EFFECTS OF PHENYLALANINE FEEDING ON FLAVONOID PRODUCTION IN *Boesenbergia rotunda* CELL SUSPENSION CULTURES

CHARUMATHI YOGARAJAN

FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2021

EFFECTS OF PHENYLALANINE FEEDING ON FLAVONOID PRODUCTION IN *Boesenbergia rotunda* CELL SUSPENSION CULTURES

CHARUMATHI YOGARAJAN

DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR

2021

UNIVERSITI MALAYA
ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **CHARUMATHI YOGARAJAN**

Matric No: **17049881/1**

Name of Degree: **MASTER OF BIOTECHNOLOGY**

Title of Thesis ("this work"):

EFFECTS OF PHENYLALANINE FEEDING ON FLAVONOID PRODUCTION IN *Boesenbergia rotunda* CELL SUSPENSION CULTURES

Field of Study:

PLANT BIOTECHNOLOGY

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(1) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(2) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(3) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

EFFECTS OF PHENYLALANINE FEEDING ON FLAVONOID PRODUCTION IN *Boesenbergia rotunda* CELL SUSPENSION CULTURES

ABSTRACT

Boesenbergia rotunda, a medicinal ginger, is reported to have flavonoids and cyclohexenyl chalcone derivatives (CCD) with anti-bacterial, anti-cancer, anti-oxidant and anti-dengue properties. These compounds are obtained from rhizomes which are susceptible to biotic and abiotic stress. Hence, cell suspension cultures grown in controlled conditions are advantageous as an alternative source. The accumulation of these compounds could be enhanced using precursor. In this study, phenylalanine was used as the precursor and a feeding strategy was designed to enhance CCD (panduratin A) and other flavonoids (pinocembrin, pinostrobin and cardamonin) production. *B. rotunda* cell suspension cultures were established in liquid Murashige and Skoog basal medium containing 1.0 mg/L 2,4-D, 0.5 mg/L BAP and 2.0% sucrose. The established cell suspension cultures were then treated with 20.0, 40.0 and 50.0 mg/L phenylalanine at day 14 and incubated for 3, 7, 14 and 21 days before harvesting. Pinocembrin, pinostrobin, panduratin A and cardamonin were quantitated using high-performance liquid chromatography (HPLC) for each treatment. It was shown that with the addition of phenylalanine, 40 mg/L phenylalanine treated cultures showed a significant increase ($p<0.05$) in the content of pinocembrin after 21 days of feeding compared to control. Meanwhile, there was no significant increase in pinostrobin, cardamonin and panduratin A in the cell cultures treated with different concentrations of phenylalanine at all incubation periods tested. The significant increase in one of the flavonoids investigated in this study, which is pinocembrin could be due to the phenylalanine feeding and additional effects from stress inflicted by dying cells or nutrient depletion over the long incubation culture period. Further studies need to be carried out to confirm the effect of

phenylalanine as a precursor that has enhanced pinocembrin accumulation in *B. rotunda* suspension cultures.

Keywords: *Boesenbergia rotunda*, cell suspension cultures, precursor feeding, phenylalanine, HPLC

KESAN PENAMBAHAN FENILALANIN TERHADAP PENGUMPULAN FLAVONOID DALAM KULTUR SEL AMPAIAN *Boesenbergia rotunda*

ABSTRAK

Boesenbergia rotunda, sejenis halia ubatan, dilaporkan mempunyai flavonoid dan chalcone (CCD) dengan ciri-ciri anti bakteria, anti-kanser, anti-oksida dan anti-denggi. Kompoun tersebut boleh didapati daripada rizom yang mana mudah terdedah kepada tekanan biotik dan abiotik. Oleh itu, kultur sel ampaian *B. rotunda* yang dihasilkan dalam persekitaran terkawal mempunyai kelebihan untuk digunakan sebagai sumber alternatif. Penghasilan kompoun ini boleh ditingkatkan dengan menggunakan prekursor. Dalam kajian ini, fenilalanin digunakan sebagai prekursor dan strategi penambahan prekursor telah direka untuk meningkatkan penghasilan CCD dan flavonoid. Sel ampaian *B. rotunda* telah dibangunkan dalam media cecair MS yang mengandungi 1.0 mg/L 2,4-D, 0.5 mg/L BAP dan 2.0% sukros. Sel ampaian tersebut kemudiannya dirawat dengan 20.0, 40.0 dan 50.0 mg/L fenilalanin pada hari ke-14 dan diinkubasi untuk 3,7,14 dan 21 hari. Pinocembrin, pinostrobin, panduratin A dan cardamonin dikuantifikasi menggunakan kromatografi cecair berprestasi tinggi (HPLC) untuk setiap rawatan. Pengumpulan pinocembrin meningkat secara signifikan ($p<0.05$) pada hari ke-21 bagi rawatan 40.0 mg/L berbandingkan sel kawalan. Sementara itu, tiada sebarang signifikasi diperhatikan dalam penghasilan pinostrobin, panduratin A, cardamonin berbandingkan kesemua kultur sel kawalan. Peningkatan yang signifikan dalam salah satu flavonoid yang disiasat iaitu pinocembrin mungkin disebabkan oleh penambahan fenilalanin dan kemungkinan ada kesan tambahan dari tekanan yang disebabkan oleh sel yang mati atau nutrien yang berkurangan disebabkan oleh masa inkubasi yang panjang. Kajian seterusnya perlu dijalankan untuk mengesahkan kesan fenilalanin sebagai prekursor yang meningkatkan pengumpulan pinocembrin dalam sel ampaian *B. rotunda*.

Kata kunci: *Boesenbergia rotunda*, kultur sel ampainan, rawatan prekursor, fenilalanin, HPLC

DEDICATION

Dedicated with much reverence and affection to my doting late grandparents; Mr. Ramachandran M. Kalappadiyar- Mrs. Anjalai A. Kandiyar who have always inspired me since young.

My precious ones; Mr & Mrs Yogarajan P. Vandayar- Sarojini Devi who have been there to shower their unconditional parental love and helped to keep up the momentum

A close to heart aunt, Mrs. Ambigai R. and her family who have always prayed for my well-being and provided goodwill, both physically and emotionally,

Ever encouraging siblings, blessings of late paternal grandparents, dear family members and my beloved husband & children.

~ This piece of page could not be made possible…

... without your love and support ~

ACKNOWLEDGEMENTS

Pranams and much gratitude to the Almighty for the celestial blessings showered on me to have completed this study successfully. I would like to extend my heartfelt gratitude to my Supervisors; Professor Dr. Norzulaani Khalid and Dr. Mahanom Jalil who have been there for me since day one, guiding through every step towards my Master's completion; paving my way to graduation, Dr. Tan Boon Chin who monitored my research throughout monthly reports, correcting my thesis draft and analysis, and Dr. Nur Ardiyana Rejab whom also came in as my saviour, after Prof's retirement. I have got no enough words to thank these good souls for their diligent guidance, insightfulness and patience throughout this course of time. Prof Lani is a definite epitome of an educator; passionate in her profession and has profound concern for her students, indeed an inspiration to me!

Not to forget Dr Lee Yean Kee for his technical guidance during the usage of HPLC at the Chemistry Lab, ever cheerful and helpful Plant Biotech Research Laboratory (PBRL)/ Plant Biotechnology Incubator Unit (PBIU) lab mates; Asniyati, Gayathri, William, Yvonne, Nazrin, Nurul Huda, Aina and Science Officer, Pn. Azlina for the conducive and healthy working environment.

Lastly, a million thanks to my dedicated babysitter Mrs. Susila Rajagopal for giving me the peace of mind, taking good care of my kids throughout these years.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

CCD	:	Cyclic chalcone derivatives
RAFD	:	Rapid Amplified Polymorphism
ALFP	:	Amplified Fragment Length Polymorphism
ITS	:	Internal Transcribed Spacer
PDMC	:	Plant Derived Medicinal Compounds
PAL	:	Phenylalanine ammonia- lyase
Phe	:	Phenylalanine
PPP	:	Phenylpropanoid pathway
SCV	:	Settled cell volume
mg/ L	:	Milligram per Liter
µM	:	Micromolar
µg/g	:	Microgram per Gram
w/v	:	Weight per volume
v/v	:	Volume per volume
nm	:	nanometer
rpm	:	Revolution per minute
min	:	Minute(s)
IAA	:	Indole acetic acid (auxin)
NAA	:	1- Naphtaleneacetic acid (auxin)
BAP	:	6- Benzylaminopurine (cytokinin)
2, 4-D	:	2,4- dichlorophenoxyacetic acid (auxin)
EA	:	Ethyl acetate
dH2O	:	Distilled water
Me OH	:	Methanol

NaCl	:	Sodium chloride
dc	:	Dense cells
vc	:	Vacuolated cells
DW	:	Dry weight

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Over the years, an array of medically important phytochemicals has been successfully isolated by researchers, using various approaches and technologies from *Boesenbergia rotunda,* a type of medicinal ginger. Bioactive compounds consisting of flavonoid, chalcone derivatives, esters, kawains, terpenes and terpenoids were isolated and elucidated (Tan et al., 2012). Flavonoids are one of the major compounds in B. rotunda which are derived from phenylalanine and acetate metabolisms. They are important for plant growth and development with a wide range of biochemical and pharmacological activities. Cardamonin, pinostrobin, pinocembrin chalcone, panduratin A which are derived all from the phenylpropanoid pathway (PPP) showed anti-HIV-1 protease inhibition, anti-inflammatory and anti-dengue-2 virus properties (Tan et al., 2006). To enhance the biosynthesis of these therapeutically important compounds, precursor feeding method and elicitors could be used. Apart from that, manipulation of the pathway by overexpressing or silencing related genes to the targeted compound could also be an alternative strategy.

1.2 Scope of Research

1.2.1 Problem statement

Despite their therapeutic potential, these flavonoids are produced in trace amount in nature. Apart from this, *B. rotunda* flavonoids are conventionally derived from field grown rhizome but are subjected to natural calamities and diseases. The production of bioactive compounds varies according to agriculture practice, location and soil type (Yusuf et al., 2013). Furthermore, chemically synthesized phyto-compounds incur high cost. Herein, *in vitro* cell cultures could help complement these challenges. Compounds

harvested from plant cell cultures are grown in aseptic and controlled environment which could allow consistent compound production. To enhance the biosynthesis of these therapeutically important compounds, precursor feeding method and elicitors could be used.

In this study Phenylalanine (Phe) treatment to the cell cultures were conducted and subsequently cells were harvested at different time points. Compounds from these cells were analyzed using high-performance liquid chromatography (HPLC). The production of *B. rotunda* flavonoids between non-fed cells (control cultures) and phenylalanine fed cell cultures were compared in order to improve targeted bioactive compound accumulation.

1.2.2 General objectives

To investigate the effect of different concentrations of phenylalanine feeding on *B. rotunda* cell suspension cultures at different time points.

1.2.3 Specific objectives

i. To establish a stable *B. rotunda* cell suspension cultures
ii. To assess the accumulation of *B. rotunda* flavonoids and cyclohexyl chalcone derivatives (CCD) *i.e.* pinocembrin, pinostrobin, cardamonin and panduratin A at different time points after phenylalanine feeding.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction of *Boesenbergia rotunda*

2.1.1 Botanical description

Boesenbergia rotunda is a ginger species from the Zingiberaceae family, a commonly used as a food ingredient and in ethnomedicinal preparations (Tan et al., 2012). This perennial herbaceous plant from the major group of Angiosperms (flowering plants), with unique finger like rhizomes; is locally known as fingerroot in English, "temu kunci" in Malay and "Kra chai" or "Kra Chai dang" in Thailand (Wong et al., 2013; Roslan et al., 2019). Due to its external heterogeneous morphology, taxonomical classification was considered tough for this plant genera. However, current molecular studies such as chloroplast DNA, Rapid Amplified Polymorphism (RAPD), Amplified Fragment Length Polymorphism (AFLP), ITS and chromosome analysis has made the classification easier (Tan et al., 2012).

2.1.2 Geographical distributions

Boesenbergia rotunda is well distributed throughout South East Asia countries like Malaysia, Thailand, Myanmar, Indonesia and the Borneo island, India, Sri Lanka, Nepal and China (Tan *et al.,* 2012; Oroun, 2017; Aryal *et al.,* 2016). The genus *Boesenbergia* comprises about 30 species and is widely spread from India to New Guinea (Mabberley, 1997). There are no native or naturalized members of this genus in Australia. Fingerroot (*Boesenbergia rotunda* (L.) Mansf. (syn. *Boesenbergia pandurata* Schltr.*, Kaempferia pandurata* Roxb., *Gastrochilus panduratus* (Roxb.) Ridl.) is used as a culinary and medicinal herb in Southeast Asia.

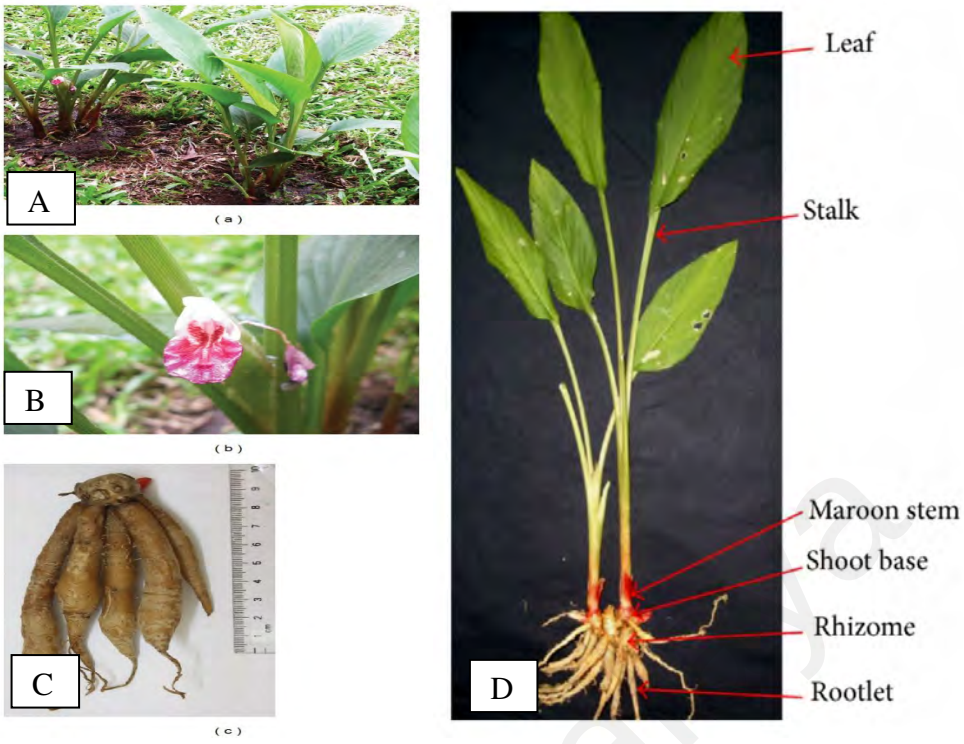


Figure 2.1: The morphology of *Bosenbergia rotunda* (A) plant on ground (B) flower and (C) rhizomes (D) whole plant (Tan et al., 2012).

2.2 Medicinal importance of *B. rotunda*

The presence of flavonoids, essential oil and chalcones in *B. rotunda* is one of the reasons the pharmacological importance has been given limelight spot among various ethnicity as well as the science community from yesteryears up to recent years (Pandji et al., 1993; Trakoontivakorn et al., 2001; Yusuf et al., 2011; Punnam et al., 2016, Nasir et al., 2020). *B. rotunda,* being the most abundant species in this region of Southeast Asia and Indo-China, has been imbibed as a daily food ingredient and as an ethno-medicine or folk medicine to alleviate food allergies and poisoning. These gingers were also found to possess anti-allergic, antibacterial, anticancer, anti-inflammatory, antioxidant, and antiulcer activities and also shown wound healing (Oraun et al., 2017)

2.2.1 Conventional usage of *B. rotunda*

B. rotunda, being the most abundant species in this region, it has been imbibed as an ethnomedicine or folk medicine to alleviate food allergies and poisoning. The rhizomes are also given as tonics, to women in mixtures after childbirth; added into lotions for rheumatism and muscular pains, and into pastes for application to the body after confinement. The leaves are used to treat fever, headache and body ache by the *Nicobarese* tribe from the Andaman Island in India (Chander et al., 2016). Escalating further, it has also been used in self-medication of AIDS patients in Thailand and also to treat several other diseases including aphthous, leucorrhea, and dysentery (Tan et al., 2012).

2.2.2 Chemical constituents and its medical importance

Scientific studies in the past have reported that the extracts isolated from the *B. rotunda* plant using various solutions (such as methanol, hexane, or chloroform) have antibacterial, antiviral, anticancer, neuroprotective and anti-feedant effects. Chemical compounds such as quercetin and kaempferol from the methanol-based extract are known to play critical roles in antioxidant and anti-inflammatory cascades or processes. When the hexane or chloroform is used in the isolation process, the resulting extract contains other important anti-oxidants: three flavanones (pinostrobin, pinocembrin, and alpinetin) and two chalcones (cardamonin and boesenbergin). Advancement in drug design and discovery research has led to the development of synthetic drugs from *B. rotunda* metabolites via bioinformatics and medicinal chemistry studies (Tan et al., 2012). Over the years, researchers have successfully isolated an array of bioactive compounds using various approaches and technologies. Nearly a hundred of compounds were isolated and elucidated, ranging from the flavonoid derivatives, chalcone derivatives, esters, kawains, esters, terpenes and terpenoids. These compounds have

been reported to exhibit great medicinal applications (Tan et al., 2012; Troung et al., 2019).

B. rotunda has attracted attention as a potential chemopreventive agent since the 1990's. A methanolic extract of fresh *B. rotunda* rhizomes showed strong inhibition of tumour promoter-induced Epstein-Barr virus activation (Murakami et al., 1993). This in vitro assay is used to screen for agents with possible anti-tumour promoting properties. The chalcone cardamonin was isolated and identified as having potent inhibitory activity in this assay (IC50 65 = 3.1 μM). Both chalcones and flavanones from this species have demonstrated anti-mutagenic activity against mutagenic heterocyclic amines in *Salmonella typhimurium* TA98 assays (Trakoontivakorn et al., 2001). An ethanolic extract of rhizomes had potent inhibitory activity (similar to that of turmeric, *Curcuma longa*) against human HT-29 colon cancer and MCF-7 breast cancer cell lines (Kirana et al., 2003). Panduratin A has also been shown to induce apoptosis in human colon cancer HT-29 cells (Yun *et al.,* 2005). The progressive oncology studies over the years also warrants further pharmacological importance of these natural compounds of *B. rotunda* on liver cirrhosis disease (Salama et al., 2012). Some of the most recent study revealed that cardamonin and pinostrobin chalcones have potentials against breast cancer and colon cancer (Mohammad et al., 2019), cardamonin against Hepatocellular carcinoma, HepG2 cells (Alshawsh et al., 2020), cardamonin is also strategized as phytochemopreventive studies (Murakami 2014) and antioxidant activity (Shindo et al., 2014). Cardamonin isolated from this species displayed anti-HIV-1 protease inhibition (Tewtrakul et al., 2003).

Flavonoids of *B. rotunda* are proven to be dengue Ns2b/ Ns3 inhibitors (Tan et al. 2006; Ahmad N. et al., 2012; Hosseini et al., 2020). Studies also revealed these compounds act as anti HIV-protein (Cheenpracha et al., 2006), proving the self-

remediation practice among HIV patients in Thailand. Another prominent compound in *B. rotunda* which is boesenbergin A exhibits ameliorative effect on oxidative stress and inflammation in ethanol induced gastric ulcer (Mohan et al., 2020).

Apart from that, a very recent studies have also provided the first evidence of cardamonin, pinostrobin and pinocembrin being a promising preventive agent of Alzheimer's via inhibition of amyloid peptide formation (Youn & Jun, 2019). Based on traditional usage of *B. rotunda* in treating pain and rheumatism, Complete Freud Adjuvant (CFA) induced rat models were tested and cardamonin was proven to have anti-arthritic properties (Voon et al., 2017). An identification of pinocembrin as an anti-glycation and alpha–glucosidase inhibitor for diabetic conditions were also studied on a tentative structure-activity towards MG trapping (Potipiranum et al., 2018). Cosmeceutical usage of *B. rotunda* as sunscreen and anti-bacterial agent were also proven in some established research in Korea (Jun *et al.,* 2019). A high content screening also revealed *B. rotunda* extract and its component panduratin A as anti SARS cov-2 agents (Kanjanasirirat et al., 2020). Further study on molecular docking and pharmacological studies on the evaluation of flavonoids at inhibiting the entry to the host cell can be explored (Bhowmik et al., 2020).

2.3 Source of secondary metabolites

Secondary metabolites used for medicament has been coined the term Plant Derived Medicinal Compounds (PDMC). Plant secondary metabolism, whose primary ecological role for plants to biosynthesize molecules to increase plants capacity to adapt to stress in the wild environment, in terms of defense or signaling (Cardoso et al., 2019) are unique sources for pharmaceuticals, food additives, flavours, and other industrial materials. These metabolites are unique sources for pharmaceuticals, food additives, flavours, and other industrial materials. Accumulation of such metabolites often occurs

when plants are subjected to stresses, including incubation with elicitors or signal molecules. Understanding the signal transduction mechanisms in secondary metabolite production (Figure 2.2) is thus important to enhance or improve their commercial values (Tan et al., 2015; Hussain et al., 2012). Most of the important secondary metabolites are currently isolated from wild or cultivated plants since chemical synthesis approach is not economically feasible.

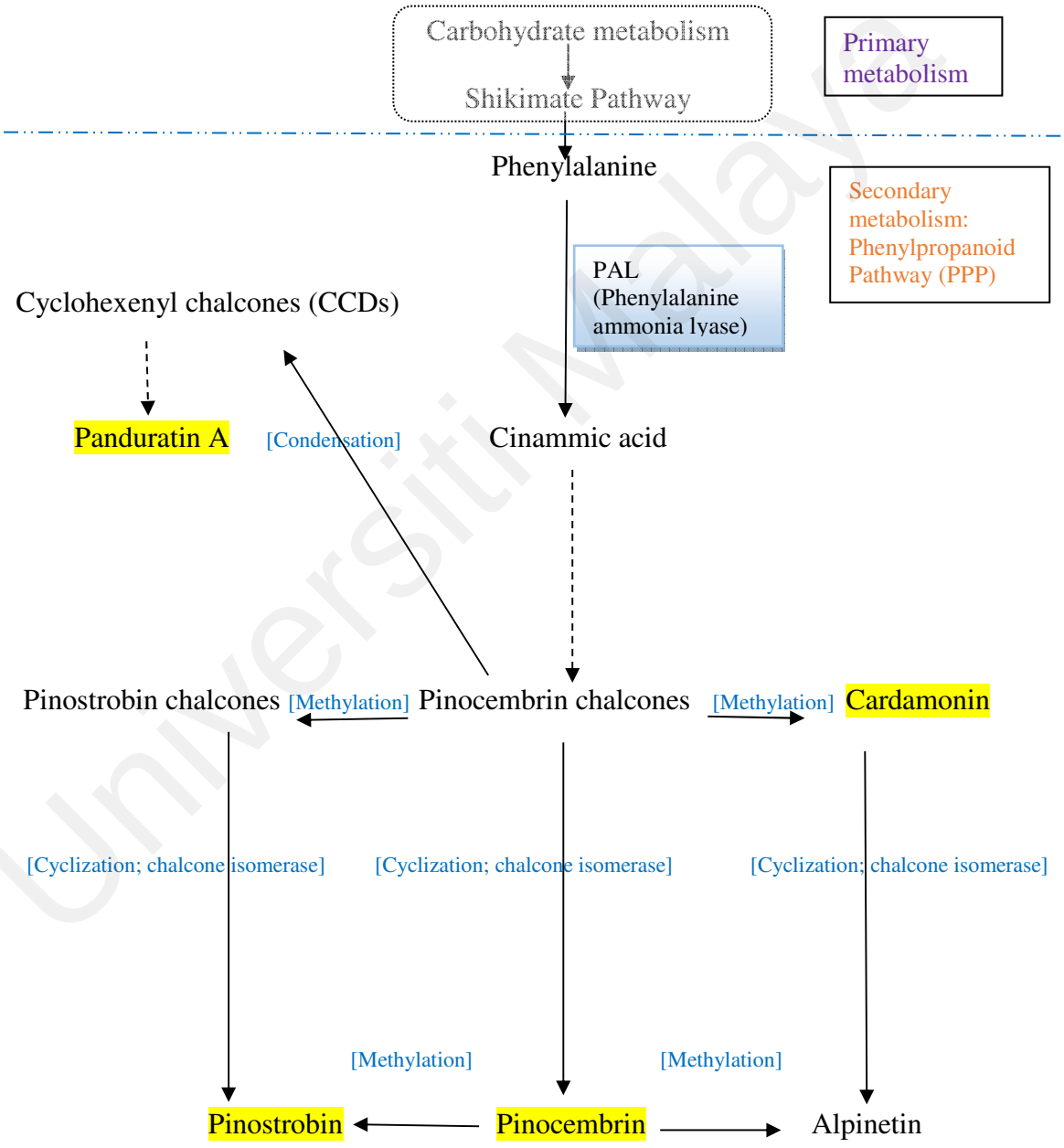


Figure 2.2: A simplified version of the biosynthetic pathway involved in the production of major flavonoid groups; flavanones and chalcones with slight modifications from Tan et al., 2015.

2.3.1 Conventional source and its challenges

B. rotunda is traditionally planted and propagated via its rhizome for large scale multiplications. Rhizomes getting rotten, lack of seed set for these ginger family, leaf spot and pathogenic diseases caused by *Coleotrichum sp.* (Chan, 2004) are some common challenges faced in conventional breeding. Soil borne pathogens permits further spread of disease from one plant or one location to another. Efforts have been made to enhance the secondary metabolite production in plant cells. However, limited success has been achieved so far, probably due to lack of understanding of how these metabolites are synthesized. Hence, alternative approaches, such as *in vitro* propagation and biotechnological techniques, metabolic engineering or intervention of the metabolic pathway of secondary metabolites via elicitation or precursor addition to enhance trace amounts of compounds, have become more attractive.

2.3.2 Current approaches and their advantages

The existence of bioactive flavonoids for *B. rotunda* has been explored in rhizomes and plant cell cultures (callus and cell suspension cultures) (Yusuf et al., 2013). The amount of flavonoids in *in-vitro* cultures were not comparable to the rhizomes, but is a pre-requisite in order to enhance the accumulation of the targeted bioactive compounds through either metabolic engineering or chemical elicitation. Although callus cultures are superior to cell suspension cultures in terms of flavonoids production, cell suspension cultures can be easily mass produced to constantly supply the desired compounds to the market (Yusuf et al., 2013). Since most of the flavonoid compounds are produced in trace amounts, an alternative approach to increase the production of these phytochemical compounds is essential. Lucrative opportunities are offered by the field of biotechnology for plant based *in vitro* systems, whereby these plants represent sources for easy and scalable production of secondary metabolites with therapeutic

importance (Efferth, 2019). Green cell factory for advanced production of secondary metabolites are the current approaches in plant biotechnology to enhance these compounds (Marchev et al., 2020).

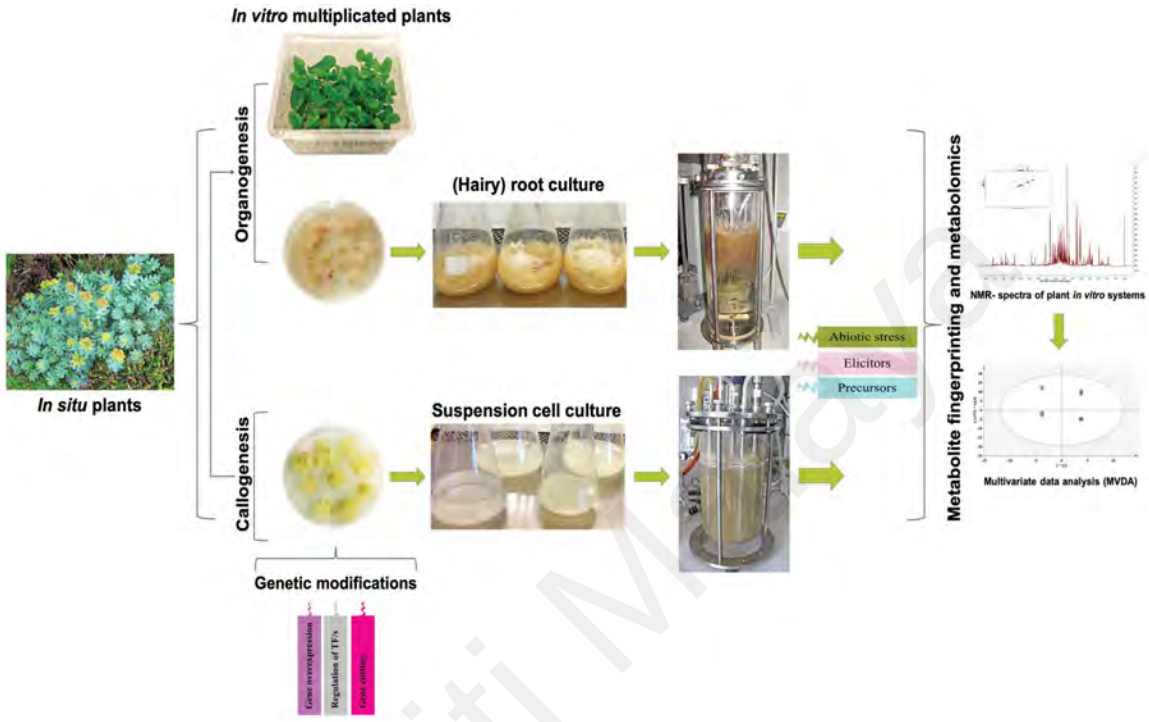


Figure 2.3: A general view on metabolic manipulation and bio-production of metabolites from *in vitro* systems (Marchev et al., 2020).

2.3.2.1 Plant tissue culture: cell and organ cultures

Cell toti-potentiality and cellular plasticity is the major physiological principle behind the plant tissue culture (Singh, 2019). Cell plasticity responses for the division and differentiation capacity of the culture cells (Gonzales et al., 2010) and the ability of the single cell to transform into a whole plant resembling exactly as the mother plant (Bhoite et al., 2014). Explants when cultured on the appropriate medium containing a particular concentration of auxin and cytokinin, develops into callus tissue, i.e. unorganised, growing and dividing mass of cells (Slater et al., 2008). Medium containing these plant growth regulators (PGRs) is known as callus inducing or

initiation medium and were extensively studied on its various concentrations manipulation to elicit development responses (Philips & Garda, 2019). Auxins and cytokinins used in combination elicit an array of tissue responses based on their relative concentrations. Higher relative auxin concentrations increase root formation, whereas higher relative cytokinin concentrations induce shoot formation. When both auxin and cytokinin concentrations are moderate to high, callus is induced. Proliferation of callus mass in a relatively unorganised way will continue for a prolonged period, if the callus tissue is maintained in the same medium through a number of subcultures. But the main objective in plant tissue culture is to regenerate a plant or plant organ from the callus culture. Scattered areas of actively dividing cells, known as meristematic centres, develop as a result of differentiation and their further activity results in the production of root and shoot primordia. Skoog and his co-workers at Wisconsin, in their studies with tobacco stem pith culture, demonstrated that the initiation and the type of organ primordia formed from the resulting callus culture could be controlled by appropriate adjust-ment of the relative levels of the auxins and cytokinins (Skoog & Miller, 1957).

The production of adventitious roots and shoots from cells of tissue culture is called organogenesis. Organogenesis means the development of adventitious organs or primordia from undifferentiated cell mass in tissue culture by the process of differentiation. It can be further defined as caulogenesis, a type of organogenesis by which only adventitious shoot bud initiation takes place in the callus tissue. The other counterpart is known as rhizogenesis, which is a type of organogenesis by which only adventitious root formation takes place in the callus tissue. (Slater, 2008). The manipulation of the growth hormones may lead to shoot, root or somatic embryogenesis which may lead to plant regeneration. Shoots culture, root as well as hairy root culture are potential secondary metabolite (Yusuf et al., 2007; Jalil, et al*.,* 2014)

Cell suspension cultures ideally consist of only single cells but, in reality, they contain a range of cell aggregates, some containing up to several hundred cells. It is much easier to use suspension cultures, since the growth rates in suspension are much higher than that of callus culture (Walton et al., 1999). These are more preferred and suitable for large scale production as it is similar to microbial culture, whereby it exhibits rapid growth cycles. Hence, it is vital to sub-culture, also known as passaging cell suspensions, to prolong the life and/or to expand the number of cells in the culture (Fu et al., 1998). Cell passaging or the splitting technique enables the cells alive and growing under the required conditions (Fu et al., 1998).

2.3.2.2 Enhancement of secondary metabolites

The evolving commercial importance of secondary metabolites which are economically significant as drugs, dyes and pigments, pesticides and food additives contributed to understanding of secondary metabolites mechanisms especially in tissue culture technology (Kumari, 2014).

There have been studies highlighting productivity increase in plant tissue culture systems by manipulating medium variation (nutrients, phytohormones, precursors and anti-metabolites), culture conditions (in terms of inoculum size, pH, temperature, light and agitation) and specialized techniques such as elicitiors, immobilization, two-phase/ two- stage systems) (Yusuf, 2011) Some of the common in vitro enhancement techniques used are nutrient supplementations such as calcium (Ca2+) and sodium (Na+) (Ahmad et al., 2016), nitrogen, phosphorus, tyrosine feeding to increase polyphenols in *Coleus bleumei* (Musbah et al., 2016), chitosan treatment to enhance phytochemical production, enzymatic and antioxidant activity in spinach (S Singh et al., 2016). Elicitors methyl jasmonate, salicylic acid and copper are also popular in enhancing these metabolites in vitro (Perez et al., 2014; Wang et al., 2015; Giri et al.,

2016; Singh et al., 2018). Combinational studies on both elicitors and precursors MeJa and phenylalanine were used (Jung et al., 2011; Saiman et al., 2012) A much hyped technique to elevate biomass elevation and secondary metabolite would be bioengineering to enhance biomass accumulation (Nartop et al., 2018). For instance, manipulation of plant specific transcription factor LrTCP4 (Chahlel et al., 2019).

2.4 Application of phenylalanine in precursor feeding strategy

Phenylalanine feeding is said to theoretically increase the production of chalcones, hence giving rise to other useful flavonoids, flavanones, flavanols, isoflavanoids, anthocyanidins anthocyanins (Masaumian et al., 2011). These strategies of enhancements include the use of precursors and the manipulation of metabolic pathways, including phenylpropanoid pathway (Tan et al., 2015). Phenylalanine produces phenolics and flavonoids by using enzymes such as phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase, and 4-coumarate-CoA ligase (Wee, 2015). Since flavanoids are derived from phenylpropanoid pathway; an essential pathway in plant secondary metabolism (Wang et al., 2016, Mustafa et al., 2012). The PAL pathway can be explained as per Figure 2.4, with the respective genes playing its role. These general phenylpropanoid enzymes would be activated by stresses like wounding, pathogen attack or UV radiation and are largely controlled at transcriptional level (Sullivan 2009; Gutierrez- Carbajal et al., 2010). Despite the available evidences, there is little understanding about the pathway-based genes that orchestrate rapid, coordinated induction of phenylpropanoid defenses in response to stress (Yadav et al., 2020)

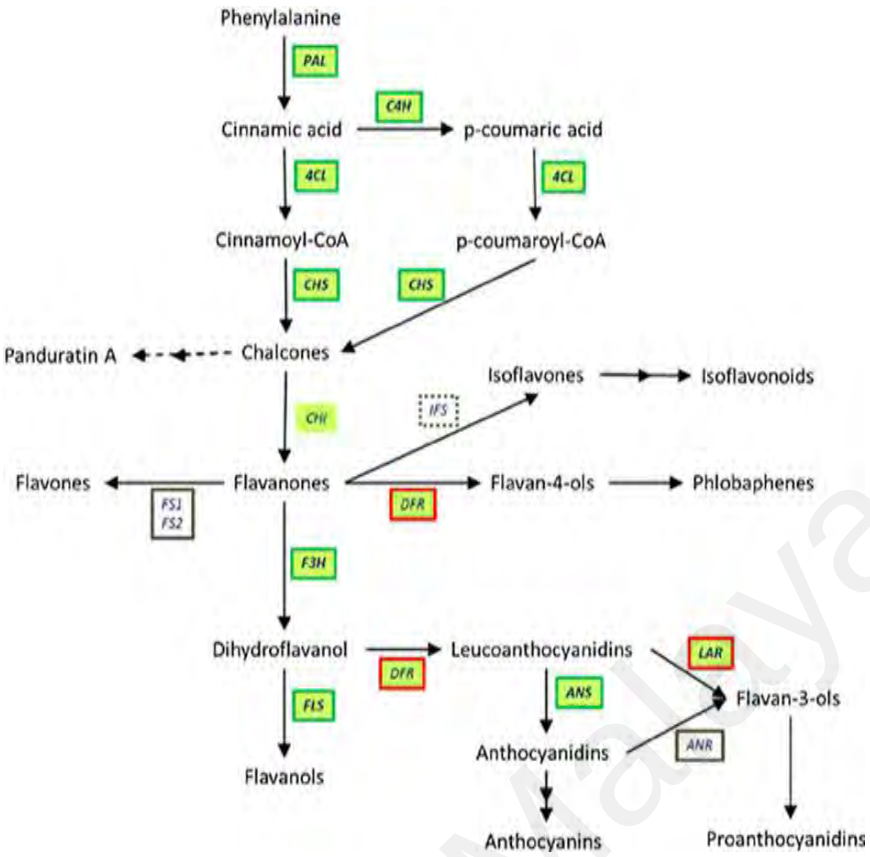


Figure 2.4: PPP, indicating role of phenylalanine in PAL activation and the regulatory genes involved in flavonoid (Mustafa *et al.*, 2012).

When these precursors were added to this culture it was found that the optimal time for supplying the precursors was at inoculation of the cells into the production medium (Whitmer et al., 2002). Precursor feeding to plant cell culture system may be promising as it showed favorable results. The key to successful protocol using precursor feeding lies in identification of cheapest by product of other processes which can be converted to desired secondary metabolite by selected plant cell line. For instance, in a study conducted by Swieca et al., 2014, significant enhancement of the antioxidant potential of sprouts that was strongly and positively correlated with total phenolic content. The highest increase of flavonoids content was found for the sprouts obtained with phenylalanine treatment (2.41 mg/g FM, 1.6-fold with respect to the control). The highest increase in the antioxidant capacity was found for the sprouts obtained with

phenylalanine (an increase of 27% after solid–liquid extraction). Though precursor feeding enhances secondary metabolism, the exact mechanism of biosynthesis is still not exactly understood thus, an intensive research in this field for exploitation of plant cells for the production of secondary metabolites is envisaged (Namdeo et al., 2007). A review paper summarized the wide usage of precursors to enhance secondary metabolites production in other plant species, as per Table 2.1. Usage of amino acids like phenylalanine, tryptophan, proline in callus, shoot or cell cultures are quite common to enhance flavonoid production. Another way to trigger the PPP pathway is by the supplementation of hormones like progesterone or other by enzymes or catalysts that that are involved in the PPP pathway itself, such as cinnamic acid or ferrulic acid which activates the genes involved in the whole production of the flavonoids as secondary metabolites in plants.

Table 2.1: Secondary metabolite-enhancing precursor applications in plant cell and tissue cultures (Nartop, 2018)

Precursor	Plant	Culture Type	Secondary Metabolite	References
Phenylalanine (20 mg/L)	*Sauropus androgynus*	Callus	Phenolics, Flavonoids, Naringenin, Quercetin, Kaempferol	Wee, 2015
Proline (50-150 mg/L)	*Verbascum thapsus* L.	Callus	Coumarin, Eugenol	Al-Jibouri et al. 2016
Tryptophan (150 mg/L)	*V. thapsus* L.	Callus	Thymol	Al-Jibouri et al. 2016
Cinnamic acid Catalpol (2.5 mg/100 mL)	*Picrorhiza kurroa*	Shoot	Picroside-I	Kumar et al. 2016
Progesterone (200–300 mg/L)	*Digitalis purpurea* L.	Shoot	Digitoxin, Digoxin	Patil et al. 2013
Arginine	*Erythroxylum coca*	Cell	Tropane alkaloids	Docimo et al. 2015
Glutamate				
Proline				
Phenylalanine				
Amyrin-enriched resins	*Centella asiatica*	Cell	Centelloside	Hidalgo et al. 2017
Ferulic acid	*Capsicum frutescens*	Plant	Vanillin	Chee et al. 2017
Phenylalanine (10 mM)	*Juniperus virginiana* L.	Callus-Cell	Podophyllotoxin	Kasparova et al. 2017

CHAPTER 3: MATERIALS AND METHODS

3.1 Plant material and surface sterilization

Mature *B. rotunda* rhizomes were purchased from an herbal supplier at a local market in Kuala Lumpur, Malaysia. These rhizomes were cleaned under running water until its completely clear or free from soil residues, drained and placed in an open basket laced with cotton to absorb excess moisture and to allow shoots to sprout. As for the shoot generation, layering the ginger root horizontally is essential to avoid overcrowding and to ensure the young shoots sprout up-right. This will ease out the manuring of the buds during the next stage, which is callus induction. Precautions were taken, so that no fungal growth is developed throughout the sprouting process. The sprouting buds with two to four cm in length were surface sterilized using 20% (v/v) Clorox® (containing 5.25% w/v active sodium hypochlorite) containing 0.1% (v/v) Tween-20® for 20 minutes with vigorous stirring using a magnetic stirrer. The buds were rinsed in distilled water, dipped in absolute alcohol for 30 s, rinsed three times with distilled water and dried on sterile filter paper in a laminar air flow cabinet (Gellman Sciences). The external leaves were removed and buds were trimmed into small pieces (0.5-0.8 mm in size) for callus induction.

Figure 3.1: (A) Buds of *Boesenbergia rotunda* at week 8 (Bar: 1cm); (B) The length of buds was ensured to be around 2-4cm (Bar: 1cm).

3.2 Callus induction

Callus cultures were induced as per the procedures of Yusuf et al. (2011) with slight modifications. The cleaned bud explants were inoculated on a sterile petri dish containing Murashige and Skoog (MS) medium supplemented with 1.0 mg/ L 2,4-Dichlorophenoxyacetic acid (2,4 D), 0.5 mg/ L α-napthaleneacetic acid (NAA), 0.5 mg/ L Indole Acetic acid (IAA), 0.5 mg/ L Biotin, 30.0 g/ L sucrose and 2.0 g/ L gelrite for 30 days. The pH was adjusted to 5.7 prior to autoclaving at 121ºC for 20 minutes and the media is poured into sterile petri dishes and let up to three days, to ensure there's no emergence of any contamination. Hence, it should be safe enough to use for callus initiation purposes. The cultures were maintained in a growth room in the dark at 25 ± 2ºC. Callus sub-culturing begins after 3-4 weeks, once the calli are formed. Sub-culturing helps to mass propagate a large stock of calli for other subsequent applications. After about 30- 60 days, the friable calli were separated and transferred to conical flasks containing 1.0 mg/ L 2,4 D, 0.5 mg/ L BAP and L-glutamine.

3.3 Initiation and maintenance of cell suspension cultures

Initiation of cell suspension culture was carried out according to Wong et al. (2013). In order to initiate cell suspension cultures, 0.2 to 0.5 g fresh weight of friable callus grown on propagation media was transferred into 50 mL Erlenmeyer flasks containing 10 mL of liquid MS basal medium supplemented with 1.0 mg/L 2,4-D), 0.5 mg/L 6-Benzylamino purine (6- BAP) and 3% (w/v) sucrose (designated as BD30 medium) and were agitated at 80.0 rpm on a rotary shaker (New Brunswick Scientific, USA). After 14 days of culture, the cell suspension cultures were scaled up by adding 30.0 ml of the same fresh medium to the 250.0 mL Erlenmeyer flasks. To obtain fine and homogenous cell suspension cultures, cells were sieved through a sterile, 425.0 µm stainless steel sieve. The filtrate was used as an inoculum for establishing fresh suspension cultures.

3.4 Growth Curve Determination and Settle Cell Volume (SCV) Technique

Settled cell volume (SCV) methods were used to study the biomass growth on the cell suspension cultures in a 2-day interval for 30 days. Randomly picked culture flasks were selected. 10.0 m into 15.0 ml Falcon tube. The cells were allowed to settle down for about 1.0 minute. The maximum growth period was used as an indicator point to treat the cells with precursor to enhance flavonoid production. The morphological changes of the cells were also observed. The cells were self-compacted by letting the cells settle at the bottom of the Falcon tube and the measurements were taken on an average of three replicates. Once the rough estimate is marked, the sample is poured back into the flask, sealed and kept in agitator at 80 rpm. Similar replicates are used throughout the growth curve study. It is essential to maintain aseptic practices during the course of this SCV technique. Sub-culturing was carried out every 6 days, as per the growth curve obtained; by replacing fresh liquid media at a 1:4 (old to fresh media) ratio. Refer to the flow chart (**Appendix A**) for detailed steps.

3.5 Phenylalanine treatment and extraction of secondary metabolites

Established cell suspension cultures were treated with a precursor, phenylalanine, at different concentrations on 14th day after subculture. 1.0 mg/ml of Phenylalanine (Phe) (Sigma Aldrich, USA) stock solution was prepared, before adding the stock at different concentrations (20.0, 40.0 and 50.0 mg/L) into the *B. rotunda* cell suspension cultures. The cells were incubated for 3, 7, 14 and 21 days respectively. Flavonoid compounds were extracted according to Yusuf et al. (2013). Cells suspended were oven dried at 38-40ºC for three days and was pulverized using mortar and pestle once dried. Powdered samples (1.0 g) were soaked in 100.0 ml of absolute methanol for 72 h and filtered through Whattman No. 1 filter paper. The filtrates were concentrated in a rotary evaporator (Buchii Rotavapo) and the slurry residue was partitioned against an equal volume of ethyl acetate (EA) and water. The EA fraction was vacuum-dried and the mass of the crude extract was recorded. The crude extract was dissolved in methanol and filtered through a 0.45 μm PTFE filter prior to analysis by high performance liquid chromatography (HPLC). A graphical step by step work flow chart on obtaining the crude extract is presented as per Figure 3.2.

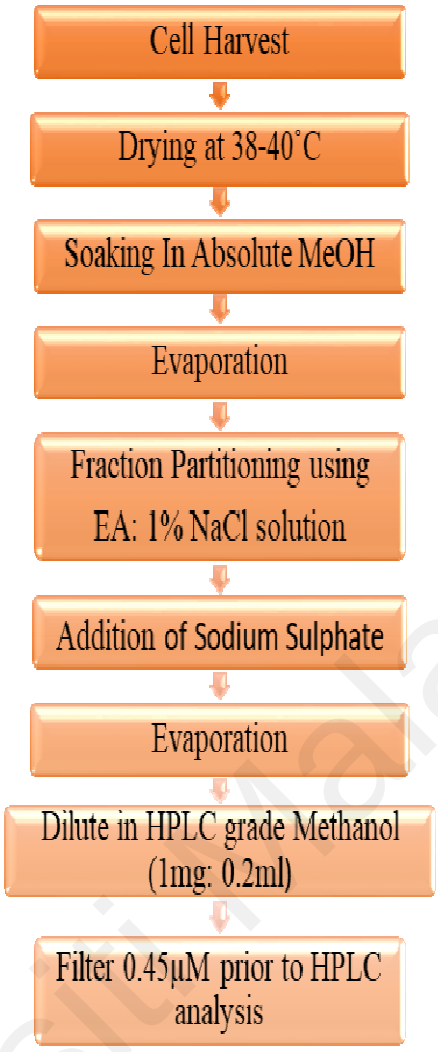


Figure 3.2: *Boesenbergia rotunda* cell extraction after phenylalanine treatment for flavonoid content analysis (detailed steps at Appendix B and C)

3.6 HPLC analysis

3.6.1. Instrument set up and solvent gradient configuration

Crude extracts which were obtained from phenylalanine-treated and untreated cells were injected into the HPLC system (Shimadzu Auto-sampler®; Liquid Chromatograph Communication Bus®, Diode Array®/Column Oven®; which is controlled by LC solution software) in order to quantify the flavonoid contents. The solvent system used 20% HPLC grade acetonitrile (Fischer Ltd.) (Solvent A) and 80% water-phosphoric acid. The gradient will be gradually increased (Solvent A) from 20% and held for 5 min

and then to 35%, 40% and later increased to 100% at and finally back to 20%, each at the designated time lapse (see Table 3.1). A total of three runs of using three biological replicates were conducted for each sample, and peak areas with less than 5% standard deviation were recorded. The compounds were then quantified by comparing the absorbance (at 285 nm & 330 nm) of the selected external standards i.e. pinostrobin, pinocembrin, panduratin A and cardamonin. Chromolith® RP-18, reverse columns were used throughout the analysis.

Table 3: HPLC Gradient Analysis Configuration

TIME (min)	FLOW (ml/min)	C (Acetonitrile)- Solvent A	D (water)- Solvent B
0.01	1.5	20.0	80.0
0.50	1.5	20.0	80.0
1.00	1.5	35.0	65.0
5.00	1.5	35.0	65.0
5.50	1.5	40.0	60.0
10.00	1.5	100.0	0.0
22.00	1.5	100.0	0.0
23.00	1.5	20.0	80.0
32.00	1.5	20.0	80.0

3.6.2 Calibration curve of standard compounds

Commercial reference standards of pinocembrin, pinostrobin, cardamonin (Sigma Aldrich, USA) and a pure compound of panduratin A synthesized *in- house* laboratory was used to derive the calibration curve. All crude extract solutions were prepared using HPLC grade methanol and the standard calibration were developed at λ = 285nm and 330nm. Peak area of each diluted solution was determined and a standard calibration curve was obtained for each compound. Flavanones (pinocembrin and pinostrobin) and chalcones (panduratin A and cardamonin) were identified by comparing their retention times with those of authentic standards. Quantification was repeated three times for each sample.

3.7 Statistical analysis

All data were analyzed statistically by analysis of variance ANOVA test followed by Duncan multiple-range test at a significance level of $p < 0.05$ using LC software.

CHAPTER 4.0: RESULTS

4.1 Source of explant

Sprouts from rhizomes were used as explants. Rhizomes were continuously sprayed with water and kept in the dark to initiate sprouts. It was also essential to keep these rhizomes in a dark area and kept upright to encourage vertical sprouting. At day 8, the sprouts began to emerge and grew to a height of less than 1 cm after 16 days (Figure 4.1 A). Sprouts were left to grow for 32 days to obtain the ideal size of about 3-5cm for culture inoculation (Figure 4.2 B). It is important to use consistent sprouts' size to ensure success in obtaining callus as basal meristematic explants have high mitotic activity which are good for callus initiation. Immature tissues or shorted shoots have better plasticity compared to mature ones. Small sprouts also succumbed to browning.

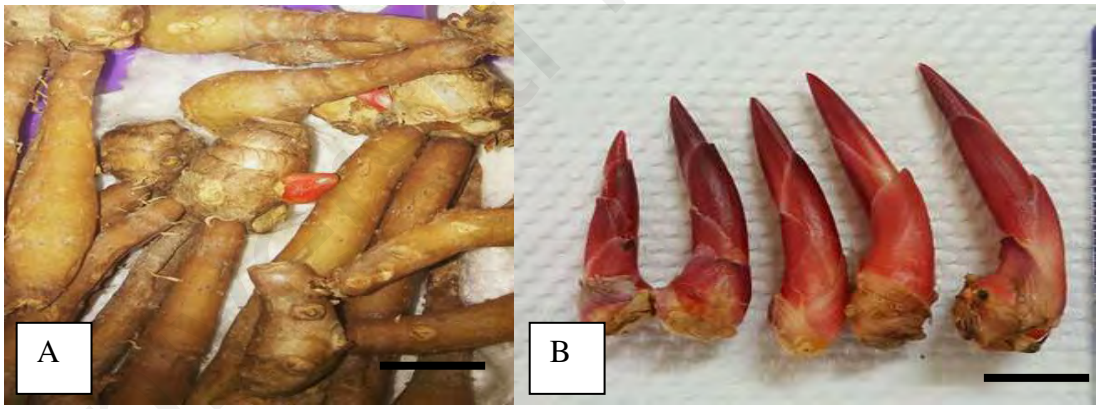


Figure 4.1: Morphology of the emerging sprouts from rhizomes. (A) size of sprouts less than 1 cm after 16 days (B) sprouts of about 3-5cm after 32 days (Bar: 1 cm).

4.2 Callus induction

Horizontally sliced sprouts were used as explants, making sure that the meristematic tissue was in contact with the media. It was observed that the proximal part was more responsive to callus initiation than the distal part of the sprout (Figure 4.2 A). Callus

was initiated from explants after 6 weeks in culture from all explants cultured except for those near the distal end (Figure 4.2 B). Calli obtained were a mixture of compact and friable callus (Figure 4.2 C). Calli were left to grow without being subcultured. Only calli that were friable and creamy in color doubled in size (about 1cm in diameter) after 12 weeks were used for cell suspension establishment. Callus cultures were subcultured not later than 12 weeks because the callus started to produce roots and turned brown after 15 weeks (Figure 4.2 D). Friable callus were transferred to liquid media for the establishment of cell suspension and some of the callus were subcultured onto solid media. Sub-culturing was discontinued once the callus ceased growing in the culture media.

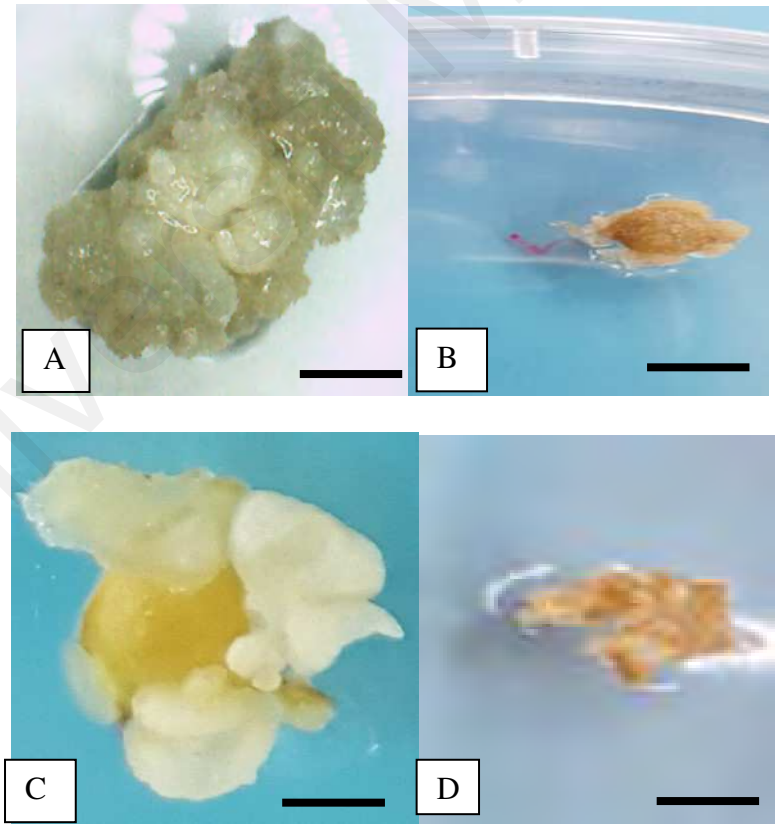


Figure 4.2: A) Friable callus formed after 6 weeks B) Explant at the distal end C) A mixture of compact and friable callus D) Rhizogenesis observed at week 15 (Bar : 1mm).

4.3 Establishment of cell suspension

The friable calli were transferred into the liquid culture containing MS media with 1.0 mg/L 2,4-D , 0.5 mg/L BAP and glutamine for initiation purposes. The ratio between callus inoculum and liquid media were maintained at about 0.5g to 10.0 ml media for 14 days. Additional 40.0 ml of fresh media were added after the 14th day and maintained in suspension form, with agitation. After about 3 months, light yellow cell suspension cultures containing aggregation of viable and vacoulated cells were established. These cells were maintained in 250.0 ml conical flasks under continuous agitation at 80 rpm to encourage cell growth. For subsequent experiments, homogenous and fine cells were desired in order to obtain vigorous and stable cell suspension cultures. Non homogeneous cell population resulted in slow growth.

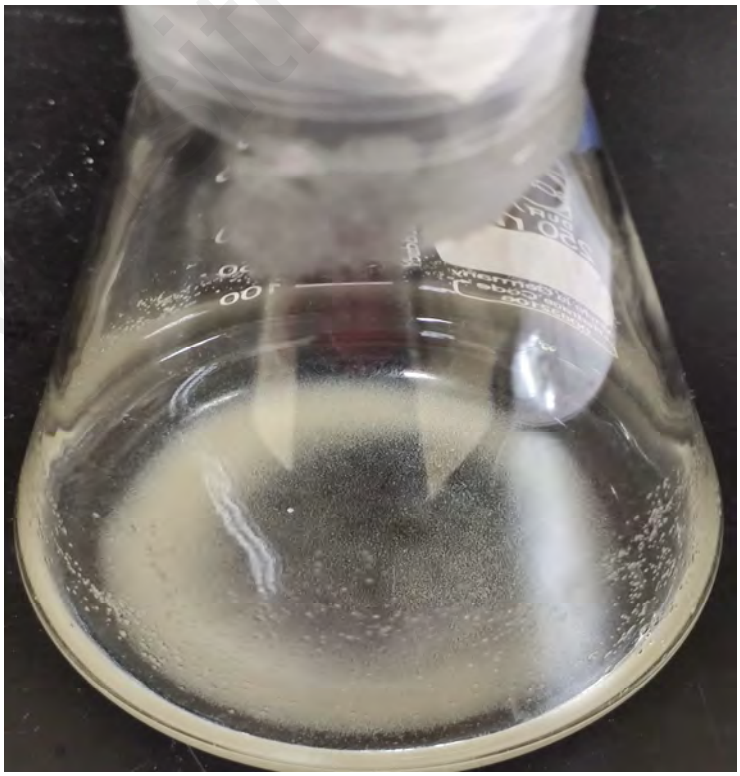


Figure 4.3: Fine and homogenous cell suspension culture in MS medium supplemented with 1.0 mg/L 2,4-D and 0.5 mg/L BAP.

Homogenous population of viable cells were obtained (Figure 4.3), by sieving using a sterile, 425µm stainless steel sieve and swirling of the cell cultures during subculturing every 14 days. Sieving the cells could separate the fine cells in the filtrate from the large vacuolated cells. Subsequently, the flasks were swirled to segregate healthy cells and cell debris. It was observed that the dense viable cells settled at the bottom of the flasks quicker than the vacuolated cell debris. These cells were observed under an inverted microscope to check its condition (Figure 4.4). The vacuolated cells were sucked out using pipettes before settling down. Dense viable cells were maintained and propagated for the next subculture. This procedure was repeated until homogenous fine and viable cell suspension cultures were established.

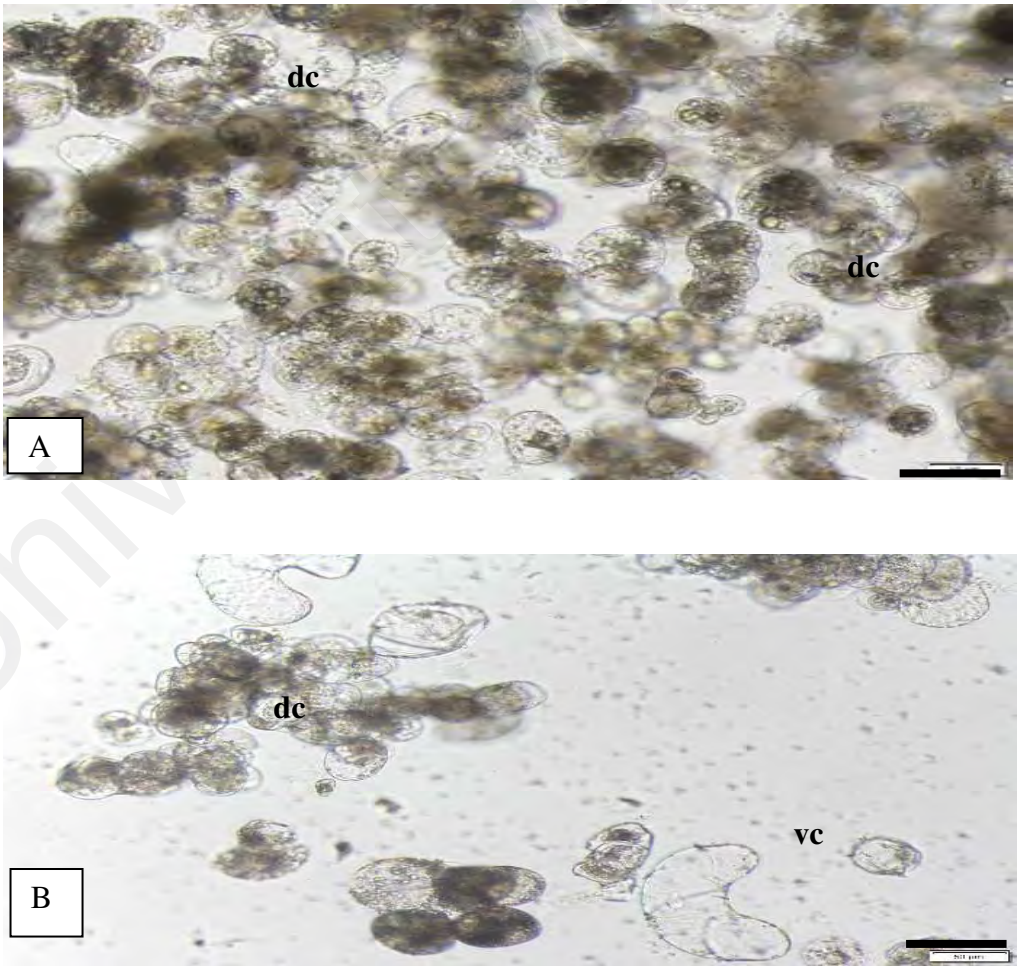


Figure 4.4: (A) Denser looking cells on the left, and its unhealthy counterpart, elongated vacuolated counterpart (B). Cells were viewed under an inverted microscope. Symbols; dc: dense cytoplasm; vc: vacuolated cell; w: wall (Bar: 20µm)

Samples of the cell cultures were also aliquoted for viability test through observation under the microscope. Cells in small clumps which were dense in cytoplasm were considered healthy, in contrary, vacuolated cells were unhealth and older cells. Only cell cultures containing viable cells were sustained for sub culturing and subjected to further treatment in subsequent experiments.

4.3.1 Growth Curve

The growth curve consists of four distinct phases. The initial phase, which is the lag phase fell in between 0-3 days. This is where the first point of treated cells was pulled for extraction purpose. The log phase fell between 3 days to 10 days. This growth curve derived contributed to the mid log phase, which is at the 6th day in this study (Figure 4.5). This point was then taken into consideration to maintain the cells i.e. the cell suspension cultures were sub-cultured. Following the log phase would be the stationary phase, between 10 to 16 days whereby the cell growth attained plateau stage. 16^{th} day onwards showed reduction in the settled cell volume (SCV). There was a continuous, gradual drop of subsequent three readings which were recorded up to 22 days. Thus, this can be concluded as the death phase.

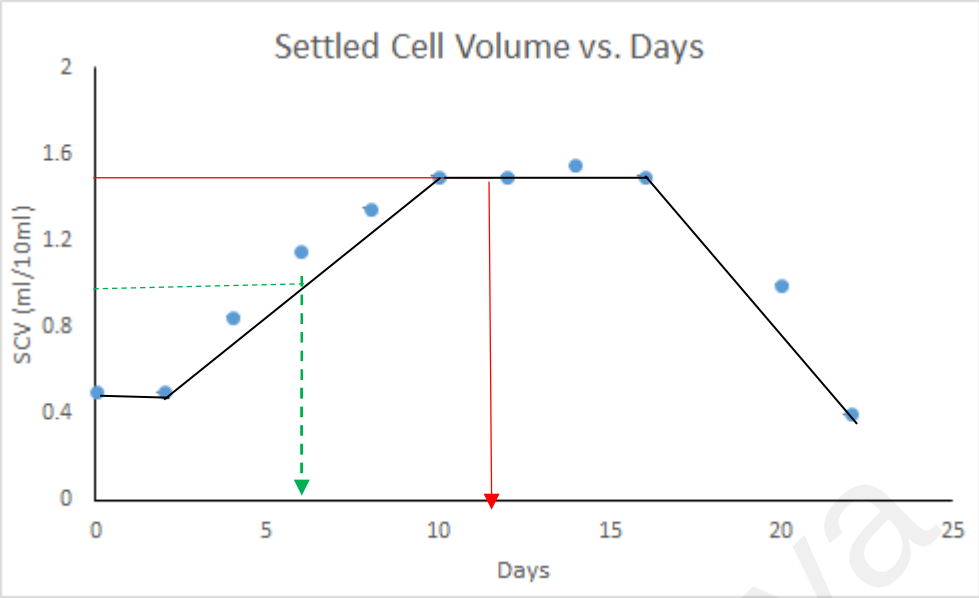


Figure 4.5: Cell growth curve indicating the lag phase (day 0-3), log phase (day 3-10), stationary phase (day 10-16), death phase (day 16-22) of *B. rotunda* cell suspension cultures.

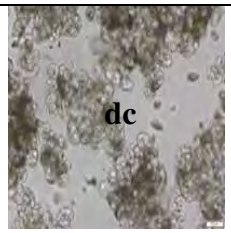
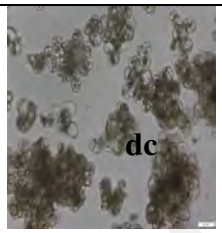
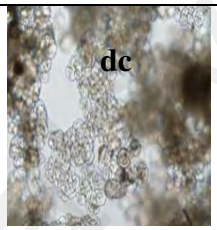
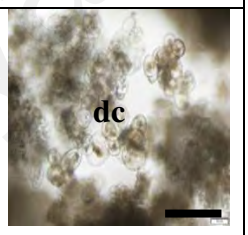
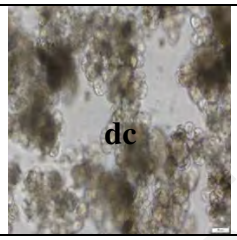
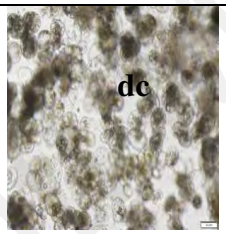
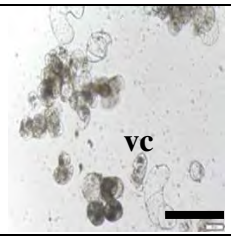
4.3.2 Phenylalanine Treatment

In the subsequent experiments, cells were harvested and observed under the microscope to determine viability of the cells before and after phenylalanine treatment (Table 4.1). The untreated cells were healthy throughout the 21 days. Upon confirming the condition of the healthy cells (intact cell wall, dense cytoplasm, spherical), the phenylalanine treatment was conducted. 14th day. The cells were treated with phenylalanine at various concentrations on the 14th day, which falls at the late stationary phase based on the growth curve. They were then harvested at 3rd, 7th, 14th and 21st days respectively. The controls were also pulled out at respective time points.

The cells seem to reveal morphological changes throughout the incubation time after the treatment whereby the density of the cell suspension samples were getting scantier as the days' progress. High density of healthy cells were observed at day 3, day 7, day 14 and day 21 pretreatment. Similar conditions were observed for post treatment cells at

day 3 and 7. However, at day 14 and 21, the cells were starting get elongated and vacuolated upon the occurrence of the treatment. A point to be noted here is that viability tests should be conducted using a standard assay kit that employs different staining colors to differentiate between viable and non viable cells.

Table 4.1: Observations of cells, before and after Phe treatment at respective incubation periods. Symbols; dc: dense cytoplasm; vc: vacuolated cells. Scale bar represents 20µm.

Incubation period	3 days	7 days	14 days	21 day
Control				
Treated cells				

4.4 Cell extraction & HPLC analysis

4.4.1 Calibration Curve

The targetted compounds i.e. pinocembrin, pinostrobin,cardamonin and panduratin A were calculated based on the calibration curve equation which were derived from each standard compound (Figure 4.7- Figure 4.10). A relatively ideal linear regression were obtained (R^2= 0.998) for all the compounds except pinocembrin and cardamonin whereby the regression value, R^2 deviated slightly by 0.0101 & 0.0112 respectively.

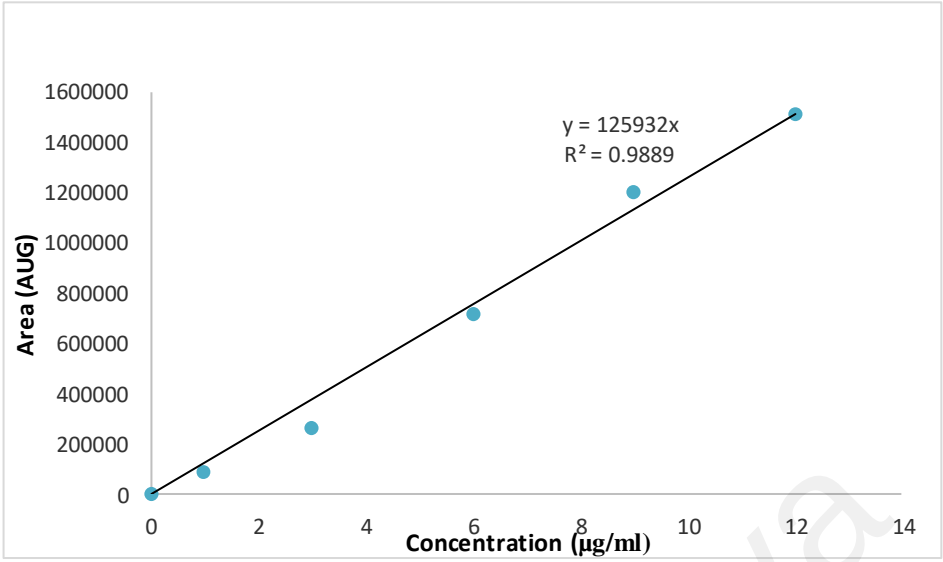


Figure 4.6: Calibration curve of pinocembrin (λ=285nm)

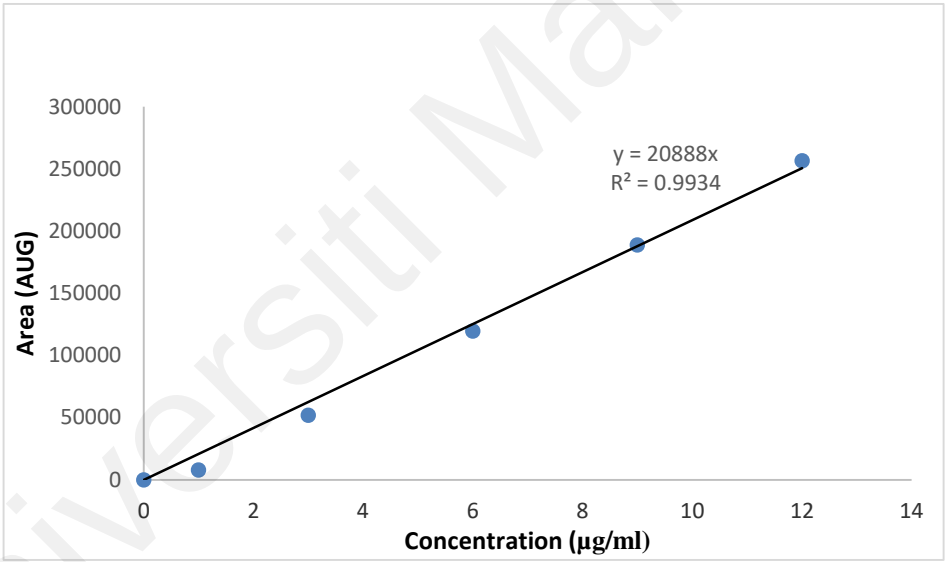


Figure 4.7: Calibration curve of pinostrobin (λ=285nm)

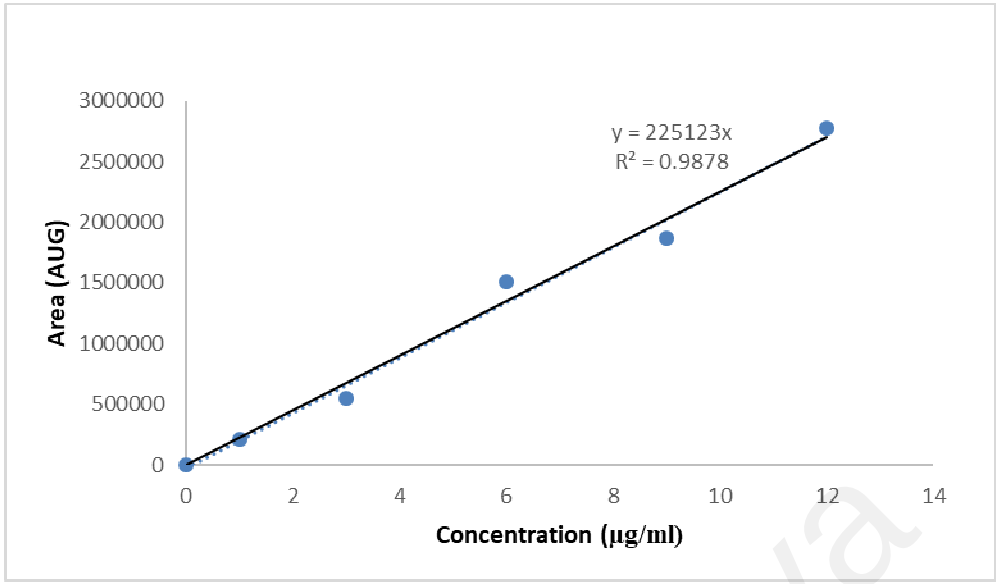


Figure 4.8: Calibration curve of cardamonin (λ=330nm)

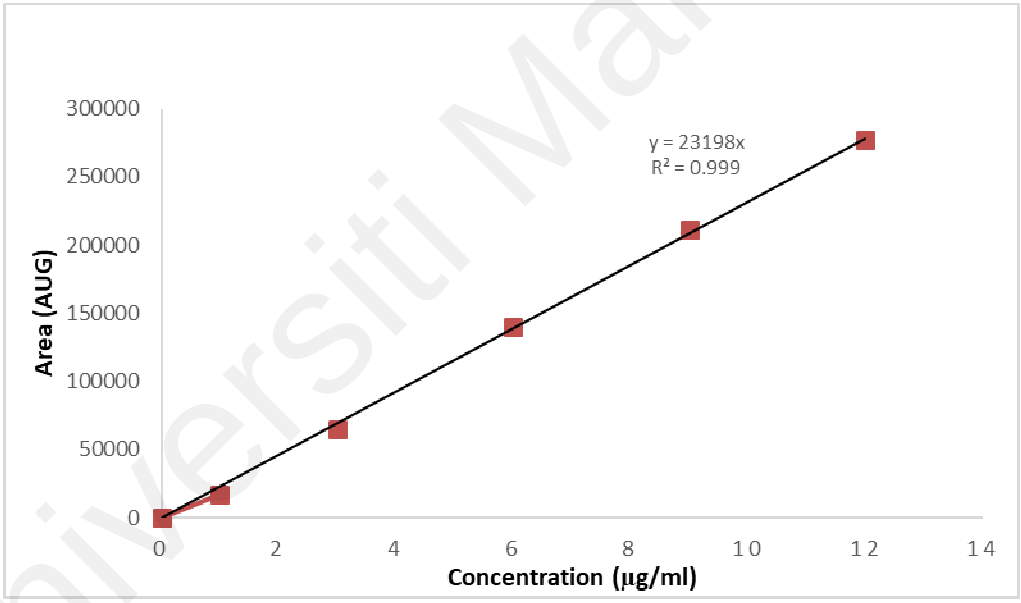


Figure 4.9: Calibration curve of panduratin A (λ=285nm)

The flavanones and the chalcone compounds had their own characteristic which absorb well at a certain wavelength. Pinocembrin, pinostrobin and panduratin A were best absorbed at 285 nm where the peaks were visible clearly (Figure 4.10) at their respective retention time (t_R) . Cardamonin were detected at the wavelenght of 330

nm, with the of t_R 12.4 minutes (Figure 4.11). The samples were also spiked with the pure standards to reconfirm the t_R.

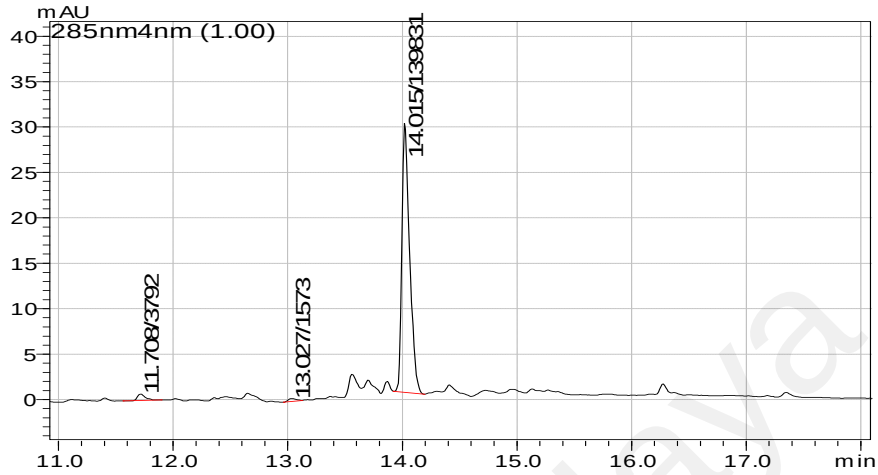


Figure 4.10: Standard mixture of pinocembrin, pinostrobin and pandurantin A being detected at 11.7 mins, 13.02 mins and 14.01 mins respectively (λ=285nm).

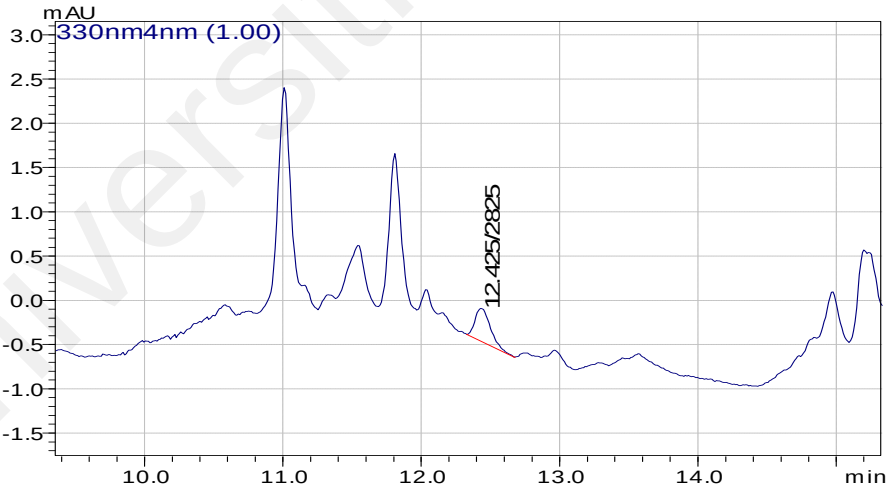


Figure 4.11: Standard compound for cardamonin detected at 12.43 min (λ= 330nm).

4.4.2 Flavonoid content

Statistical analysis is based on ANOVA test coupled with Duncan mulitple range test ($p<0.05$); whereby same letter indicates are not significantly diffferent, i.e “a” and “a” or “ab” are the same.

4.4.2.1 Pinocembrin

The results shows that the highest level of pinocembrin (0.257 µg/g), dry weight (DW) after Phe feeding was produced at day 21, when treated under 40 mg/L phenylalanine treatment (Figure 4.12). Hence it also gives credit to the significance of the treatment. However there were no significance for other days of incubation and the concentration of PAL treatment, in comparison to the baseline control, C0.

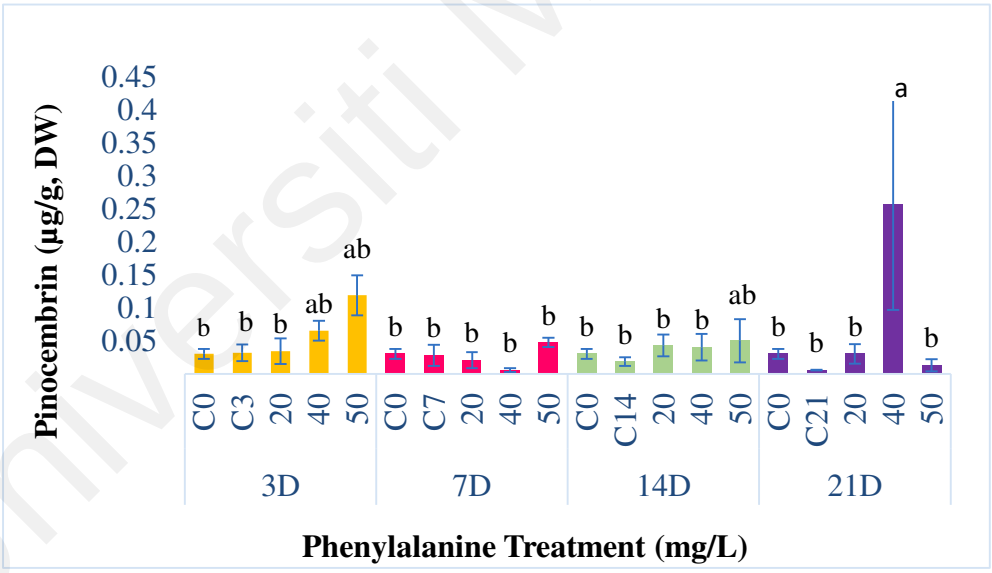
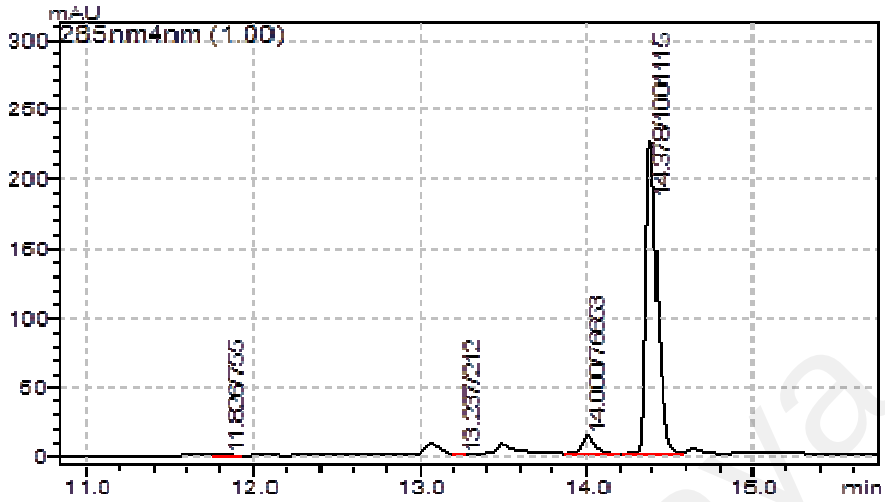


Figure 4.12 : The content of pinocembrin in different Phe treatment (20.0, 40.0, 50.0 mg/L) and incubation time (3, 7, 14 and 21 days). Bars represent the means and standard error, SE of three replicates.

(A) Control (Day 21, C21)



(B) Treatment (40.0 mg/ L phenylalanine)

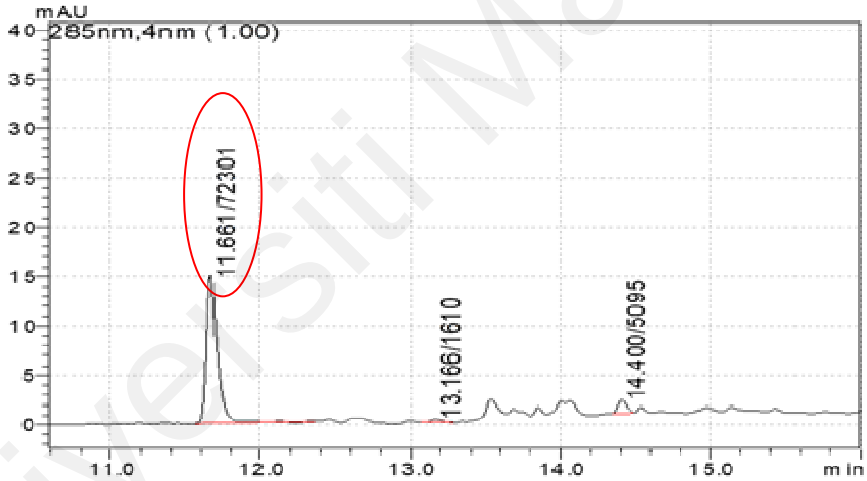


Figure 4.13: Chromatograms show the detection of pinocembrin in both (A) control and (B) upon precursor feeding, Phe (40.0 mg/ L) at day 21 of *B. rotunda* cell suspension culture.

4.4.2.2 Panduratin A

For the CCD compound, which is the panduratin A, at a glance, there is no significance in terms of incubation days and PAL concentration. The highest amount of total panduratin was recorded as 6.25 µg/g for 40.0 mg/L of treatment on day 7 (Figure 4.14). The control sample, C0 exhibited high level of panduratin A content (4.95 µg/g) for this particular cyclic aromatic compound. Chromatogram as shown in Figure 4.15. However, the controls of the incubation point, C3, C7, C14 and C21 were very much lower .

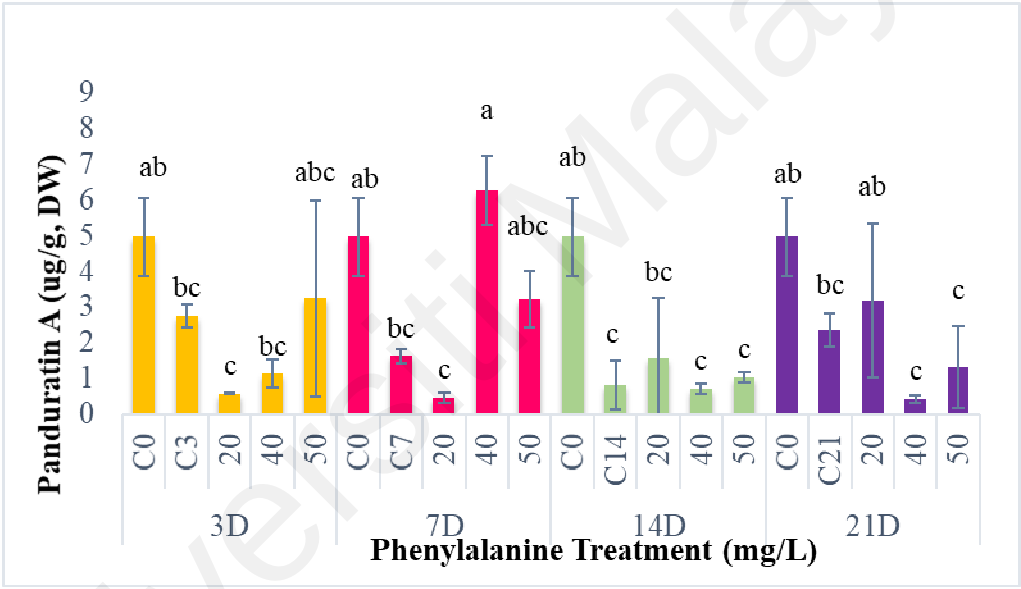
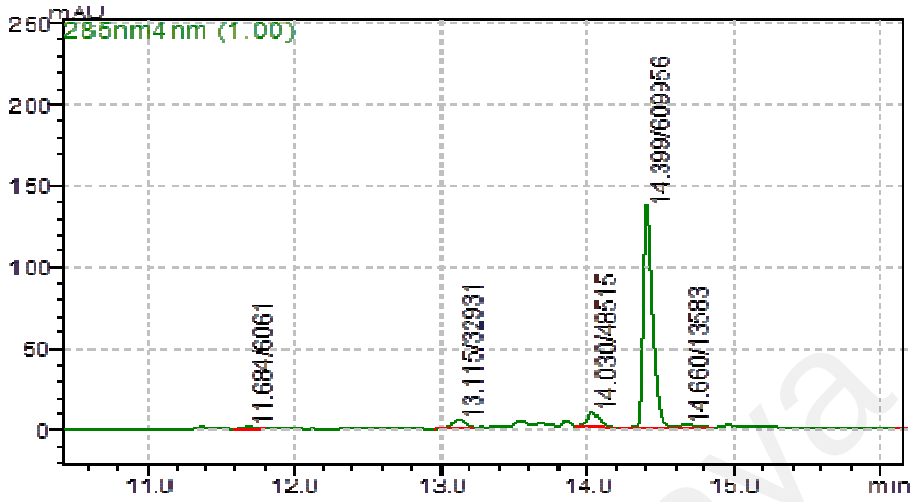


Figure 4.14 : The content of panduratin A in different Phe treatment (20, 40, 50 mg/L) and incubation time (3, 7, 14, 21 days). Bars represent the means and standard errors, SE of three replicates.

(A) Control (Day 7, C7)



(B) Treatment (40.0 mg/ L phenylalanine)

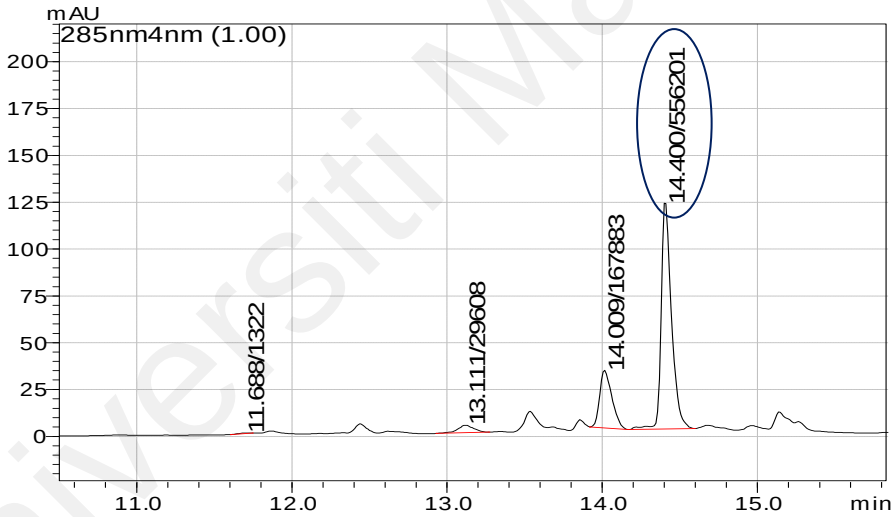


Figure 4.15: Chromatograms show the detection of panduratin A (A) control (B) upon precursor feeding, Phe (40.0 mg/ L) at day 7 of *B. rotunda* cell suspension culture.

4.4.2.3 Pinostrobin

The third flavonoid that was studied was pinostrobin. There was a significant increase after 40mg/L treatment at day 3 when compared to C0. The maximum content recorded was 2.71µg/g at day 3 (Figure 4.16). Chromatogram as shown in Figure 4.17. The rest of the reading did not exhibit any significance when the samples were compared to the control readings.

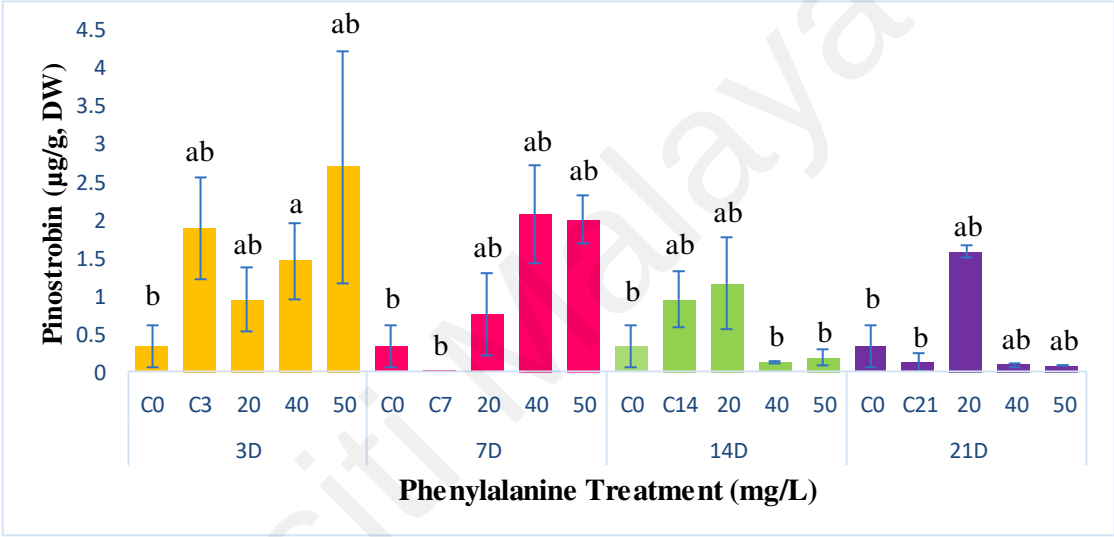
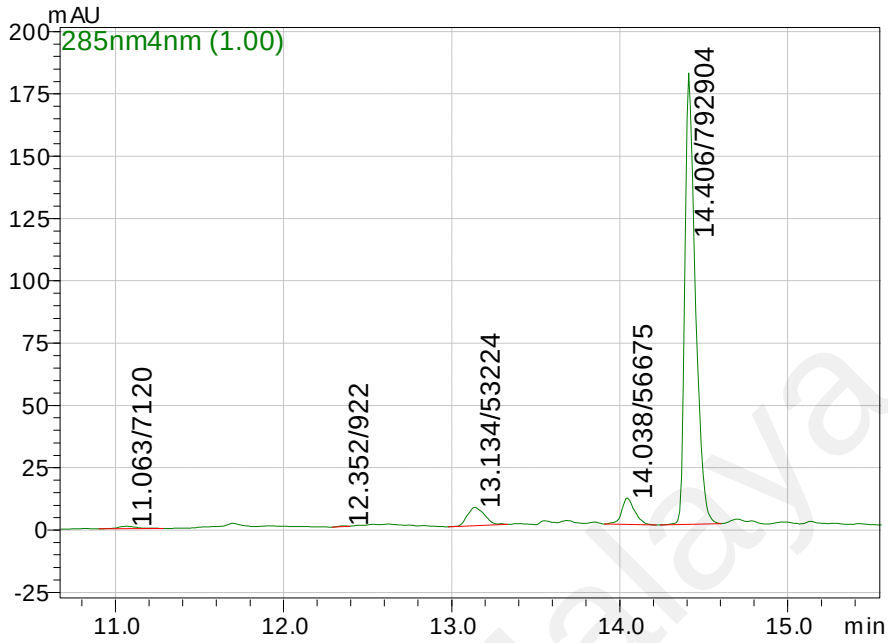


Figure 4.16 : The content of pinostrobin in different Phe treatment (20.0, 40.0, 50.0 mg/L) and incubation time (3, 7, 14 and 21 days). Bars represent the means and standard error, SE of three replicates.

(A) Control (Day 3, C3)



(B) Treatment (50.0 mg/ L phenylalanine)

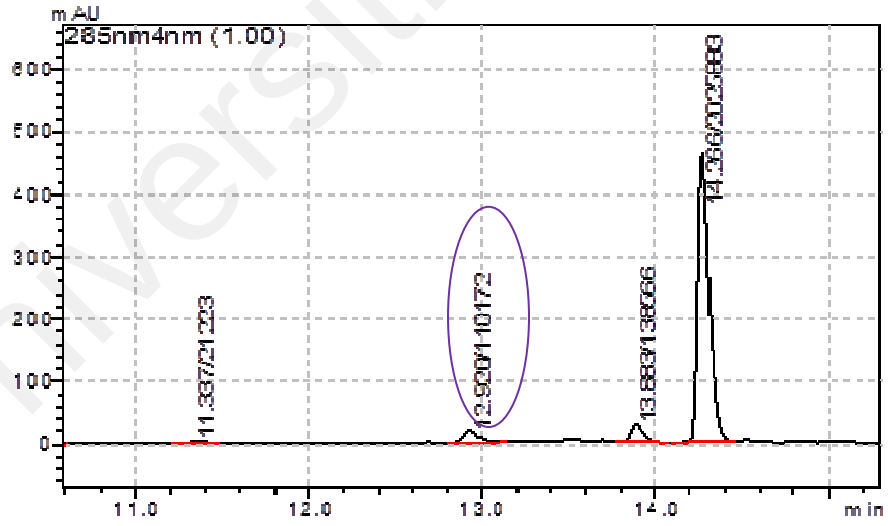


Figure 4.17: Chromatogram shows the detection of pinostrobin (A) control; (B) upon precursor feeding, Phe (50.0 mg/L) at day 3 of *B. rotunda* cell suspension culture.

4.4.2.4 Cardamonin

The cardamonin on the other hand, exhibits the highest amount (0.033 µg/g) at 20 mg/L phenylalanine day 21 (Figure 4.18). Chromatogram as per Figure 4.21. When compared to in terms of the trend of the flavonoid content, the reading decreased for day 3 and day 7 for all concentration when compared to C_0.

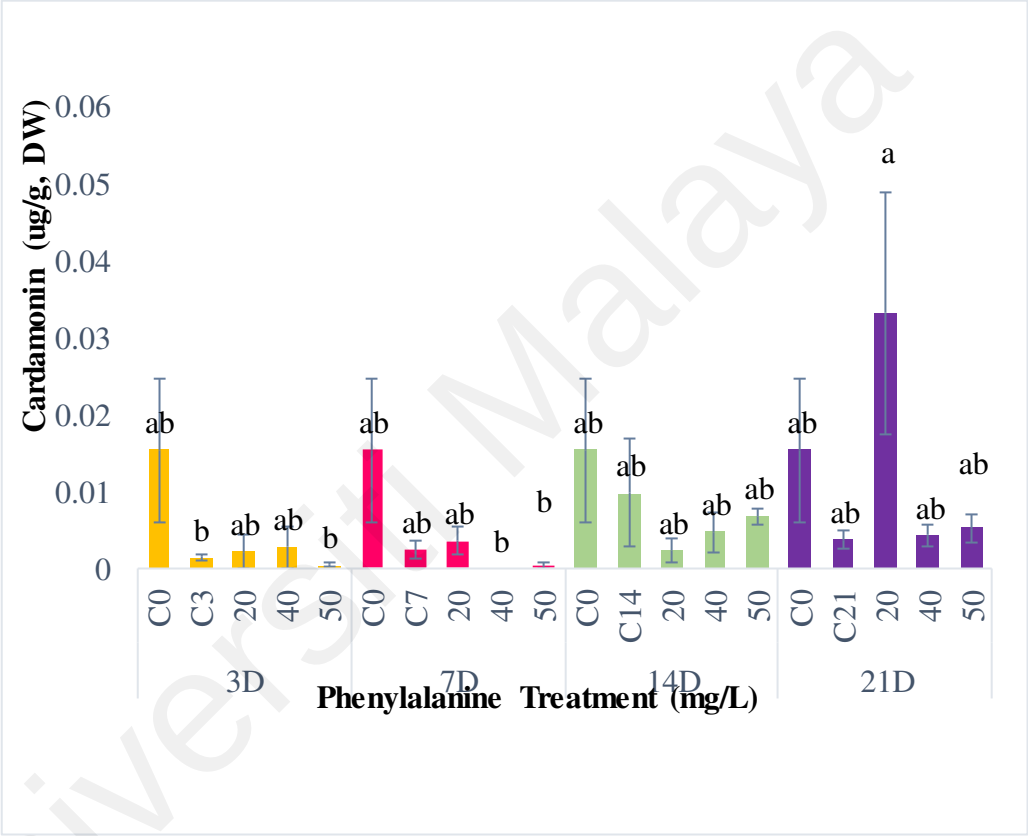
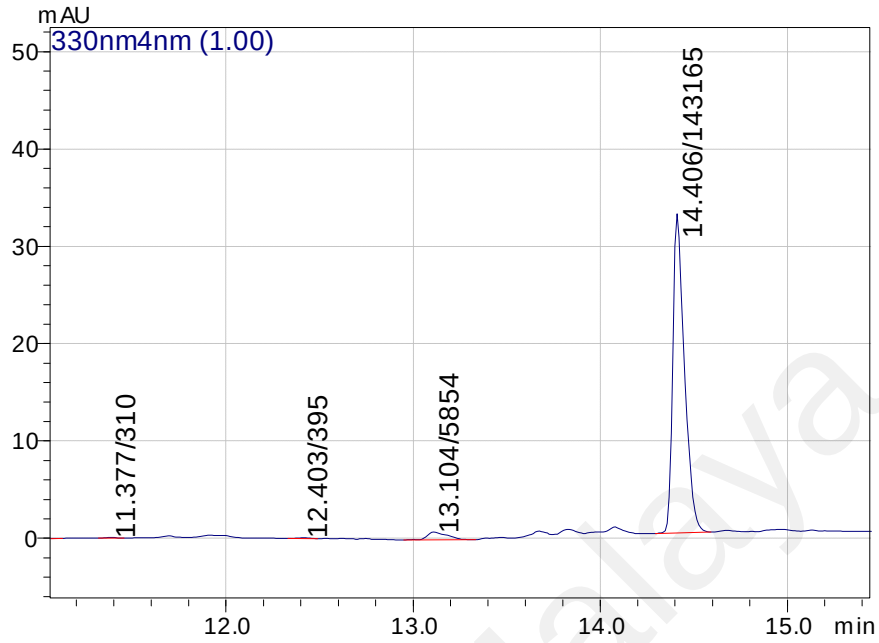


Figure 4.18 : The content of cardamonin in different Phe treatment (20.0, 40.0, 50.0 mg/L) and incubation time (3, 7, 14 and 21 days). Bars represent the means and standard error, SE of three replicates.

(A) Control (Day 3, C3)



(B) Treatment (20.0 mg/ L Phe)

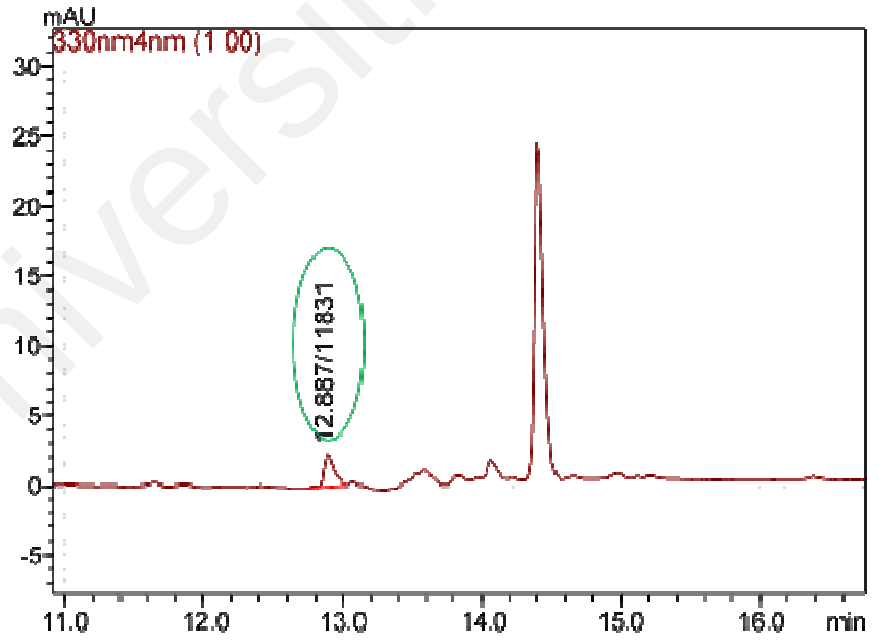


Figure 4.19: Chromatograms show the detection of cardamonin (A) control (B) upon precursor feeding, Phe (20.0 mg/L) at day 3 of *B. rotunda* cell suspension culture.

CHAPTER 5: DISCUSSION

5.1 Source of explant

Emergence of sprouts from rhizomes of *B. rotunda* were initiated as a source of shoot base explant prior to callus initiation for the establishment of cell suspension cultures. Rhizomes of *B. rotunda* were bought from a wet market based on availability and the harvest period was not taken into consideration. According to published reports, the accumulation of bioactive compounds in rhizomes of certain ginger species may differ based on the harvest period (Rudragouda et al, 2007). It was found that the agricultural practices may have impact on bioactive contents in the *B. rotunda* (Yusuf, 2011).

The length of the *B rotunda* sprouts was also kept within 2-5cm in length before excising the shoot base explants for culture. This was based on the results from an investigation on the relative concentrations of the four secondary metabolites in the different sections of the *B. rotunda* sprout that is T1 and T2 sections taken from 1–5 cm and 6–10 cm distant from the shoot base respectively (Ng et al., 2016). It was revealed that the shoot base (T1) had significantly higher concentrations of all secondary metabolites, decreasing from the shoot base and along the more distal sprout samples. This was consistent with the activity of biosynthesis in the rhizome and diffusion of its products along the shoot towards the growing tips. The shoot base samples contained meristem cells that were capable of differentiation into organs and self-multiplication. Thus, shoot base cells were actively involved in metabolism of carbohydrates, fat and proteins to form the ATP needed for growth (Ng et al., 2016). After the sprouts were excised, they were surface sterilized to remove contaminants.

In this study, it was crucial to surface sterilize the sprouts of *B rotunda* using a combination of disinfectants that is Clorox® and ethanol, with Tween 20® as surfactants to combat microbial contamination and particle aggregation (Holliester et

al.,1994). Clorox®, is a disinfectant containing hypochlorite as an active ingredient whereas ethanol is a powerful sterilizing agent but extremely phyto-toxic while Tween 20® acts as a mild non-ionic surfactant which is a highly hydrophilic wetting agent to reduce the surface tension; hence making cleansing more effective. Sterilized sprouts were dissected aseptically by removing the layers of sheath to expose the shoot base explants to be cultured. The contact time of the explants with the disinfectants and surfactants were imperative to ensure high survival rates of the explants.

In addition, the sterility of the media, tools, culture vessels used for this study were autoclaved at high temperature and pressure to ensure that even heat resistant microbes were eradicated. The autoclaved media were cooled to reduce condensation and kept up to at least three days in case of any microbe growth emergence. Prior to autoclaving, the pH of the media was adjusted to a range of 5.5-5.7 as a normal practice. However, repeated adjustment of pH by adding NaOH and HCl could lead to the increase in Na^+ and Cl^- concentration and decreased in Mg^{2+} and Ca^{2+}concentration in the medium due to precipitation (Bhatia, 2015). Hence, a study suggested that pH range of 5.5-7.5 in a medium could be directly used for apple tissue culture without adjusting pH (Shi et al., 2017).

5.2 Callus induction and cell suspension establishment

The preliminary basis of establishing a successful cell suspension lies in merging of various biotic and abiotic factors like inoculum size, pH, growth regulators, agitation speed, temperature, carbon sources and its concentrations. Hence, the conditions for the establishment of Zingiberaceae cell suspension cultures were well studied over the years in our laboratory (Tan et al., 2006, Yusuf et al., 2012, Wong et al., 2013).

The shoot base explants were sliced horizontally into 3-5mm size to ensure maximum surface area in contact with the media and efficient nutrient uptake. A mixture of compact and pale, yellowish friable calli was observed after 6 weeks in

culture. Callus initiation was first observed in the meristematic region of the shoot base explants. The proximal end was more responsive than the distal end of the shoot base explant. Similar finding was observed in Yang et al., (1991). This was due to the presence of the younger meristematic cells in the proximal than the distal end. Presence of young meristematic cells are crucial for cell differentiation (Ikeuchi et al., 2013) since these cells are physically and metabolically active. This finding was supported by a transcriptomics study in maize showing up-regulated transcripts in ATP synthesis in the newly formed shoot meristem, compared to mature meristem tissue (Takacs et al., 2012). Callus was left to propagate without subculturing due to the possible presence of growth promoting factors released by the actively growing cells which has promotive action on the adjacent cells (Slater, 2008). However, after 15 weeks, callus ceased growing and roots started to emerge from the explants probably due to depletion of PGR and stressed caused by phenolic oxidation.

Even though there was a mixture of compact and friable callus, only the friable, loose calli propagated profusely. Friability of the callus could be improved either by reducing the gelling agent or via repeated subculture (Slater et al., 2008). This friable calli were chosen to establish the cell suspension cultures. Wong et al., (2013) concurred with this finding. According to Yusuf et al., (2012), the establishment of cell suspension, the inoculum size used in this study was 2% (1.0 ml of SCV added into 50.0 ml of media). This inoculum size was optimum and apparently gave significant accumulation of the flavonoid content. Low inoculum size led to higher bioavailability of the cells towards the nutrient which might lead to a negative impact to the cells (Carvalho & Curtis, 1999). On the other hand, higher inoculum size limited the nutrient and oxygen availability (Haida et al., 2019).

5.3 Phenylalanine feeding and growth phases of the cell suspension culture

Phenylalanine has been shown to induce the expression of flavonoid-related genes of the general phenylpropanoid route leading to the generation of flavonoids essential for developmental and non-developmental processes (Sutela et al., 2014). Hence, in this study, the effect of phenylalanine on flavonoid production was investigated. Growth curve serves as reference point as to determine the precursor feeding of the cells. Phenylalanine is used as a precursor to enhance the accumulation of pinocembrin, cardamonin, panduratin A and pinostrobin in the *B. rotunda* cell suspension cultures.

In this study, the sigmoidal growth curve of the *B rotunda* cell suspension cultures showed that the lag phase was between 0-3 days. It was suggested that there was a correlation between pH and the cell growth phases (Muhammad Puad & Abdullah, 2019). During lag phase, the pH was said to have dropped forcing cells to adapt to the new environment in the culture media. Only competent cells were able to survive, whereas the rest of the non-competent cells started undergoing necrosis, releasing phenolic compounds which further decreased the pH of the cultures.

Log phase where the cells were actively growing was achieved between 3-10 days in the *B rotunda* cell suspension cultures in this study. The introduction of phenylalanine was considered ideal at this stage to stimulate the PPP and enhanced the flavonoid accumulation (Ata et al., 2015; Jalil et al., 2011). Previous studies have shown that feeding PAL to the suspension cultures during the mid log phase has enhanced the flavonoid accumulation (Ata et al., 2015; Yusuf et al., 2011; Tan et al., 2006). Some groups have focused on feeding the cultures during stationary phase where maximum biomass of the suspension cultures were already achieved. Jaishankar & Srivastava (2017) have shown that the cells during the stationary phase were still metabolically active. In addition, some reports have shown an increased in the bioactive compound

accumulation after precursor feeding during the stationary phase (Morgan & Shank 2000; Gaviraj & Veereshem 2008).

Mid log phase was achieved on the 6th day after initiation of *B rotunda* suspension cultures. Subculturing was performed on the 6th day to avoid cells dying or browning. According to Bhatia & Dahiya (2015), mid log and early stationary phase was ideal for sub culturing of suspension cultures to split the actively growing cells in the cultures. During log phase, actively growing cells were competing for nutrient, hence a depletion of nutrient started to occur at this point. The growth curve reached a plateau after 10 days of culture, showing stagnation of cell growth.

Based on the growth curve, phenylalanine was fed to the *B. rotunda* cultures during the stationary phase where a maximum biomass (settled cell volume at 1.6ml/10.0 ml) was already achieved. Microscopic observation showed that the cells were densely cytoplasmic and viable. After treatment, cells were incubated beyond stationary phase to assess whether the enhanced accumulation of pinocembrin, cardamonin, panduratin A and pinostrobin in the *B. rotunda* cell suspension cultures were due to phenylalanine treatment or stress caused by prolonged duration in cell suspension (nutrient depletion takes place over time).

5.4 The flavonoid content quantification and the statistical findings

Analysis of variance (ANOVA) test, coupled with Duncan test was done to determine the significance of the treatment at different concentrations, with $p < 0.05$. All compound quantitation readings were compared between treated and non-treated control cultures for 0, 3, 7, 14 and 21 days. Pinocembrin is being accorded for the highest content of flavonoids produced at day 21, in samples pretreated with 40.0 mg/L of phenylalanine in this study. This may be due to stress inflicted by dying cells or nutrient depletion over the long incubation culture period (Mosa *et al.,* 2017). The aromatic

zamino acid, phenylalanine from the Shikimate pathway, enters PPP and acts as the precursor for the *de novo* flavanoid biosynthesis. This precursor acts in activating one of the enzymes, namely PAL and triggers off the production of myriads of flavanoid (Tan et.,2015). Negative feedback or the inhibiton of the pathway takes place, when the overexpression of the flavanoids suppresses the action of the precursor, thus lowering the flavanoid production. In particular, phenylalanine, as the committed step into the phenylpropanoid pathway, has been shown to be metabolically regulated through negative feedback by cinnamic acid on *PAL* transcription and on enzyme activity (Yin *et al.,* 2012). Thus, the addition of the precursor into the *B. rotunda* cultures would have prompted the stress to the cells directing to high production of the pinocembrin.

As for the panduratin A, the total flavonoid content of the controls, ranges from 1.63 µg/g to 4.95 µg/g for the non-treated cell (C0, C3, C7, C14 and C21). With the baseline control cells, C0, carrying the highest content of flavonoid, 4.95 µg/g. On the other hand, the treated cells ranges from up to 0.42 µg/g to 6.259 µg/g of panduratin A at various concentrations of phenylalanine. This led to the insignificance in terms of both treated and untreated cells, the controls are relatively on the higher note (Figure 4.6) whereby the control cells indicate denser population of cells throughout the planned incubation days. Whereas the cells debris and vacuolated cells were seen in treated cultures at day 14 and day 21. The nutrient depletion took place over long incubation period, causing stress and provoke the flavonoid release. Long incubation may also cause cells to die; leaving the samples at day 14 and 21 mostly low in cell count.

Pinostrobin rose up visually, from as low as 0.35 µg/g for the control samples up to 2.69 µg/g in treated cells (day 3, at the concentration of 50 mg/L PAL). But this particular compound failed to exhibit any form of significance in this study. The increasing trend for day 14 and day 21 was observed at concentration of 20 mg/L

phenylalanine in particular. However, at high concentration, a decrease in flavonoid content was observed.

Similar trend of was observed in cardamonin. The highest amount was 0.033 µg/g at day 21 under 20 mg/L PAL. The controls ranged from 0.004 to 0.015 µg/g, hence when compared with the treated cells, the significance of the study for this compound seem void. There is a possibility for the dedifferentiated cell mass unable to complete the biosynthetic pathway of these medically important plant metabolites in *in vitro* cultures (Rahpeyna *et al.,* 2014) This condition impact would impact the effective regulations of the genes and the enzyme machinery in plants (Qian *et al.,* 2006). In *B. rotunda*, relatively low concentrations of secondary metabolites in calli and cell suspension cultures compared to that in shoot base, raises the question of whether the compounds are biosynthesized at low levels in the tissues or are residual form the explant. Another example of cell compartmentation and enzyme localization were studied in *Catharantheus roseus, whereby the* overexpression of *CrGES* impacts primary metabolism differently if expressed in the plastids or cytosol. The levels of valine, leucine, and some metabolites derived from the shikimate pathway, i.e. phenylalanine and tyrosine were significantly higher in the plastidial- but lower in the cytosolic-*CrGES* overexpressing cell lines. This result shows that overexpression of *CrGES* in the plastids or cytosol caused alteration of primary metabolism that associated to the plant cell growth and development. A comprehensive omics analysis is necessary to reveal the full effect of metabolic engineering (Saiman *et al.,* 2018).

CHAPTER 6: CONCLUSION

In this study, the cell suspension cultures obtained were homogenous, with high density of cytoplasm. The growth curve obtained for these cell lines consisted the essential four growth phases *i.e.* the lag phase, log phase, stationary phase and finally the death phase. The conditions of the medium, pH, PGR, nutrient composition from previous studies are deemed suitable and further contributed to the stability of the cell cultures for future manipulations.

Besides the establishment of a stable cell suspension cell cultures, this study mainly emphasized on the effect of phenylalanine in magnifying the trace amounts flavonoids; which falls under the subgroups of chalcones (panduratin A and cardamonin) and the flavanones (pinocembrin and pinostrobin), at different concentrations and incubation period. The flavanoid quantification was analyzed using HPLC.

In general, high concentrations of the precursor (40 mg/L and 50 mg/L phenylalanine) increased the flavonoid content regardless of the days of incubation except for cardamonin. The significant accumulation of pinocembrin was 8.6 times higher in treated cells when compared to control, C0 at the concentration of 40 mg/L at day 21. The addition of phenylalanine showed panduratin A decreased the flavonoid content for all treatments except for the cells treated with 40 mg/L at the incubation period of 7 days, which is 1.26 fold higher than control. As for pinostrobin, the feeding of precursor resulted in high flavonoid content for all concentrations of treatment for day 3 and day 7. The highest content of pinostrobin was elucidated at a much earlier incubation, day 3 at 50 mg/L of phenylalanine. While, for cardamonin an increase of 2 fold higher when compared to the control cells and the treated cells at day 21 under 20mg/ L treatment.In conclusion from this

study, the phenylalanine feeding showed insignificant results of pinostrobin, panduratin A and cardamonin as compared to the control. In contrary, the concentrations of flavanones namely pinocembrin increased at 21 days after feeding with 40.0 mg/ L phenylaalanine. In accordance to that, further studies need to be carried out based on these findings to enhance the flavonoid content. Future conformational study on the effect of phenylalanine that had significant finding of this study, *i.e.* pinocembrin accumulation in *B. rotunda* suspension cultures also need to be looked into. Thus, systemic approach in exploring the transcriptomic, proteomics and the metabolomics study will be useful to confirm the apparent pattern of the flavonoid biosynthetic pathways in these cultures.

REFERENCES

Ahmad, P., Abdel Latef, A. A., Abd_Allah, E. F., Hashem, A., Sarwat, M., Anjum, N. A., & Gucel, S. (2016). Calcium and potassium supplementation enhanced growth, osmolyte secondary metabolite production, and enzymatic antioxidant machinery in cadmium-exposed chickpea (*Cicer arietinum* L.). *Frontiers in Plant Science*, *7*, Article#513.

Akira M., Hajime O., Koichi K. (1999). Chemoprevention: Insights into biological mechanisms and promising food factors. *Food Reviews International* 15:3, 335-395.

Ali, M., Abbasi, B.H., Ahmad, N. (2016). Sucrose-enhanced biosynthesis of medicinally important antioxidant secondary metabolites in cell suspension cultures of *Artemisia absinthium* L. *Bioprocess Biosystems Engineering* 39, 1945–1954

Ata, N., Yusuf, N.A, Tan B.C., Husaini, A., Mohd-Yusuf, Y., Abd. Majid N., Khalid N. (2015). Expression profiles of flavonoid-related gene, 4-coumarate: coenzyme A ligase, and optimization of culturing conditions for the flavonoid production in *Boesenbergia rotunda*. *Plant Cell Tissue Organ Culture.*

Barrientos, C. H., Pérez, C., Zúñiga, G., & Mahn, A. (2014). Effect of methyl jasmonate, sodium selenate and chitosan as exogenous elicitors on the phenolic compounds profile of broccoli sprouts. *Journal of the Science of Food and Agriculture*, *94*(12), 2555-2561.

Bemami, E., Ghanati, F., Rezaei, A., Jamshidi, M. (2012) Effect of phenylalanine on Taxol production and antioxidant activity of extracts of suspension cultured hazel (*Corylus avellane L.*) cells. *Journal of Natural Medicine* vol. 67: 446-451

Bhatia S. & Dahiya R. (2015) Concepts and Techniques in Tissue Culture Science in: Bhatia S., Dahiya R. *Modern Applications of Plant Biotechnology in Pharamaceutical Sciences,* 121-156

Bhoite, H.A. and G.S. Palshikar (2014). Plant tissue culture: A review. *World Journal of Pharmaceutical Sciences*., 2: 565-572.

Cardoso, J.C., Maria O., Eduarda B.S., & Cardoso, F. CI. (2019). Advances and challenges on the in vitro production of secondary metabolites.

Chahel, A. A., Zeng, S., Yousaf, Z., Liao, Y., Yang, Z., Wei, X., & Ying, W. (2019). Plant-specific transcription factor LrTCP4 enhances secondary metabolite biosynthesis in *Lycium ruthenicum* hairy roots. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *136*(2), 323-337.

Cheenpracha S., Karalai C., Ponglimanont C, Subhadhirasakul S., & Tewtrakul S. (2006). Anti HIV-1 protease activity of compounds from *Boesenbergia pandurata*. *Bioorganic & Medicinal Chemistry* 14, 1710-1714

Dailey, R. M. (2017). Development of a Plant Cell Culture Platform for Recombinant Protein Expression. https://digitalcommons.wpi.edu/mqp-all/1596 metabolites from medicinal plants. *Horticultura Brasileira*, *37*(2), 124-132.

Garcia-Gonzales, R., K. Quiroz, B. Carrasco and P. Caligari, (2010) Plant tissue culture: Current status, opportunities and challenges. *Ciencia Investigacion Agraria*, 37: 5-30. 7.

George E.F., Hall M.A., Klerk GJ.D. (2008) The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems. in: George E.F., Hall M.A., Klerk GJ.D. (eds) Plant Propagation by Tissue Culture.

Georgiev, M.I., Weber J., Maciuk A. (2009) Bioprocessing of plant cell cultures for mass production of targeted compounds. *Appl Microbiol Biotechnol*ogy, 83 (5):809–23.

Giri, C. C., & Zaheer, M. (2016). Chemical elicitors versus secondary metabolite production in vitro using plant cell, tissue and organ cultures: recent trends and a sky eye view appraisal. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *126*(1), 1-18.

Faridah, Q. Z., Abdelmageed, A. H. A., Julia, A. A., & Hafizah, R. N. (2011). Efficient in vitro regeneration of Zingiber zerumbet Smith (a valuable medicinal plant) plantlets from rhizome bud explants. African Journal of Biotechnology, 10(46), 9303-9308.

Hollister, K. R., Ladd, D., McIntire, G. L., Na, G. C., Rajagopalan, N., & Yuan, B. O. (1994). U.S. Patent No. 5,352,459. Washington, DC: U.S. Patent and Trademark Office.

Hosseini, Samira; Muñoz-Soto, Rodrigo B.; Oliva-Ramírez, Jacqueline; Vázquez-Villegas, Patricia; Aghamohammadi, Nasrin; Rodriguez-Garcia, Aida; Martinez-Chapa, Sergio O. (2020). Latest Update in Dengue Therapeutics: Natural Marine & Synthetic Drugs. *Current Medicinal Chemistry,* Volume 27(5), 719-744(26).

Ikeuchi, M., Sugimoto, K., & Iwase, A. (2013). Plant callus: mechanisms of induction and repression. *The Plant cell*, *25*(9), 3159–3173.

Jacobs, D. I., Snoeijer, W., Hallard, D., & Verpoorte, R. (2004). The *Catharanthus* alkaloids: pharmacognosy and biotechnology. *Current medicinal chemistry*, *11*(5), 607-628.

Jalil M, Annuar M.S.M, Tan B.C, Khalid N (2015). Effects of selected physiochemical parameters on Zerumbone Production of *Zingiber zerumbet* Smith cell suspension culture. *Evidence Based Complementary and Alternative Medicine*

Kanjanasirirat, P., Suksatu, A., Manopwisedjaroen, S., Munyoo, B., Tuchinda, P., Jearawuttanakul, K., ... & Thitithanyanont, A. (2020). High-content screening of Thai medicinal plants reveals *Boesenbergia rotunda* extract and its component Panduratin A as anti-SARS-CoV-2 agents. *Scientific reports*, *10*(1), 1-12.

Karuppusamy, S. (2009). A review on trends in production of secondary metabolites from higher plants by in vitro tissue, organ and cell cultures. *Journal of Medicinal Plants Research*, *3*(13), 1222-1239.

Kim, H. J., Park, K. J., & Lim, J. H. (2011). Metabolomic analysis of phenolic compounds in buckwheat (*Fagopyrum esculentum* M.) sprouts treated with methyl jasmonate. *Journal of Agricultural and Food Chemistry*, *59*(10), 5707-5713.

Kirana C, Jones GP, Record IR, McIntosh GH (2007). Anticancer properties of panduratin A isolated from *Boesenbergia pandurata* (Zingiberaceae) Journal of Natural Medicines. 2007;61(2):131–137.

Kumari, M (2014). Enhancement of some pharmaceutically important secomdary metabolites by in vitro techniques. *Journal of Herbal Science, 3(1)*

Md-Mustafa N. D, Khalid N (2014). Transciptome profiling shows gene regulation patterns in a flavonoid pathway in response to exogenous phenylalanine in *Boesenbergia rotunda* cell culture, *BMC Genomics,15:* 984.

Moreno, P. R. H., Van der Heijden, R., & Verpoorte, R. (1993). Effect of terpenoid precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of Catharanthus roseus. Plant Cell Reports, 12(12), 702-705

Murthy, H. N., Lee, E. J., & Paek, K. Y. (2014). Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell, Tissue and Organ Culture* (PCTOC), 118(1), 1-16.

Nasir, N., Shahari, R., & Tajudin, N. (2020). The Effect of Different Growing Medium Combination on Growth and Yield of *Boesenbergia rotunda,* Malaysian Journal of Halal Research (published online ahead of print)

Ng T.L.M, Karim R., Tan Y.S., Teh H.F., Danial A.D., Ho L.S., et al. (2016). Amino Acid and Secondary Metabolite Production in Embryogenic and Non-Embryogenic Callus of Fingerroot Ginger (*Boesenbergia rotunda*). *PLoS ONE* 11(6): e0156714.

Othman R, Kiat T.S, Khalid N, (2008). Docking of noncompetitive inhibitors into dengue virus type 2 protease: understanding the interactions with allosteric binding sites. *Journal of Chemical Information and Modeling.;48(8):1582–1591.*

Ongwisespaiboon, Oranun & Jiraungkoorskul, Wannee. (2017). Fingerroot, *Boesenbergia rotunda* and its Aphrodisiac Activity. *Pharmacognosy Reviews. 11. 27. 10.4103/phrev.phrev_50_16.*

Phillips, G.C., Garda, M. (2019) Plant tissue culture media and practices: an overview. *In Vitro Cell.Dev.Biol.-Plant* **55,** 242–257

Pinar Nartop, (2018) in Plant Metabolites and Regulation Under Environmental Stress

Punnam Chander M., Vinod Kumar K., Chandan Lall, R. Vimal Raj & Vijayachari P. (2016). GC/MS profiling, *in vitro* anti-leptospiral and haemolytic activities of *Boesenbergia rotunda* (L.) Mansf. used as a medicinal plant by Nicobarese of Andaman and Nicobar Islands, Natural Product Research, 30:10, 1190-1192

Saiman, M. Z., Mustafa, N. R., Schulte, A. E., Verpoorte, R., & Choi, Y. H. (2012). Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *Gynura procumbens*. Plant Cell, Tissue and Organ Culture (PCTOC), 109(3), 465-475.

Saiman M. Z., Miettinen K, Mustafa N. R., Choi Y. H., Verpoorte R., Schulte A.E. (2018) Metabolic alteration of *Catharanthus roseus* cell suspension cultures overexpressing *geraniol synthase* in the plastids or cytosol. Plant Cell Tissue Organ Cult. 2018;134(1):41-53.

Singh, S. (2016). Enhancing phytochemical levels, enzymatic and antioxidant activity of spinach leaves by chitosan treatment and an insight into the metabolic pathway using DART-MS technique. *Food chemistry*, *199*, 176-184.

Shi. X, Yang L, Yan G, Du G (2017). Medium pH between 5.8 to 7.5 has minimal effects on apple tissue culture, *American Society for Horticulture Science*, 53(3): 475-478

Skoog, F., & Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured. In Vitro Symp Soc Exp Biol.

Springer, Dordrecht. https://doi.org/10.1007/978-1-4020-5005-3_4

Stevenson PC, Veitch NC, Simmonds MSJ. Polyoxygenated cyclohexane derivatives and other constituents from *Kaempferia rotunda L*. Phytochemistry. (2007) 68(11):1579–1586. [PubMed] [Google Scholar]

Sullivan, M. L. (2009). Phenylalanine ammonia lyase genes in red clover: expression in whole plants and in response to yeast fungal elicitor. Biologia Plantarum, 53(2), 301-306.

Sutela S, Hahl T, Tiimonen H, Aronen T, Ylioja T, Laakso T, Saranpa¨a¨ P, Chiang V, Julkunen-Tiitto R, Ha¨ggman H (2014). Phenolic compounds and expression of 4CL genes in silver birch clones and Pt4CL1a lines. PLoS One 9(12): e114434

Swieca, Michał & Surdyka, Magdalena & Gawlik-Dziki, Urszula & Złotek, Urszula & Baraniak, Barbara. (2013). Antioxidant potential of fresh and stored lentil sprouts affected by elicitation with temperature stresses. International Journal of Food Science & Technology. 49. 10.1111/ijfs.12489.

Takacs E.M, Li J, Du C, Ponnala L, Janick-Buckner D, Yu J, et al. (2012). Ontogeny of the maize shoot apical meristem. *The Plant Cell* 24(8):3219–34. pmid:22911570

Tan B.C, Tan S.K, Wong S.M, Ata N, Abd. Rahman N, Khalid N (2015). Distribution of flavanoids and cyclohexenyl chalcone derivatives in conventional propagated and in-vitro derived field grown *Boesenbergia rotunda* (L) Mansf., *Evidence Based Complementary and Alternative Medicine*

Tan E.C, Foo G.T, Wong S.M, Abd. Rahman N, Khalid N, Karsani S.A, Othman S, Yusof, R. (2011). Optimization of two-dimensional gel electrophoresis protocols for ***B. rotunda*** in vitro suspension culture, Journal of Medicinal Plants Research, 5-16: 3777- 3780.

Tan E.C, Karsani S.A, Foo G.T, Wong S.M, Abdul Rahman N, Khalid N, Othman S, Yusof R (2012). Proteomic analysis of cell suspension cultures of ***Boesenbergia rotunda*** induced by phenylalanine: identification of proteins involved in flavonoid and phenylpropanoid biosynthesis pathways. Plant Cell Tiss Org vol. 111-2: 219-229

Tan E.C., Lee Y. K., Chee C. F., Heh C.H., Wong S. M., Thio C., Foo G.T., Khalid N, Abd Rahman N, Karsani S.A., Othman S, Yusof N. (2012). Review Article, *Boesenbergia rotunda*: From *Ethnomedicine to Drug Delivery, Evidence Based Complementary and Alternative Medicine*, 1-25

Trakoontivakorn G, Nakahara K, Shinmoto H, Takenaka M, Onishi-Kameyama M, Ono H, Yoshida M, Nagata T, Tsushida T (2001) Structural analysis of a novel antimutagenic compound, 4-hydroxypanduratin A, and the antimutagenic activity offlavonoids in a Thai spice, fingerroot ***(Boesenbergia pandurata***Schult.) against mutagenic heterocyclic amines. J Agric FoodChem 49:3046–3050

Voon, F. L., Sulaiman, M. R., Akhtar, M. N., Idris, M. F., Akira, A., Perimal, E. K., ... & Ming-Tatt, L. (2017). Cardamonin (2′, 4′-dihydroxy-6′-methoxychalcone) isolated from ***Boesenbergia rotunda (L.) Mansf. inhibits CFA-induced rheumatoid arthritis in rats. European journal of pharmacology, 794, 127-134.***

Wang, J., Qian, J., Yao, L., & Lu, Y. (2015). Enhanced production of flavonoids by methyl jasmonate elicitation in cell suspension culture of *Hypericum perforatum*. *Bioresources and Bioprocessing*, *2*(1), 1-9.

Wong S.M, Salim S, Harikrishna J.A, N. Khalid (2013). Highly efficient plant regeneration via somatic embryogenesis from cell suspension cultures of *Boesenbergia rotunda*. *In Vitro Cell Dev. Biology*: vol. 49: 665-673.

Wu CH, Dewir YH, Hahn EJ, Paek KY (2006) Optimization ofculturing conditions for the production of biomass and phenolicsfrom adventitious roots of Echinacea aungustifolia. J Plant Bio 36:133-138

Yali Li, Tingting Meng, Yuxi Wang & Xiaoli Zhang (2016) Study on enzymatic browning in suspension cultures of licorice cells, *Biotechnology & Biotechnological Equipment*, 30:2, 277-283

Yadav, V., Wang, Z., Wei, C., Amo, A., Ahmed, B., Yang, X., & Zhang, X. (2020). Phenylpropanoid pathway engineering: An emerging approach towards plant defense. *Pathogens*, *9*(4), 312.

Yang, M., Jia, S. & Pua, E. (1991) High frequency of plant regeneration from hypocotyl explants of *Brassica carinata* A. *Plant Cell Tiss Organ Cult* **24,** 79–82

Yin R., Messner B., Kessler T., Hoffmann T., W. Schwab W., Hajirezaei M.R., Saint Paul V., W. Heller H, Schäffner A.R (2012) Feedback inhibition of the general phenylpropanoid and flavonol biosynthetic pathways upon a compromised flavonol-3-O-glycosylation, *Journal of Experimental Botany,* 63(7):2465-2478

Youn, K.; Jun, M. (2019). Biological Evaluation and Docking Analysis of Potent BACE1 Inhibitors from *Boesenbergia rotunda*. *Nutrients*, *11*, 662.

Yusuf N. A., (2011). Biomass and selected flavonoids productionm in cell suspension cultures of *Boesenbergia rotunda* (L) mansf. (Doctoral dissertation).

Yusuf N. A, Annuar M. S. M, Khalid N. (2013). Physical stress for overproduction of biomass and flavonoids in cell suspension cultures of *B. rotunda, Acta Physiol Plant, 5:*1713-1719

Yusuf N.A, Annuar M.S.M, Khalid N (2013). Existence of bioactive flavonoids in rhizomes and plant cell cultures of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl, Australian Journal of Crop Science: vol. 7-6: 730-734.

Yusuf, N. A., Annuar, M. S., & Khalid, N. (2011). Rapid micropropagation of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (a valuable medicinal plant) from shoot bud explants. African Journal of Biotechnology, 10(7), 1194-1199.

Yusuf, N. A., M Annuar, M. S., & Khalid, N. (2013). Existence of bioactive flavonoids in rhizomes and plant cell cultures of *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. Australian Journal of Crop Science, 7(6), 730.