00[a]
4JA	0.6 mg/L TDZ + 4 mg/L JA	224.73 ± 1.94[b]	4.20 ± 0.12[b]	5.25 ± 0.00[a]	44.50 ± 0.00[b]

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Independent samples t-test.

**CM*: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid

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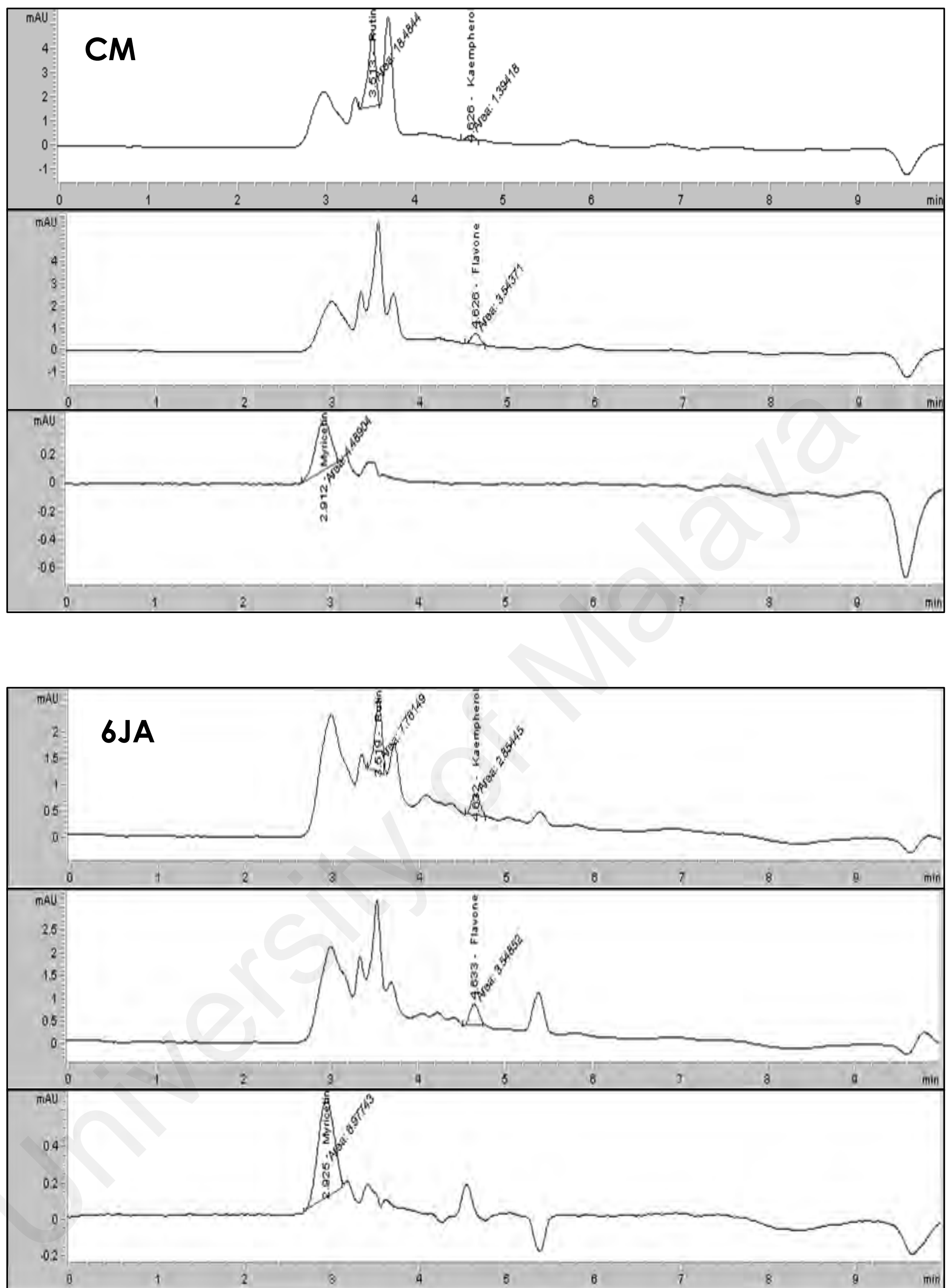

Figure 4.5: Flavonoids HPLC chromatograms showing the presence of various flavonoids in green callus extracts of 4-week-old CM and 6JA samples.

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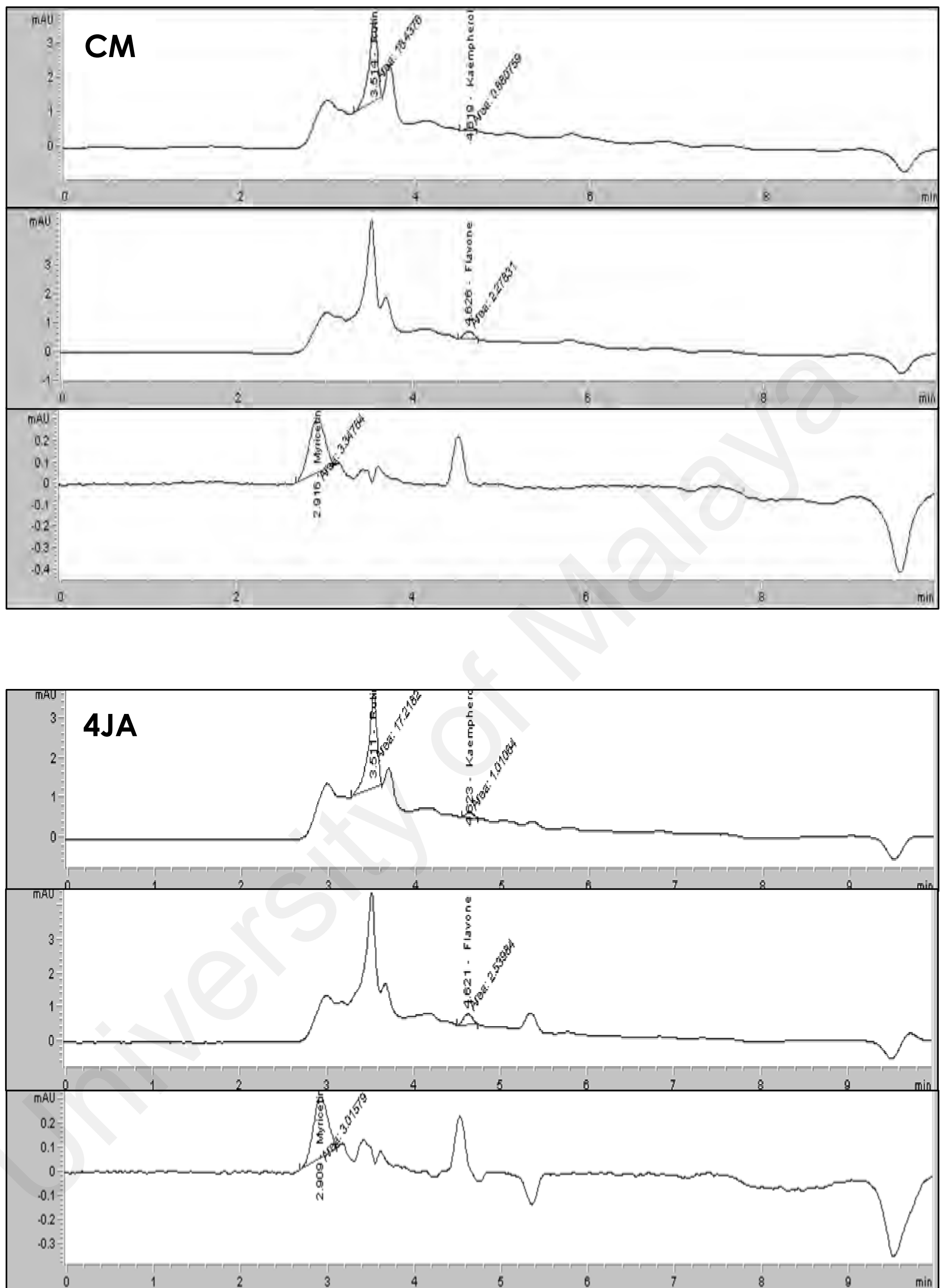

Figure 4.6: HPLC chromatograms showing the presence of various flavonoids in green callus extracts of 8-week-old CM and 4JA samples.

4.4 Determination of Antioxidant Potential of *A. indica* Methanolic Extracts by using ABTS and FRAP Assays

Antioxidant activities of all extracts against ABTS (2, 2-azino-bis (3-ethylbenzotiazoline-6-sulfonic acid) radical and reducing ability of *A. indica* methanolic extracts by FRAP (ferric reducing antioxidant power) assay were recorded in Table 4.14 and Table 4.15. The ABTS free radical scavenging activity of *A. indica* methanolic extracts were represented as IC_{50} mg per ml of each extract. The IC_{50} values defined the concentration of antioxidant needed to reduce the initial ABTS concentration by 50%. Meanwhile, FRAP reducing activity was expressed as g per g $FeSO_4$ equivalent of each extract. Data analysis for 4-week-old samples revealed that the highest ABTS radical scavenging and FRAP reducing activities were shown by 6JA extract, with IC_{50} of 8.29 ± 0.10 mg/ml and FRAP value of 90.60 ± 1.55 g/g of sample, respectively (Table 4.14). The ABTS IC_{50} values for 4-week-old sample extracts were found to decrease with increasing JA concentration. The same trend was observed for FRAP values of 4-week-old sample extracts. FRAP value of 2JA extract was slightly higher when compared to FRAP value of 4JA extract. However, all extracts of 4-week-old JA-treated samples showed higher FRAP values compared to control. On the other hand, extract of 8-week-old 4JA sample showed the highest ABTS radical scavenging potential and FRAP reducing activity, with ABTS IC_{50} of 7.73 ± 0.03 mg/ml and FRAP value of 74.59 ± 3.91 g/g of dried callus, respectively (Table 4.15). Overall, elicitation with JA was found to yield higher antioxidant activity in the samples, as shown by the lower ABTS IC_{50} and higher FRAP values in JA-treated callus samples, compared to callus produced on CM media (control).

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Table 4.14: Effect of different concentration of jasmonic acid treatments on antioxidant potential of 4-week-old *A. indica* green callus; CM is control media.

Sample ID	Treatment	IC_{50} ABTS (mg/mL)	FRAP value ($FeSO_4$ in g/g of dried callus)
CM	0.6 mg/L TDZ	10.38 ± 0.06^{b}	66.44 ± 3.35^{a}
2JA	0.6 mg/L TDZ + 2 mg/L JA	8.62 ± 0.07^{a}	82.69 ± 3.93^{b}
4JA	0.6 mg/L TDZ + 4 mg/L JA	9.10 ± 1.00^{ab}	81.80 ± 3.37^{b}
6JA	0.6 mg/L TDZ + 6 mg/L JA	8.29 ± 0.10^{a}	90.60 ± 1.55^{b}

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT);

CM: callus induction media composed of 0.6 mg/L TDZ;* TDZ*: thidiazuron;* JA*: jasmonic acid; ABTS*: 2,2-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid); *FRAP*: ferric reducing antioxidant power.

Table 4.15: Effect of different concentration of jasmonic acid treatments on antioxidant potential of 8-week-old A. indica green callus; CM is control media.

Sample ID	Treatment	IC_{50} ABTS (mg/mL)	FRAP value ($FeSO_4$ in g/ g of dried callus)
CM	0.6 mg/L TDZ	11.09 ± 0.02^{d}	56.45 ± 5.30^{a}
2JA	0.6 mg/L TDZ + 2 mg/L JA	9.05 ± 0.01^{b}	53.01 ± 2.24^{a}
4JA	0.6 mg/L TDZ + 4 mg/L JA	7.73 ± 0.03^{a}	74.59 ± 3.91^{b}
6JA	0.6 mg/L TDZ + 6 mg/L JA	8.99 ± 0.01^{b}	73.12 ± 5.44^{b}

Data expressed as mean values ± standard error (SE) of three replicates. Means with different letters in the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT);

*CM: callus induction media composed of 0.6 mg/L TDZ; *TDZ*: thidiazuron; *JA*: jasmonic acid; *ABTS*: 2,2-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid); *FRAP*: ferric reducing antioxidant power.

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4.5 Effects of Sample Age on Synthesis of Pigments and Bioactive Compounds in *A. indica* Methanolic Extracts

4.5.1 Anthocyanins, Phenols, and Flavonoids

In this study, the TAC, TPC and TFC of methanolic extracts of *A. indica* callus, with and without JA elicitation after 4 and 8 weeks of incubation were evaluated and compared. The differences in amount of total anthocyanin, phenolic, and flavonoid contents between extracts of both sample ages (4 weeks and 8 weeks) of the same treatment were shown in Figure 4.7.

Generally, comparison between 4-week-old and 8-week-old samples revealed that 4-week-old samples of CM, 2JA, 4JA, and 6JA had significantly greater amount of TAC, TPC, and TFC in comparison to 8-week-old samples of the same treatments, except for TAC and TFC of 4JA. For sample age of 4 weeks, TAC of 4JA extract was found to be 0.25 ± 0.01 mg/g DW while the TAC of 8-week-old 4JA sample was 0.32 ± 0.01 mg/g DW. The TAC of 8-week-old 4JA sample was higher by 0.07 ± 0.01 mg/g DW. The TFC of 4-week-old 4JA sample extract was 0.43 ± 0.01 g QE/g DW, whereas TFC of 8-week-old sample under the same treatment was higher (0.52 ± 0.01 g QE/g DW).

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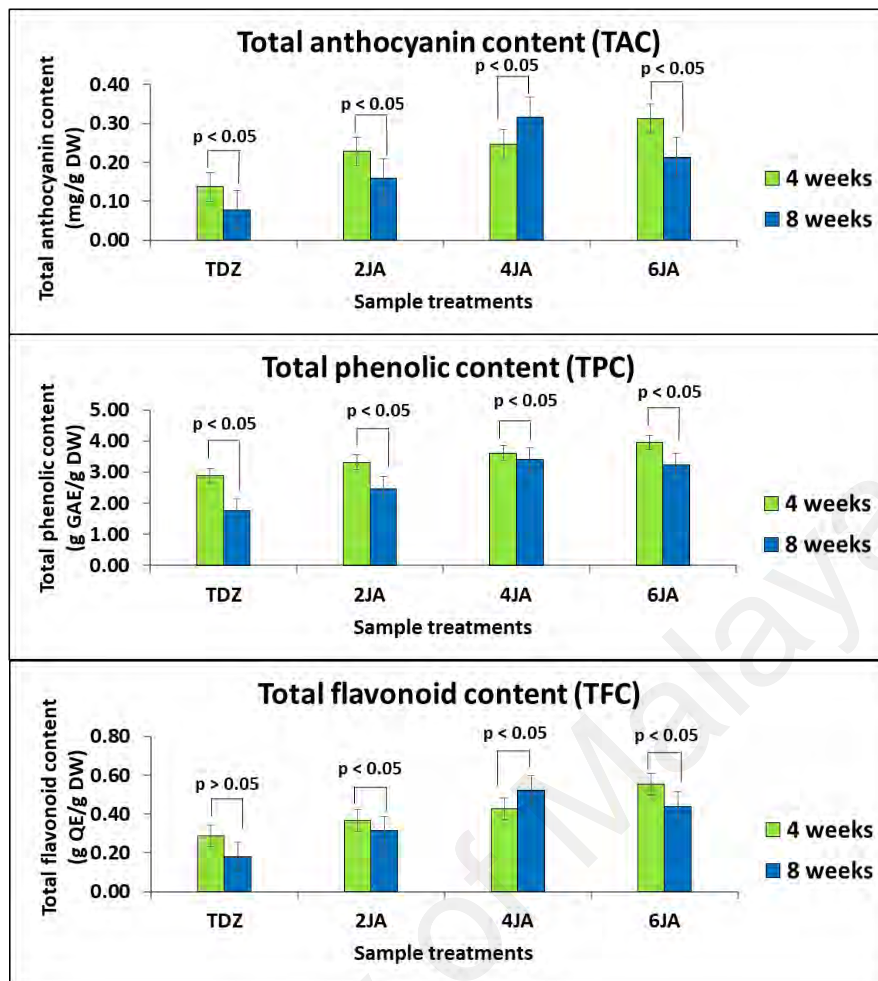

Figure 4.7: Effect of sample age on TAC, TPC, and TFC of the methanolic extracts of *A. indica* supplemented with different concentration of jasmonic acid. Notes: 2JA: 2 mg/L; 4JA: 4 mg/L; and 6JA: 6 mg/L of jasmonic acid. Data expressed as mean values ± standard error (SE) of three replicates. Means with value $p \leq 0.05$ are significantly different according to Duncan's multiple range test (DMRT).

4.5.2 Chlorophylls and Carotenoids

In this study, total chlorophylls and carotenoid content of *A. indica* methanolic extracts after 4 and 8 weeks of incubation on control media (CM) and JSM were also evaluated. As shown in Figure 4.8, 4-week-old sample of all treatments exhibited significantly higher amount of chlorophyll a, chlorophyll b as well as total chlorophyll a and b and carotenoid contents compared to extracts from 8-week-old samples. Longer incubation time was found to significantly affect the amount of total chlorophylls and carotenoids of

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the extracts. On the other hand, Figure 4.9 shows that the Ca+Cb/C(x+c) ratio of 4-week-old samples decreased with increasing JA concentration, in contrast to 8-week-old samples.

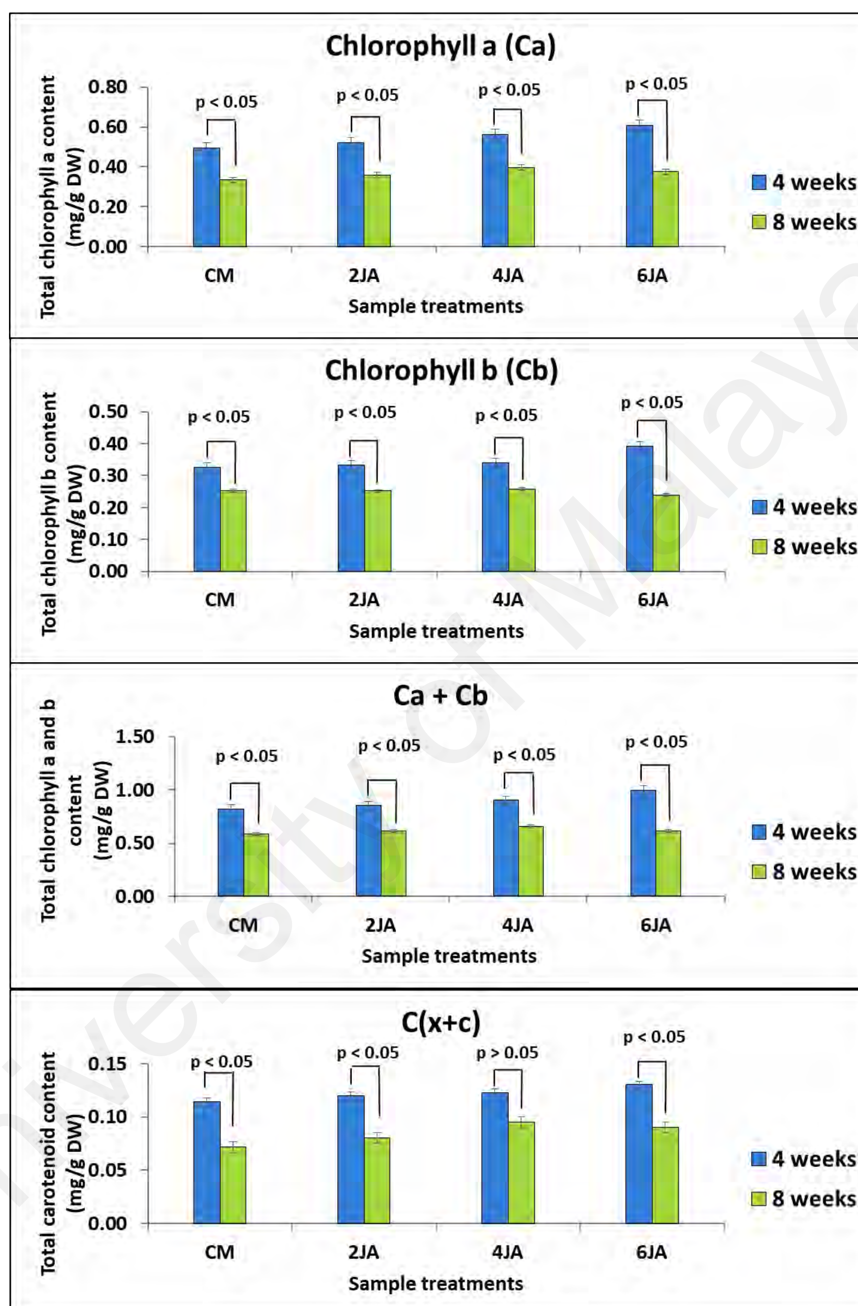

Figure 4.8: Effect of sample age on the total amount of photosynthetic pigments (chlorophylls and carotenoids) in the methanolic extracts of *A. indica* supplemented with different concentration of jasmonic acid. Notes: 2JA: 2 mg/L; 4JA: 4 mg/L; and 6JA: 6 mg/L of jasmonic acid. Data expressed as mean values ± standard error (SE) of three replicates. Means with value $p \leq 0.05$ are significantly different according to Duncan's multiple range test (DMRT).

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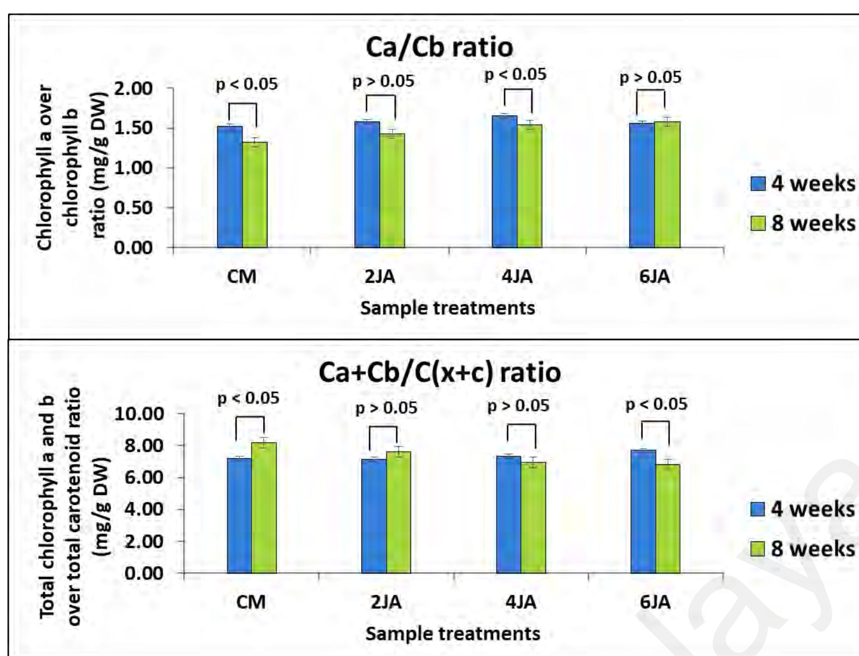

Figure 4.9: Effect of sample age on Ca/Cb ratio and Ca+Cb/C(x+c) ratio in the methanolic extracts of *A. indica* supplemented with different concentration of jasmonic acid. Notes: 2JA: 2 mg/L; 4JA: 4 mg/L; and 6JA: 6 mg/L of jasmonic acid. Data expressed as mean values ± standard error (SE) of three replicates. Means with value $p \leq 0.05$ are significantly different according to Duncan's multiple range test (DMRT).

4.6 Effect of Sample Age on Antioxidant Potential of *A. indica* Methanolic Extracts

The effect of sample age on antioxidant potential of the extracts was also evaluated and compared (Figure 4.10). The scavenging activities of the extracts against ABTS radicals were found to be significantly higher than that shown by 8-week-old samples, except for 4JA. Results revealed that the IC_{50} value of 4-week-old 4JA extract was higher than that of 8-week-old sample. However, these differences were found to be not statistically significant. Therefore, it could be deduced that in general, extracts of 4-week-old samples had better ABTS radical scavenging activities than extracts of 8-week-old samples. Similarly, all 4-week-old samples also showed greater FRAP values compared to 8-week-old samples. Results revealed that shorter culture incubation period (4 weeks) resulted in production of callus with better FRAP (Fe^{3+} to Fe^{2+}) reducing activities.

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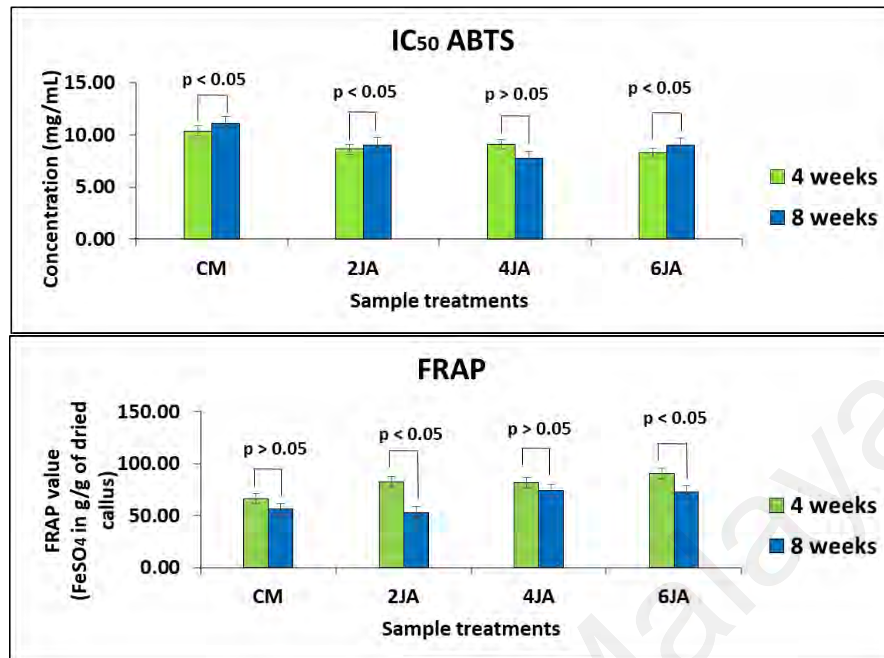

Figure 4.10: Effect of sample age on the radical scavenging activity of ABTS and FRAP activity in the methanolic extracts of *A. indica* supplemented with different concentration of jasmonic acid. Notes: 2JA: 2 mg/L; 4JA: 4 mg/L; and 6JA: 6 mg/L of jasmonic acid. Data expressed as mean values ± standard error (SE) of three replicates. Means with value $p \leq 0.05$ are significantly different according to Duncan's multiple range test (DMRT).

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4.7 Correlation between Bioactive Compounds Content and Antioxidant Potential of *A. indica* Extracts

To comprehend the relationship between the antioxidant properties and bioactive pigments of green callus extracts of *A. indica*, correlation analysis was conducted for both 4-week-old and 8-week-old samples (Table 4.16). Negative and significantly strong correlations were found between ABTS IC_{50} with TAC, TPC and TFC of the extracts. This show that the ABTS IC_{50} values would decrease with increasing TAC, TPC and TFC amounts, indicating that ABTS radical scavenging activities would increase parallel to the increase in anthocyanin, phenolic and flavonoid contents in the samples. In contrast, weak correlations were found between ABTS IC_{50} with TCh and TC, but these correlations were found to be insignificant. Moreover, positive and significantly strong correlations were observed between FRAP values and the bioactive pigments (TAC, TPC, TFC, TCh and TC), indicating that the ferric reducing potential of the extracts would increase with increasing pigments content (Table 4.16).

Table 4.16: Pearson's correlation coefficients between the variables.

Correlations							
	TAC	**TPC**	**TFC**	**TCh**	**TC**	**ABTS**	**FRAP**
TAC	1						
TPC	0.886**	1					
TFC	0.923**	0.871**	1				
TCh	0.480*	0.719**	0.421*	1			
TC	0.555**	0.794**	0.511*	0.919**	1		
ABTS	-0.806**	-0.697**	-0.765**	-0.252	-0.373	1	
FRAP	0.716**	0.834**	0.662**	0.720**	0.754**	-0.476**	1

** Correlation is significant at $p < 0.01$

** Correlation is significant at $p < 0.01$

* Correlation is significant at $p < 0.05$

TAC: total anthocyanin content; *TPC*: total phenolic content; *TFC*: total flavonoid content; *TCh*: total chlorophyll content; *TC*: total carotenoid content; *ABTS*: 2,2-azino-bis(3-ethylbenzotiazoline-6-sulfonic acid); *FRAP*: ferric reducing antioxidant power.

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CHAPTER 5

DISCUSSION

5.1 Effects of TDZ and Jasmonic Acid on the Growth of *A. indica* Green Callus

The culture of explants MSO did not yield in callus formation due to absence of TDZ as a growth regulator. No sign of callus formation was observed from the leaf explants when cultured on MSO, even after 8 weeks of incubation. Initiation of green callus formation from leaf explants of *A. indica* can only be observed when the explants were cultured on CM media, which contained TDZ. Similar observations were recorded in a previous study where callus was successfully induced from different blueberry plant genotypes by utilizing TDZ as the only growth regulator in the basal media (BM) (Ghosh et al., 2017). Leaf explants of all genotypes started to develop into calli after 4 weeks of incubation on BM supplemented with different concentrations of TDZ (0.1, 0.5, and 1.0 mg/dm^3) and fully formed calli after 12 weeks of culture. It was also reported that cultures cultivated on BM without TDZ did not form any callus and therefore, were excluded from data analysis (Ghosh et al., 2017). In addition, formation of green callus from *A. indica* leaf explants by using TDZ as growth regulator was also supported by findings of a previous study done by Ashokhan et al., (2018).

In the present study, successfully developed calli on CM after 8 weeks of culture cultivation were subcultured on JSM. It appeared that combination of TDZ with JA did not exert any significant effect on callus fresh weight and growth index. Also, the application of JA as a stress factor resulted in no negative effects on the growth of *A. indica* green callus, thus it is suitable to be used as an elicitor to boost the production of bioactive pigments in the callus. Besides, TDZ alone was apparently very efficient in stimulating biomass accumulation in *A. indica* callus culture, which is in agreement with several reports on other medicinal plant species. Zhang et al., (2005) reported that callus

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formation of *Scutellaria baicelensis*, a Chinese medicinal plant, was well induced by exogenous application of TDZ. Besides, higher concentrations of TDZ (1.0, 2.0, and 5.0 mg/L) generated multiple shoots from the induced calli but only after being subcultured on hormone free medium whereas TDZ, at lower concentration (0.3 mg/L) produced shoots directly from calli on the induction medium. There was also a report on TDZ effects on stem, petiole and leaf explants from 4-week-old *Scutellaria bornmuelleri* seedlings during callus formation induction. MS and half-strength MS media employed in the study were supplemented with 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0 mg/L of TDZ. Results revealed that, 4 days were needed for the stem and petiole explants to initiate callus formation but no callogenesis was observed from leaf explants. Moreover, type of explant and medium used were shown to have different influences on the callus induction frequency. However, all stems were found to form callus within 2 weeks of culture, regardless of the medium used (Gharari et al., 2019).

5.2 Phytochemical Screening

All treatments including CM, 2JA, 4JA, and 6JA crude extracts were subjected to phytochemical screening by following the standard method as described by Solihah, (2012). In this study, alkaloids, flavonoids, phenols, tannins, and terpenoids were detected from the screening analysis. Several studies had reported the presence of different phytochemicals in crude methanolic extract of *A. indica* that have valuable therapeutic application in various fields. In one study, methanolic extract of *A. indica* leaves had shown to contain alkaloids, tannins, flavonoids, as well as other phytochemicals such as saponins, glycosides, and reducing sugar (Dash et al., 2017). In another study, methanolic extracts from fresh and shade dried leaves of *A. indica* reported the presence of alkaloids but flavonoids and tannins were not detected (Cheenickal & Mendez, 2017).

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5.3 Detection of Bioactive Pigments

5.3.1 Total Anthocyanin, Phenolic, and Flavonoid contents

In the present study, the total anthocyanin, phenolic, and flavonoid contents of *A. indica* green callus produced in extracts of CM, 2JA, 4JA, and 6JA were determined. Compared to the control (CM media), total anthocyanin, phenolic, and flavonoid contents in green callus extracts of *A. indica* were higher in the media elicited with JA. This finding is in agreement with a previous study, where low total anthocyanin, phenolic, and flavonoid contents were determined in *A. indica* green callus extract produced on MS media with TDZ but without the presence of any elicitor (Ashokhan et al., 2018). Besides, when compared to the control, the significant increase in the amount of bioactive pigments content of plant extracts when JA was added suggests that JA is a good stress-induced elicitor to enhance the biosynthesis of bioactive pigments in green callus of *A. indica*. Also, results obtained from the present study showed that total anthocyanin, phenolic, and flavonoid increased significantly with the increase of JA concentration for 4-week-old samples. However, the amount of TAC, TPC, and TFC of 8-week-old samples increased in 2JA and 4JA extracts only and then decreased in 6JA extract. Previous cell culture studies reported that, elicitation of bioactive pigments in plants was influenced by different parameters, for examples, the elicitor specificity, concentration, and time of exposure (Vasconsuelo & Boland, 2007). Another study reported that even though plants respond to JA by altering their defense mechanisms as well as secondary metabolites production, the induced levels are commonly not stable after a long time (Kim et al., 2006). Therefore, treating sample with higher concentration of elicitor does not necessarily increase the amount of accumulated secondary metabolites. It was shown that treating plants with different JA concentration can be done as a mean to select for an optimum concentration of JA that could induce the maximum amount of bioactive compounds (Kim et al., 2007). Jasmonates such as JA and MeJA were reported to increase

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the accumulation of phenolics in *Mentha* x *piperita*. However, the observed changes were different depending on elicitor's concentrations and the time of exposure (Krzyzanowska et al., 2011).

Based on the results, total flavonoid contents in each treatment was found to be the highest compared to total anthocyanins and phenolics. Therefore, HPLC analysis was done to determine individual flavonoids present JA-treated samples that showed the highest TFC amounts, compared to control sample (CM). Flavonoid analysis performed by HPLC system detected four flavonoid peaks which were rutin, kaempherol, flavone, and myricetin. All compounds were scanned using three different wavelengths ranging from 250 to 350 nm to increase the efficiency of peak separation, hence avoiding the peak from overlapping (Seo et al., 2016). The major flavonoid constituent in *A. indica* green callus was found to be rutin. According to the study done by Vergallo et al. (2019), the qualitative-quantitative composition of flavonoids was exhibited to be depending on the extraction solvent used which means different organic solvents may have different efficiency in extracting particular group of flavonoids and in the present study, acetonitrile was shown to have better efficiency in extracting rutin hence it was found to be the highest compound screened followed by myricetin, kaempherol, and flavone. Meanwhile in another study, HPLC analysis of ethanolic extracts obtained from leaves and bark of *A. indica* was shown to contain several compounds of phenolics and flavonoids. Major compounds found in the extract were quercetin (14.09 ± 0.01 μg/mL), kaempherol (8.15 ± 0.03 μg/mL), and coumarin (7.48 ± 0.01 μg/mL) while other compounds detected included gallic acid (2.65 ± 0.01 μg/mL), catechin (1.79 ± 0.03μg/mL), chlorogenic acid (1.81 ± 0.01 μg/mL), rutin (3.72 ± 0.02 μg/mL), quercitrin (7.46 ± 0.01 μg/mL), and luteolin (3.74 ± 0.01 μg/mL) (Cristo et al., 2016). In another study, two flavonoids extracted from *A. indica* leaves, quercetin and kaempherol, were successfully characterized and isolated by HPLC and Preparative-HPLC (P-HPLC)

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respectively and were shown to have potential antimicrobial properties as they exhibited valuable anti-MRSA activity (Ullah et al., 2018).

5.3.2 Total Chlorophylls and Carotenoids Content

The supplementation of JA at different concentrations also influenced the regulation of photosynthetic pigments (chlorophylls and carotenoids) production in green callus of *A. indica.* Similar to TAC, TPC, and TFC analysis, the highest amount of chlorophylls and carotenoids were exhibited by extract of 4-week-old 6JA and 8-week-old 4JA samples, respectively. Thus, at this juncture it could be deduced that 6 mg/L and 4 mg/L were the optimum concentration for JA to elicit bioactive pigments production in the present study, depending on the duration of culture incubation (or callus age).

Results showed that, the amount of photosynthetic pigments of 8-week-old samples was significantly lower compared to 4-week-old samples. Most of studies on the effects of JA on plants showed that, JA exerted inhibitory effect on the growth and photosynthetic pigments content of the plant samples grown *in vitro* and *in vivo* (Golovatskaya & Karnachuk, 2008; Kovač & Ravnikar, 1998; Kovač & Ravnikar, 1994; Ueda et al., 1981). A study had demonstrated that JA and its methyl ester, methyl jasmonate (MeJA) strongly inhibited the accumulation of chlorophylls as time increased and the inhibitory effect of MeJA was greater than JA (Ananiev et al., 2004). Another previous study had reported the negative effect of JA on plant pigments could be explained by the degradation of chloroplast proteins and pigment–protein complexes in chloroplasts (Kumari & Sudhakar, 2003). These observations suggested that JA concentration may increase or decrease the amount of photosynthetic pigments of plant samples treated and most of the time JA exhibited an inhibitory effect that reduce the photosynthetic pigment amount as time increases.

Besides, both 4-week-old and 8-week-old controls were observed to have lower photosynthetic pigments amount than their respective treatments. Therefore, jasmonic acid is a potent elicitor in inducing the biosynthesis of photosynthetic pigments in plants though the induced levels was commonly unstable for a long time (Kim et al., 2007). Moreover, elicitation of *Lactuca sativa* L. with jasmonic acid in one study was proven to significantly increase the amount of carotenoids in the plant compared to the control. Interestingly, 8-week-old control had lower amount of total chlorophylls and carotenoids than 4-week-old control while both of the samples were not subjected to jasmonic acid stress elicitation. This implied that 8-week-old control experienced greater deterioration of total chlorophylls and carotenoids as the control hardly survived a longer culture period with the same amount of limiting macronutrients as 4-week-old control. Chlorophylls and carotenoids are photosynthetic apparatus involve in photosynthesis which is the key process in plant metabolism. Deprivation of macronutrients hinders plant metabolism and affects plant growth and development (Sitko et al., 2019; Samborska et al., 2018; Thuynsma et al., 2016).

The samples were also screened for the presence of eight major carotenoids using HPLC. In this part of the study, the extract from 4-week-old CM sample was used for optimization and was found to show good separation of compounds, thus the same protocol was used for all other samples. In contrast to the results gathered for 4-week-old CM samples, extract of 4-week-old 6JA samples were found to contain only lutein. Meanwhile for 8-week-old samples, only lutein was detected in CM extract and none was detected in the extract of 4JA sample. This is the first time changes on lutein during treatment with jasmonic acid was observed since previous literature has not reported similar finding. Nevertheless, there may be other unknown carotenoids present in the samples, but they could not be identified due to the presence of unresolved peaks in the HPLC chromatograms. According to Dworkin (2011), compounds do not split up well

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chromatographically due to the occurrence of chromatographic co-elution which happens when compounds involved have retention time that differ by less than the resolution of the method. Besides, degradation of column used and mass or volume overload on the column may also lead to peak distortion (Ahmad, 2017). Another possible cause of peak distortion is incompatibility of diluent and mobile phase where the elution strength of mobile phase used is significantly weaker than the diluent or there is other possible immiscibility issues (Wahab et al., 2017).

The ratio of chlorophyll a to chlorophyll b (Ca/Cb) and total chlorophylls to total carotenoids Ca+Cb/C(x+c) were measured at 4 and 8 weeks of samples incubation in all extracts. Based on the results obtained, samples of 8-week-old green callus showed lower Ca/Cb ratio as compared to 4-week-old green callus samples. In all sample extracts, chlorophyll a content is greater than chlorophyll b and Ca/Cb ratio is also higher compared to previous findings by Ashokhan et al. (2018). This is because when plant is under stress, both chlorophyll a and b decreased while, the Ca/Cb ratio tend to increase due to greater reduction in chlorophyll b compared to chlorophyll a. In any situation, plants have more chlorophyll a than b because of maintenance of more chlorophyll a than b is important for survival. Therefore, under the influence of a stress factor, chlorophyll b may be converted into chlorophyll a during the chlorophyll degradation process that lead to the increased in chlorophyll a contents (Ashraf & Harris, 2013; Eckardt, 2009; Fang et al., 1998). A previous study using two types of potato plants grown *in vitro* under the influence of JA also showed higher content of chlorophyll a than b. In JA treated leaves of potato cv. Sante the amount of chlorophyll a and b were 2.92 ± 0.16 µg/mg DW and 1.17 ± 0.05 µg/mg DW respectively. Meanwhile, the amount of chlorophyll a and b were reported to be 6.25 ± 0.45 µg/mg DW and 2.39 ± 0.14 µg/mg DW respectively for JA treated leaves of potato cv. Ulster (Kovač & Ravnikar, 1994). Also, fennel plants treated with 50 µM JA methyl-ester, methyl jasmonates, showed the amount of

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chlorophyll a of 3.25 ± 0.41 mg/g FW which was also greater than amount of chlorophyll b which was recorded as 1.10 ± 0.17 mg/g FW (Parmoon et al., 2018).

Other than that, the ratio of chlorophylls a and b to total carotenoid, Ca+Cb/C(x+c) was also determined in the present study to demonstrate the greenness of the samples (Lichtenthaler & Buschmann, 2001). Generally, Ca+Cb/C(x+c) ratio values of all samples were close to each other thus, representing green colour of callus samples used in the present study. Besides, Ca+Cb/C(x+c) ratio values in this study was lower than Ca+Cb/C(x+c) ratio in the study done by Ashokhan et al. (2018). It was reported that, greater degradation of chlorophylls and carotenoid happened due to stress, senescence, and damage of the plant photosynthetic apparatus resulting in lower Ca+Cb/C(x+c) ratio values (Lichtenthaler & Buschmann, 2001). Other than that, development of chromoplast also may decrease Ca+Cb/C(x+c) ratio which can be observed through the change in plant colour (Giusti & Wrolstad, 2001).

5.4 Antioxidant Activities of *A. indica* Extracts

The chemical reaction between radicals and antioxidant compounds are different depending on the mechanism of actions employed by the molecules involved. Hence, a number of assays have been developed to measure the free radicals scavenging activity of the available antioxidants appropriately (Marathe et al., 2011). Developed methods are made on the basis of hydrogen atom transfer (HAT) and also electron transfer (ET). HAT methods antioxidant activity marker is the extent of the scavenging by hydrogen- or electron-donating of a pre-formed free radical in inhibition assays. Meanwhile, ET methods employed an antioxidant system during radical generation where when antioxidants reacts with the chromogenic reagent, the absorbance increase at a prespecified wavelength (Moharram & Youssef, 2014). ABTS and FRAP assays are one of available HAT and ET methods, respectively. Therefore, to increase the accuracy, both

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ABTS and FRAP assays were used in this study to measure the antioxidant capacity of the plant extracts.

ABTS is a type of decolorization assay where ABTS radical cation ($ABTS^{\bullet+}$) was produced in the stable form using sodium persulfate in which appears to be blue or green $ABTS^{\bullet+}$ chromophore. ABTS is reduced to an extent and on a time-scale which influenced by the antioxidant activity, the concentration of the antioxidant, and the duration of the reaction when antioxidants is added to the pre-formed radical cation (Re et al., 1999). In the present study, ABTS scavenging activity is expressed as IC_{50} value which is defined as concentration of antioxidants required to scavenge 50% of ABTS free radicals in the sample extract. Meanwhile, FRAP assay is based on the reduction of Fe^{3+} TPTZ complex (colourless complex) to Fe^{2+}-TPTZ (blue coloured complex). This complex is formed by the action of electron donating antioxidants at low pH where an intense blue colour complex is formed when ferric tripyridyltriazine (Fe^{3+} TPTZ) complex is reduced to ferrous (Fe^{2+}) form (Rajurkar & Hande, 2011). A higher antioxidant activity can be observed if there is greater amount of Fe^{3+} being reduced to Fe^{2+} and vice versa (Benzie & Strain, 1999).

Analysis of variance was used to determine the effects of JA elicitor treatment on antioxidant activity of *A. indica* green callus. Among all four extracts, 6JA showed the lowest concentration of antioxidants (8.29 ± 0.10 mg/mL) needed to scavenge 50% of ABTS free radicals in the extract hence had the highest antioxidant activity and the results were revealed to be statistically significant when compared to the control. Meanwhile, 4JA extract had the highest ABTS radical scavenging activity among the four extracts of 8-week-old samples with ABTS IC_{50} value of 7.73 ± 0.01 mg/mL in comparison to the control. Similar observations can be seen in FRAP reducing activity of *A. indica* green callus extracts, where the highest FRAP value for 4-week-old and 8-week-old sample

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extracts were produced by 6JA (90.60 ± 1.55 g/g of dried callus) and 4JA (74.59 ± 3.91 g/g of dried callus), respectively.

A recent study had revealed the effect of two different concentrations of an elicitor which was analyzed on day 3, 7, and 15 post-elicitation in shoot cultures of *Knautia sarajevensis*. The highest antioxidant activity were exhibited by 50 and 100 mM elicitor-treated extract harvested on day 7 and 3 respectively, while the lowest antioxidant activity was exhibited by both 50 and 100 mM elicitor-treated extracts harvested on day 15 (Karalija et al., 2019). Another antioxidant potential study using lettuce and endive showed that, 1 µM of JA treated samples had the highest antioxidant activity after being stored for 4 days while 100 µM of JA treated samples had the highest antioxidant activity for lettuce and endive after being stored for 0 and 2 days respectively (Złotek, 2017). Interestingly, for 4-week-old and 8-week-old samples, green callus extract treated with 6.0 mg/L of JA and 4.0 mg/L of JA, respectively, gave the highest TAC, TPC, and TFC as well. Thus, at this juncture, it could be deduced that 6 mg/L and 4 mg/L of JA were the optimum concentration for maximum secondary metabolites production and antioxidant activities in *A. indica* green callus in the present study for 4-week-old and 8-week-old samples, respectively. Also, cultures of *A. indica* elicited with JA exhibited greater antioxidant potential as compared to other parts of the plants such as the flowers and leaves (Nahak & Sahu, 2011; Sithisarn et al., 2005).

Higher IC_{50} value shown by all 8-week-old sample extracts indicated for lower antioxidant activity than 4-weeek-old extracts. Similarly, 8-week-old sample also had lower FRAP values compared to 4-week-old sample extracts. Therefore, it could be deduced that increased duration of elicitor exposure may reduce or increase the antioxidant activity of the extracts, depending on type of elicitor used. Moreover, this observation is supported by a study done using methyl jasmonates, salicyclic acid and combination of both as elicitors, where antioxidant activity of some cell suspension

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cultures of *Thevetia peruviana* were evaluated at 24, 48, 72, 96, and 120 h post-elicitation. The results exposed that antioxidant activity of samples treated with methyl jasmonate increased from 48 to 96 h post elicitation but decreased after 120 h post elicitation (Mendoza et al., 2018). One previous study showed that, antioxidant activity of methyl jasmonate-treated *Lactuca sativa* var. *capitata* increased when evaluated on pre-harvest day 1, 3, 5, and 7 but decreased on day 15 (Moreno-Escamilla et al., 2017).

5.5 Correlation between Bioactive Compounds and Antioxidant Activities

It was reported that bioactive phytochemicals present in the plants will influence its antioxidant activities (Hsu et al., 2006). It was observed that both ABTS radical scavenging and FRAP reducing activity of the green callus extracts would increase with increased amounts of bioactive pigments. Free radical scavenging activity of anthocyanins is associated with the number of sugar residues, oxidation state of carbon ring, and hydroxylation and methylation pattern of the compounds (Jordão & Correia, 2012). Also, their peculiar chemical structure of electron deficiency caused anthocyanins to be particularly reactive toward reactive oxygen species and reactive nitrogen species (Dai & Mumper, 2010). Besides, the inhibitory activity of anthocyanins towards lipid peroxidation was found to be comparable to Vitamin E (Hwang et al., 2012). Strong and significant correlation between anthocyanins and antioxidants activities from the results is supported by a previous study where, anthocyanins were found to be major active components that contributed to high antioxidant activity in *Elatostema rugosum* (Neill et al., 2002).

Phenolics can carry out their antioxidative activities during termination reaction in which, the generation cycle of new radicals is broken down through a reaction between a hydrogen-donating antioxidant and reactive oxygen and nitrogen species. Phenolics are known as good hydrogen-donating antioxidants because they possess hydroxyl groups in

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their structures (Heim et al., 2002; Payá et al., 1992). Moreover, previous studies using extract from neem leaves suggested that phenolic compounds are excellent free radical scavengers and reducing agents because there were strong correlations between phenolic content and antioxidant activities in the extract (Dhakal et al., 2016; Pandey et al., 2014). Meanwhile, flavonoids antioxidant potential enable them to protect against oxidative diseases, activate or inhibit various enzymes bind specific receptors, and protect against cardiovascular diseases by reducing the oxidation of low-density lipoproteins (Aron & Kennedy, 2008). Chlorophylls and carotenoids that are present in the methanolic extracts of *A. indica* were also observed to be correlated to their ABTS and FRAP activities. Carotenoids such as lutein, lycopene, β-carotene, and zeaxanthin are well known as free radical scavengers and strong antioxidants, particularly vital in human diets (Johnson, 2002). Meanwhile, chlorophylls were reported to be responsible for breaking the chain reaction in cellular oxidation caused by free radicals by acting as the hydrogen donor (Endo et al., 1985). However, correlation of chlorophylls and carotenoids to ABTS activity was rather weak and not significant as compared to their correlation to FRAP activity. Moreover, this result was in contrast to a study where chlorophylls were reported to have a high antioxidant activity in inhibiting free radicals through DPPH and ABTS assays (Silva-Beltrán et al., 2015).

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CHAPTER 6

CONCLUSION

6.1 Conclusion

The present study showed that elicitation with JA enhanced the biosynthesis of secondary metabolites in green callus of *A. indica*. These metabolites were shown to have the ability to scavenge free radicals generated by oxidative stresses. The optimum JA concentration that gave the highest antioxidant activities and amount of anthocyanins, phenolics, flavonoids, chlorophylls and carotenoids for 4-week-old and 8-week-old samples were found to be 6 mg/L and 4 mg/L, respectively. Furthermore, Pearson's correlation analysis also showed positive correlations among the total anthocyanin content, total phenolic content, total flavonoid content, total chlorophyll and carotenoid content and antioxidant activities of the extracts. These findings suggested that the exogenous application of JA into culture medium boosted the production of secondary metabolites production in the samples, which was responsible for the antioxidant activities in JA-induced green callus cultures of *A. indica*.

6.2 Recommendation for Future Research

Further studies involving advanced spectroscopic methodology for structural elucidation of secondary metabolites are essential to identify novel pharmaceutical compounds in *A. indica* green callus. The HPLC chromatograms showed a large number of unknown and unresolved peaks detected in JA-induced green callus cultures. This showed that tissue culture of *A. indica* has the potential to synthesize a relatively huge number of secondary metabolites, which could be responsible for the medicinal properties such as antioxidant, antimicrobial and anticancer activities of neem plant. Therefore, in order to thoroughly identify the unknown secondary metabolites present in tissue cultures of *A. indica,* a high-throughput screening technique such as liquid chromatography-mass

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spectrometry (LC-MS), gas chromatography (GC) and capillary electrochromatography (CEC) can be used in future studies. Meanwhile, nuclear magnetic resonance (NMR) can be used for complete structure elucidation of flavonoids, the major constituent in the callus extracts produced in this study and to identify novel pharmaceutical compounds present in *A. indica* green callus elicited with JA.

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