CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Plants have been extensively used for treating human illness for thousands of years. Plant-based remedies have always been an integral part of traditional medicine. About 80% of worldwide natural pharmaceuticals products are of plant origin. Herbal industries in the world are estimated to have a potential market value around USD 50 billion and the global trade of pharmaceuticals is increasing at a percentage of 20% per year (Patwardhan *et al*., 2004). The increase in the use of herbal medicine is probably due to their insignificant side effects compared to synthetic drugs and antibiotics. Hence, substitute remedy incorporating herbal drugs are increasingly popular (Kupiec and Raj, 2005). Based on the demand, it is expected that the market value will further increase over the coming years.

1.1.1 Medicinal plant industry in Malaysia

Malaysia is known to be among the world's mega-biodiversity center with prosperous flora and fauna for medicinal genetic resources with approximately 3 200 species with medicinal value (Ibrahim, 2004). Acanthaceae, Annonaceae, Apocynaceae, Araceae, Compositae, Dioscoriaceae, Lauraceae, Leguminosae, Myrtaceae, Rutaceae and Zingiberaceae are among those families that have medicinal properties (Soepadmo, 1991). The Malaysian pharmaceutical market increased from USD 1.22 billion in 2009 to USD 1.40 billion in 2010 (Biospectrum, 2011).

1.1.2 Medicinal plant research in Malaysia

Studies on medicinal plants in Peninsular Malaysia have been explored since 1892. This information includes studies carried out on poisonous, economic and traditional usage of plants. Currently, the importance of traditional medicine as alternative treatment for reliable types of illness or health care purposes continues. Substitute medicine using herbal remedies are popular as public perceived it to be safer than modern medicine. Owing to this rapid economic potential, research activities on medicinal plants in public universities as well as research institutions are actively carried out (Ibrahim, 2004).

The most researched medicinal plants in Malaysia are halia (*Zingiber officinale*), followed by tongkat ali (*Eurycoma longifolia*), pegaga (*Centella asiatica*), mengkudu (*Morinda citrifolia*), kunyit (*Cucurma domestica*), hempedu bumi (*Andrographis paniculata*), lengkuas (*Alpinia galangal*), sireh (*Piper betel*), misai kucing (*Orthosiphon stamineus*) and kacip fatimah (*Labisia pumila).* The major scopes of research include raw materials, traditional medicines, phytomedicines, pharmaceuticals, nutraceuticals and essential oils (Maziah *et al.*, 2003).

Various medicinal plant species of Malaysia have been investigated previously for their valuable properties such as anti-inflammatory, antimicrobial, antioxidant, antihypertensive, anti-dengue and anticancer. In general, traditional applications for the precious medicinal properties are concurring with the scientific findings.

1.2 SPECIES STUDIED (*BOESENBERGIA ROTUNDA* (L.) MANSF.)

Boesenbergia rotunda (L.) Mansf. is a medicinal and culinary herb believed to have been originated from India, South-East Asian and South China. *B. rotunda* has many synonyms. It was known scientifically as *Kaempferia pandurata* Roxb. in the past and more recently *Boesenbergia pandurata* Roxb. Schltr. Locally in Malay, it is called 'Temu Kunci'. Fingerroot is the best English name that can be given to this spice, which has become generally known in the West only in the recent years.

1.2.1 Morphological descriptions

Boesenbergia rotunda (L.) Mansf. is a small perennial plant with very attractive light green foliage and grows to a height of 20 to 40 cm (Figure 1.1 a-c). The whole ensemble of the plant resembles the *Curcuma* species except for the inflorescence that is covered by the leaf sheaths and has distichous bracts. Its flower is pink with a prominent lip in darker shade pink, extremely delicate and short-lived, usually appearing one at a time and expansions along the stem. *B. rotunda* is widely cultivated in South East Asia for its small underground rhizome.

Figure 1.1: a) Whole plant of *B. rotunda* with maroon stem. b) Shoots of *B. rotunda* usually comprises of 3 to 5 leaves with maroon sheaths. c) Leaf of *B. rotunda* is about 7 to 9 cm broad and 10 to 20 cm long.

The underground portion of the plant consists of a small globular central rhizome (1.5 to 2 cm in diameter) from which several tubers (slender and long tubers) sprout all in the same direction like the fingers of the hand, thus the common name, fingerroot (Figure 1.2 a-b). The tubers are about 1.0 to 1.5 cm thick in diameter and 5 to 10 cm long. The tissue of the tuber is looser, softer and more watery than the central rhizome. Both the central rhizome and the tubers are yellow for the yellow variety but their fragrance differs. Other varieties have either red or black rhizomes. *B. rotunda* has a strong, dominating flavor where the tubers and the central, globular part of the rhizome have different odor.

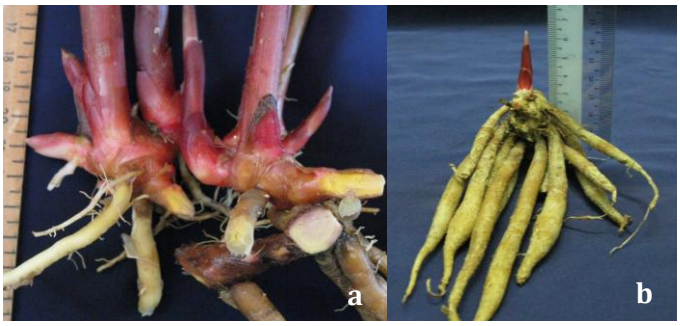


Figure 1.2: a) Rhizome of *B. rotunda.* b) Tubers sprout from the globular rhizome all in the same direction.

1.2.2 Common uses

In several countries in South East Asia, *B. rotunda* is used as both folk medicine and food ingredient (either as spice or condiments). Traditionally the Chinese, Thai and Malay use it for medicinal purposes. They believe that it possesses anti-inflammatory effects, provides relief from bacterial dysentery, stomach ache, and anti-flatulence and helps to promote appetite. The tubers are widely used as local application for tumors, swellings and wounds. They are also used for treating colic disorder such as diarrhea, worms, removing blood clots and as an aphrodisiac (Burkill, 1966).

1.2.3 Pharmacological properties

B. rotunda contains 1 to 3% of essential oil and several aroma components have been identified such as 1-8 cineol, camphor, d-borneol and methyl cinnamate. Trace components present are *d*-pinene, zingiberene, zingiberone, curcumin and zedoarin. Among the non-volatile constituents, flavones and flavonoids (pinostrobin, alpinetin, pinocembrin), chalcones (cardamonin) and dihydrochalcones (boesenbergin A) have been identified. Several pharmacological findings and publications have established where *B. rotunda* compounds such as flavonoids and chalcones are pharmaceutically

active. *B. rotunda* extracts also have been reported to yield various chalcones (Trakoontivakorn *et al*., 1999), flavonoids (Jaipetch *et al*., 1983), flavones (Jaipetch *et al*., 1982) and essential oil (Lawrence *et al*., 1971, Pandji *et al*., 1993). Compounds and pharmacological properties of *B. rotunda* are listed in Table 1.1.

Table 1.1: *Boesenbergia rotunda* pharmacological properties and significance.

Compounds	**Pharmacological properties**	**Researchers**
Cardamonin	Exhibited appreciable anti-HIV-1 protease inhibition	Tewtrakul *et al*., 2003
Cardamonin Hydroxypanduratin A Panduratin A	Anti-inflammatory	Tewtrakul *et al*., 2009
Crude extract	*In vitro* anti-tumor promoting activity using tumor promoter-induced Epstein-Barr virus activation assay.	Murakami *et al*., 1994
Crude extract	Analgesic and antipyretic	Pathong *et al*., 1989
Panduratin A	Anticancer properties	Kirana *et al*., 2007
Panduratin A	Antibacterial	Rukayadi *et al*., 2010
Panduratin A	Antibacterial	Yanti *et al*., 2009
Panduratin A	Strong inhibition of both nitric oxide and prostaglandin production through the suppression of NF-kappa B activation.	Yun *et al*., 2003
Panduratin A and 4'-hydroxypanduratin A	Topical anti-inflammatory activity	Tuchinda *et al*., 2002
Panduratin A and 4-hydroxypanduratin A	Inhibitory activity towards dengue-2 virus protease	Kiat *et al*., 2006
Pinostrobin (flavonone)	Elevate the activity of quinone reductase (an antioxidant enzyme)	Fahey and Stephenson, 2002
Pinostrobin, Pinocembrin chalcone, pinocembrine, cardamonin, panduratin A and 4'-hydroxypanduratin A	Strong anti-mutagen	Trakoontivakorn *et al*., 2001

1.3 SCOPE OF THE RESEARCH

Five flavonoid compounds were successfully identified from field grown rhizomes, callus and cell suspension cultures. As an alternative source for these compounds, cell suspension cultures were established with superior formulation for biomass growth and accumulation of these metabolites. The production of these compounds were enhanced through physical and chemical factors such as medium strength, initial inoculation volume, initial pH, agitation, culture temperature, carbon sources and concentration and plant growth regulators (PGRs).

Applications of *in vitro* cultures in large scale production of both biomass and targeted metabolites were developed in three types of bioreactors. Protocols for direct regeneration, callus and cell suspension cultures were also established.

General objective:

To compare the enhancement strategies of flavonoid compounds accumulation and biomass of suspension cultures in shake flasks and bioreactors.

Specific objectives:

1. To establish protocols for direct regeneration, callus and cell suspension cultures for *B. rotunda.*
2. To study the distribution of 5 flavonoids in rhizomes, callus and cell suspension cultures.
3. To study the effects of various culture conditions and PGR on the biomass growth and accumulations of selected flavonoids in cell suspension cultures.

4. To study the *B. rotunda* cell suspension cultures growth and their metabolites production in four types of reactors with different geometries.

Hypothesis:

Physical and chemical factors can enhance the accumulation of flavonoid compounds and biomass of suspension cultures of *B. rotunda*.

CHAPTER 2

LITERATURE REVIEW

2.1 PROPAGATION OF *BOESENBERGIA ROTUNDA*

2.1.1 Conventional propagation

Boesenbergia rotunda is traditionally propagated by vegetative techniques using a rhizome segment which is protracted for large-scale multiplication. The lack of seed set in Zingiberaceae family makes conventional breeding methods inapplicable. Furthermore, many of the Zingiberaceae species are susceptible to rhizome soft rot diseases, leaf spot and pathogens such as *Coleotrichum* species (Balachandran *et al*., 1990; Chan, 2004). Moreover, conventional method allows transmission of soil borne pathogen and may spread the disease either to other plant or place. Another factor was the rhizomes size, where *B. rotunda* consist only a small central rhizomes therefore only a few buds could be produced. Conversely, large size rhizome is the best source of planting material for plant growth and production. The other weakness of conventional propagation is the requirement for appropriate maintenance and protection of seed rhizome throughout the dormancy stage before next replanting process. In addition, *in vitro* plant regeneration is a simple and cost-effective way to get abundantly uniform plant within a relatively short time (Çirak *et al*., 2007). Therefore, it is necessary to develop methods for *in vitro* propagation.

2.1.2 *In vitro* culture techniques

2.1.2.1 Micropropagation

Propagation technique was extensively used after the invention of plant growth regulators, auxins and cytokinins (Pierik, 1997). Micropropagation or plant tissue culture refers to a tissue culture technique for plant propagation where plants are cloned from tissue of a single plant on formulated semisolid or in liquid media under aseptic and controlled condition. This technique could produce high yield of plants after a few times of subculture.

There are five major phases for successful micropropagation of plantlets. The first phase, a suitable starting material, secondly establishment of sterilization procedure in aseptic condition, subsequently initiation of culture from explants on a suitable medium followed by multiple shoot formation. As a final point, the *in vitro* plantlets were acclimatized to improve survival when transferred to the soil conditions.

Some *Zingiberaceae* spp. had been reported to be propagated by *in vitro* culture techniques either from buds, shoot tips or meristems (Bhagyalakshmi and Singh, 1988; Illg and Faria, 1995; Borthakur *et al*., 1998). Even *Curcuma* from Zingiberaceae family has been recently investigated for their propagation by *in vitro* culture techniques (Salvi *et al*., 2002, Loc *et al*., 2005, Yusuf *et al*., 2007). The studies include the effect of PGR for the induction of multiple shoots formation (Nayak, 2000, Loc *et al*., 2005, Yusuf *et al*., 2007), field performance including variation and genetic stability in micropropagated plants (Salvi *et al*., 2002, Das *et al*., 2010, Siju *et al*., 2010). However, to date there are no reports on the rapid micropropagation of *B. rotunda*, which will be discussed in this study.

2.1.2.2 Callus cultures

According to Street (1997), a callus culture is an unorganized plant tissue growing on a solidified medium. Heinstein and Emery (1988) meanwhile defined the initial undifferentiated growth obtained from the explant upon transfer to the agar containing nutrient solution as callus. When newly formed callus tissue is removed from the explant and transferred to new agar medium, new growth will form on the outside of the transferred callus. Thus, callus tissue is a heterogenous accumulation of old and young cells (Heinstein and Emery, 1988). Callus culture need to be transferred to fresh agar medium periodically, depending on plant variety.

In theory, any plant part obtained from any species can be employed to induce callus, however, successful production of callus depends on many factors such as culture conditions, genotypes and media components. Mostly callus formed from the same explant can normally be grown on the same medium. A suitable medium for initiation and maintenance of callus can only be obtained by trial and error. According to Street (1997), dicotyledons are rather amenable for callus tissue induction as compared to monocotyledons whilst callus of woody plants generally grow slow. Mature stem, leaves, roots, flowers, seeds and any other parts of plants can be used for callus initiation, but younger meristematic tissue and fresh explants are preferable as explant materials.

Callus cultures are tremendously important in plant biotechnology. It has been shown that shoot organogenesis *via* an intermediate callus phase can be used as an effective method for multiplication of other Zingiberaceae species (Malamug *et al*., 1991, Kackar *et al*., 1993, Salvi *et al*., 2001, Tan *et al*., 2005). Similar accomplishment using other explants and hormone hitherto has not been published, although the first report on somatic embryogenesis of *B. rotunda* is available (Tan *et al*., 2005).

2.1.2.3 Cell suspension cultures

Cell suspension cultures are initiated from callus cultures by transferring pieces of callus into liquid medium (Street, 1997). This requires a large amount of callus and the callus should be carefully divided to prevent the accumulation of large clumps of cells (Heinstein and Emery, 1988). As an initial starting point, the same nutrient composition as the callus medium can be used before optimum growth media is obtained. 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 (Walton *et al*., 1999).

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). The time required to establish cell suspension culture varies greatly and depends on the tissue of the plant species and the medium composition (Fu, 1998). Depending on the doubling time of the cell culture, subculturing should be done when the culture acquires a thick mass of cells (Heinstein and Emery, 1988).

Cell suspension is the preferred type of culture for large-scale production because it is similar to microbial cultures and has rapid growth cycles. Cell suspension culture has more immediate potential for industrial application than plant tissue or organ cultures, due to the extensive growth of young meristematics cells and less risk of contamination (Fu, 1998).

2.2 PLANT SECONDARY METABOLITES

Plant secondary metabolite is a basic name used for thousands of constituents exclusively produced by plants. It is well known that plants generate a variety of economically important secondary metabolites, with approximately 4,000 new discoveries every year, adding to over 100,000 known compounds (Verpoorte *et al*., 1999).

Plant secondary metabolites have multiple functions throughout the plant's life cycle. The plants develop secondary metabolites in order to protect against pests, as coloring, aroma, or attractants (Verpoorte and Memelink, 2002). Some of the examples of these secondary metabolites are antimicrobial-phytoalexins such as stilbene, pigments such as anthocyanins or carotenoids and antifeedents such as alkaloids (Zhang *et al*., 2002b).

Secondary metabolites can act as defensive compounds in the human body. Plant secondary metabolites can increase the immune system, protect the body from free radicals and kill pathogenic germs (Verpoorte and Memelink, 2002).

An important source of active pharmaceuticals species were listed in Table 2.1. Due to their large biological activities, plant secondary metabolites have been used for centuries in traditional medicine. A number of current medicinal plants generally cultivated in Malaysia for commercialization purposes are shown in Table 2.2.

Studies on plant secondary metabolites have been increasing over the last 50 years and have been receiving much attention nowadays particularly in pharmaceutical and agricultural areas (Huang and Chou, 2000). Although advancements have been made in synthetic chemistry, about 25% of all prescription medicines are constituted of plant origin (Arroo *et al*., 2002). However, decreased plant resources, increased labor cost

and other problems associated with obtaining high-value added substances from natural plants have led to the use of plant cell culture for production.

With the cell culture method, production is more controllable in terms of quality and quantity of the product and also less dependent on geographical and climatic factors (Kieran *et al*., 1997). Cell culture may also offer better selectivity and yield for the desired bioactive products, since the cell strains may be selected from tissues or organs, which can be more productive than other parts of the plant (Misawa, 1994).

Table 2.1: Important plant derived pharmaceuticals.

Product	**Use**	**Plant species**
Ajmalicine	Antihypertensive	*Catharanthus roseus*
Artemisinin	Antimalarial	*Artemisia annua*
Berberine	Intestinal ailment	*Coptis japonica*
Camptothecine	Antitumor	*Camptotheca acuminate*
Capsaicin	Counterirritant	*Capsicum frutescens*
Castanopermine	Glycoside inhibitor	*Castanospermum australe*
Codeine	Sedative/Analgesic	*Papaver somniferum*
Colchicine	Antitumor	*Colchium autumnale*
Digoxin	Heart stimulant	*Digitalis lanata*
Diosgenin	Steroidal precursor	*Dioscorea deltoidea*
Ellipticine	Antitumor	*Orchrosia elliptica*
Emetine	Emetic	*Cephaclis ipecaccuanha*
Forskolin	Bronchial asthma	*Coleus forskolii*
Ginsenosides	Heath tonic	*Panax Ginseng*
Morphine	Sedative/Analgesic	*Papaver somniferum*
Podophyllotoxin	Antitumor	*Podophyllum petalum*
Quinine	Antimalarial	*Cinchona ledgeriana*
Shikonin	Antibacterial	*Lithospermum erythrorhizon*

Table 2.1, continued.

Product	**Use**	***Plant species***
Taxol	Anticancer	*Texus brevifolia*
Vincristine	Antileukemic	*Catharanthus roseus*
Vinblastine	Antileukemic	*Catharanthus roseus*

Source: Ramachandra Rao and Ravishankar, 2002.

Table 2.2: Popular medicinal plants with economic value.

Species	**Part (s) used**	**Treatment**
Alpinia galanga	Rhizome	Indigestion, flatulence, colic, dysentery, skin disease
Andrographis paniculata	Leaves	Snake bites, insect stings, fever, diabetes, skin disease, flatulence, antihypertensive
Centella asiatica	Leaves or whole plants	Ulcers, cooling, rheumatism, cough, bronchitis, asthma, gastric catarrh, dysentery
Curcuma domestica	Rhizome	Stomachic, stimulant, carminative, treat minor wound, ulcers, abscesses, inflammations, diarrhea, dysentery, flatulence, rheumatism
Curcuma xanthorrhiza	Rhizome	Postpartum medicine, dysentery, bloody diarrhea, infected wounds, treatment for liver affection (jaundice, gall stone and promoting the flow of bile)
Cymbopogon nardus	Leaves, roots	Postpartum bath, a diuretic, treatment of kidney stone, oil used to relieve rheumatic pains
Eurycoma longifolia	Roots	Treatment for fever, wounds, ulcers, tonic for postpartum medicine, decoction to relieve pain in the bones
	Leaves	Decoction to reduce fever, paste for treat dermatitis, wounds and relieve itches
	Bark	Relieve lumbago, treat jaundice, fever, diarrhea, wounds and ulcers
	Fruit	Dysentery
	Stem	Coughs

Table 2.2, continued.

Species	Part (s) used	Treatment
Labisia pumila	Whole plant	Decoction used before childbirth to expedite labor
	Leaves	Colic
	Roots	Treatment muscle pain, constipation
Morinda citrifolia	Leaves	Heated leaves for treatment of coughs, nausea, abdominal colic, fever
	Fruits	Stomachic, laxative, treatment for diabetes
Orthosiphon stamineus	Leaves	Diuretic, high blood pressure
	Leaves and roots	Decoction for treatment of diabetes
	Whole plant	Decoction for treatment kidney stone
Piper betle	Leaves	Dysentery, bronchitis, rheumatism, chew as stimulant, heated leaves relieve cough and asthma
Zingiber officinale	Rhizome	Diuretic, stimulant, carminative, decoction for treatment of flatulence, antinausea, treatment for cold, rheumatism, cataracts, lumbago, menstrual pains

Sources: Ibrahim, 2004.

2.2.1 Plant cell cultures as a source of secondary metabolite

Plant cell cultures are an attractive alternative source to whole plant for the production of high-value secondary metabolites (Verpoorte and Memelink, 2002; Zhang *et al*., 2002b). Plants cells are biosynthetically totipotent, which means that each cell in culture retains complete genetic information and hence are able to produce the range of chemical found in parent plant (Ramachandra Rao and Ravishankar, 2002).

The advantages of plant cell culture technology over the conventional agricultural production outlined by Ramachandra Rao and Ravishankar (2002) are (a) it is independent of geographical and seasonal variations and various environmental factors (b) it offers defined production system, which ensures the continuous supply of

products, uniform quality and yield (c) it is possible to produce novel compounds that are not normally found in parent plant (d) it is independent of political interference (e) efficient downstream recovery and product (f) rapidity of production (g) it performs stereo- and region specific biotransformation for the production of novel compounds from cheap precursors.

Biosynthesis of secondary metabolites is not related to cell growth or division and high rates of their production usually occur during low rates of growth, often under conditions of significant physiological or biochemical stress on the cells (Linden *et al*., 2001). However, some secondary plant products are known to be growth-associated with undifferentiated cells, such as betalains and carotenoids (Bourgaud *et al*., 2001). When secondary metabolites are synthesized in plant cell cultures, they are either secreted into the surrounding medium or stored intracellularly (Zhang *et al*., 2002b).

2.2.2 Sources of secondary metabolites

2.2.2.1 Conventional approach

Secondary metabolites are obtained by extraction from field grown plants especially for commercial purposes. Plant sources comprise a variety of secondary metabolites which scientifically proven effect on health for example protects the body against cancer and cardiovascular illnesses (Frishman *et al*., 2009; Genovese *et al*., 2009).

Flavonoids are an example of secondary metabolites widely distributed in the vegetable kingdom. Flavonoids inhibit the growth of bacteria and viruses, protect the cells against the damages of free radicals and have a repressive effect against inflammations (Tewtrakul *et al*., 2009; Rukayadi *et al*., 2010).

2.2.2.2 *In vitro* derived source of secondary metabolite

Plant tissue culture especially cell culture system is an attractive alternative compared to the traditional mode of plantation. It offers various advantages including controlled supply of biochemical independent of plant availability (cultivation season, pests and politics), well defined production systems which results in higher yields and more consistent quality of the product.

Different strategies, using *in vitro* system, have been extensively studied with the objective of improving the production of secondary plant compounds. Undifferentiated cell cultures have been widely studied, but a large interest has also been shown in hairy root and other organ cultures.

2.2.2.2.1 Shoot cultures

Productions of secondary metabolites were found at higher level in differentiation tissues. Since the production of secondary metabolites is mostly higher, there are a number of efforts to develop organ cultures such as shoot and root cultures aiming for overproduction of pharmaceutically significant compounds. Interest in producing organ culture is due to the stability of the produced culture. In recent times, shoot culture derived from medicinal plants have been intensively exploited for metabolites studies (Table 2.3).

Table 2.3: Shoot cultures for secondary metabolite production.

Plant species	Product	Reference
Artemisia annua L.	Artemisinin	Paniego and Giulietti, 1996
Echinacea angustifolia	Alkamides	Lucchesini *et al*., 2009
Harpagophytum procumbens	Phenolic and gallotannin	Bairu *et al*., 2010
Hypericum perforatum L.	Hypericins	Kornfeld *et al*., 2007

Table 2.3, continued

Plant species	Product	Reference
Hypericum perforatum L.	Hypericin and pseudohypericin	Kirakosyan *et al*., 2000
Hypericum perforatum L.	Hypericins, hyperforins and flavonoids	Pasqua *et al*., 2003
Pelargonium tomentosum	Essential oils	Charlwood and Moustou, 1988
Polygonum tinctorium	Indirubin	Shim *et al*., 1998

2.2.2.2.2 Root cultures

Root cultures system were not extensively studied compared with shoot cultures since the root systems of higher plants generally exhibit slower growth and are difficult to harvest (Ramachandra Rao and Ravishankar, 2002). The use of root derived compounds may possibly require an adaptation of cultured scale-up technologies. Up till now, there are a few studies comprise of scale up process of root culture in bioreactor as an alternative for root-derived compounds (Bondarev *et al*., 2003; Cui *et al*., 2010). Following are a number of medicinal plants whose root cultures have been studied for metabolites (Table 2.4).

Table 2.4: Root cultures for secondary metabolite production.

Plant species	Product	Reference
Artemisia annua L.	Artemisinin	Paniego and Giulietti, 1996
Echinacea purpurea	Caffeic acid derivatives	Wu *et al*., 2008
Hemidesmus indicus	2-hydroxy-4-methoxybenzaldehyde	Sreekumar *et al*., 1998
Hypericum perforatum L.	Chlorogenic acid and Hypericins	Cui *et al*., 2010
Hypericum perforatum L.	Hypericins, hyperforins and flavonoids	Pasqua *et al*., 2003
Panax ginseng	Ginsenosides	Wu *et al*., 2008
Panax ginseng	Linoleic and α-linolenic	Wu *et al*., 2009
Polygonum tinctorium	Indigo, indirubin	Shim *et al*., 1998

2.2.2.2.3 Hairy root cultures

A hairy root is a fine adventitious root grows from tissue which has been wounded and inserted by the T-DNA from *Agrobacterium rhizogenes* plasmid (Choi *et al*., 2000). Currently, the usage of *A. rhizogenes* received enormous attention in production of secondary metabolite research. The capability of *A. rhizogenes* in inducing hairy roots in diverse plants species has pooled interest to study on this system as a promising alternative source of root-derived pharmaceutically importance (Flores *et al*., 1999). Furthermore, the availability of these hairy roots to propagate rapidly without an external supply of auxins adds to the interest. The genetic stability of this hairy roots also produce stable metabolite yield (Ramachandra Rao and Ravishankar, 2002). Due to these benefits, several of the root-derived plant metabolites for production by *in vitro* culture were reconsidered for production using the hairy root cultures system. Some studies in hairy roots production from medicinal plants are in Table 2.5.

Table 2.5: Hairy root cultures producing pharmaceutical products of interest.

Plant species	Product	Reference
Ammi majus	Furanocoumarins	Królicka *et al*., 2001
Azadirachta indica A.	Azadirachtin	Satdive *et al*., 2007
Panax ginseng	Ginsenoside	Yu *et al*., 2005
Rauvolfia micrantha	Ajmalicine	Sudha *et al*., 2003
Rudbeckia hirta L.	Pulchelin E	Łuczkiewicz *et al*., 2002
Solanum chrysotrichum	Saponin	Caspeta *et al*., 2005

2.2.2.2.4 Callus cultures

Apart from intact plants, unorganized tissue or cell cultures have the ability to produce metabolites. Callus culture is crucial material as source of suspension cultures. Even though callus culture is not as advantageous as cell suspension cultures for secondary

metabolite production, yet, successful secondary metabolite production in callus cultures has also been reported in several plant species summarized in Table 2.6.

Table 2.6: Callus culture as a source of secondary metabolite.

Plant species	Product	Reference
Arnebia hispidissima L.	Alkannin	Shekhawat and Shekhawat, 2010
Echinacea angustifolia	Caffeic acid derivatives	Lucchesini *et al*., 2009
Nothapodytes foetida	Camptothecin	Thengane *et al*., 2003
Rhazya stricta	3-Oxo-rhazinilam	Gerasimenko *et al*., 2001
Solanum tuberosum L.	*glycoalkaloids*	Al-Ashaal, 2010
Zataria multiflora	Rosmarinic acid	Mohagheghzadeh *et al*., 2004

2.2.2.2.5 Cell suspension cultures as a source of secondary metabolite

Cell suspension cultures are unorganized cells similar to callus culture but grown in liquid media. Cell suspension cultures propagate more rapid than callus due to close proximity to the media and agitation causes aeration which supplies oxygen to the cells. In recent years, numerous researches were conducted in order to investigate the production of secondary metabolite in cell suspension culture technology as listed in Table 2.7.

Table 2.7: Cell suspension cultures as an alternative source of secondary metabolite.

Plant species	Product	Reference
Arnebia hispidissima (Lehm).	Alkannin	Shekhawat and Shekhawat, 2010
Cassia acutifolia	Anthraquinones	Nazif *et al*., 2000
Catharanthus roseus	Catharanthine	Zhao *et al*., 2001b
Eucommia ulmoides	Chlorogenic acid	Wang *et al*., 2003
Hypericum perforatum	Hypericins, hyperforins and flavonoids	Pasqua *et al*., 2003
Larrea divaricata	Phenolic	Palacio *et al*., 2011
Podophyllum hexandrum royle	Podophyllotoxin	Chattopadhyay *et al*., 2002
Stizolobium hassjoo	L-Dopa	Huang *et al*., 2002
Torreya nucifera var. *radicans*	Diterpenoids	Orihara *et al*., 2002
Zataria multiflora	Rosmarinic acid	Mohagheghzadeh *et al*., 2004

2.3 CELL SUSPENSION CULTURES ENHANCEMENT STRATEGIES

In optimized culture conditions, several compounds in plant cell culture systems could produce higher yield than in the intact plants (Table 2.8). Conversely more reports on lower accumulation of secondary compounds in tissue culture system such as callus and cell suspension cultures were discovered. With the purpose of obtaining higher level of compounds, various efforts were taken into consideration. Several approaches to increase productivity in plant cell cultures systems have been investigated (Table 2.9).

Table 2.8: An example of compounds accumulated in higher amount in culture cell system.

Plant species	**Compounds**	**Culture**
Catharanthus roseus	Ajmalicine	Suspension
Coleus blumeii	Rosmarinic acid	Suspension
Coptis japonica	Berberine	Suspension
Dioscorea deltoides	Diosgenin	Suspension
Lithospermum erythrorhizon	Shikonin	Suspension
Morinda citrifolia	Anthraquinones	Suspension
Nicotiana tabacum	Ubiquinone-10	Suspension
Nicotiana tabacum	Nicotine	Callus
Panax ginseng	Ginsenoside	Callus
Tripterygium wilfordii	Tripdiolide	Suspension

Table 2.9: Several factors influence accumulation of secondary metabolite production in plant cells.

Strain improvement	**Screening and selection**
Medium variation	Nutrients, phytohormones, precursors and antimetabolites
Culture conditions	lnoculum size, pH, temperature, light and agitation
Specialized techniques or Selection	Elicitors, immobilization, permeabilization, two-phase systems and two-stage systems

Source; Dörnenburg and Knorr, 1995

2.3.1 Strain improvement

Cell lines chosen for studies aim for overproduction of secondary metabolite are essential. The source of plant material or mother plant is crucial in order to obtain high content of secondary metabolite production. The parent plant also played an important role in obtaining intermediate stage to produce high yielding cell lines.

In secondary metabolite enhancement research, high producing parent plant as a marker in order to introduce high producing cell lines eventhough variable in production level in cell lines was observed (Deus and Zenk, 1982). Assortment and separation of elite clone were required during cultivation phase due to the genetic and epigenetic instability in plant cell (Dörnenburg and Knorr, 1995).

Variability occurring in cell cultures can cause a decrease in metabolites productivity through subculturing which might result to mutation in the culture. These conditions likewise lead to physiological changes in the culture condition. The physiological characteristics of individual plant cells are not always uniform. So, early selection for a desired cell lines from the overall cell population is needed in plant cell cultures studies. For example, Yamada *et al*. (1981) obtained an elite strain of *Coptis japonica* cell suspension culture which could grow faster and at the same time could produce high quantity of berberine. This condition was achieved after a number of subcultures. This particular cell lines increased around 6-folds within 3 weeks in biomass with 1.2 g/l alkaloid of the medium. Even after 27 times of subcultures, stability of the strain proven in producing a high production of berberine.

2.3.2 Medium optimization

A number of physical and chemical factors that might influence desired product should be optimized. Generally, the hormone level and mixtures are frequently applicable.

Auxin and cytokinin affecting the production of secondary metabolite in plant cell cultures have been extensively investigated. The type and concentration of auxin and cytokinin, either alone or in combination has been known to profoundly influence growth as well as product formation in cultured cells.

Excessive level of auxin which claims to be good for cell growth however could adversely affect the secondary metabolite production. Production of anthocyanin in carrot cells was remarkably suppressed by auxins (Narayan *et al*., 2005). Nevertheless, productions of anthocyanin by *Camptotheca acuminate* cell cultures were increased in presence of kinetin than in the presence of other cytokinin (Pasqua *et al*., 2005).

Cytokinin also plays a major role in determining the success of overproduction of secondary metabolite. The combinations of auxin and cytokinin resulted in a significant decrease of *Morinda citrifolia* root biomass, but increased the secondary metabolite production (Baque *et al*., 2008). Same combination of growth regulators (BAP and NAA), gave the maximum alkaloid production by *Hyoscyamus muticus* suspension culture (Aly *et al*., 2010).

Gibberelin is usually not added to culture medium, however, there are a few reports describing its effect on secondary metabolite production. It was discovered that the callus growth of *Taxus cuspidata*, which produce taxol was extensively encouraged by the addition of gibberellic acid into the solid medium (DiCosmo and Misawa, 1995).

Production of secondary metabolite also could be affected by basal media. Some researchers work intensively to test various well known basal media in order to study the desired product. It was found that production of serpentine from *Catharanthus roseus* cell suspension cultures was basal medium dependent, where MS basal medium found to be the best to produce high level of this particular alkaloid (Zenk *et al.,* 1977).

2.3.3 Culture conditions

Modifications of nutrient concentrations, light intensity and inoculums density may also affect growth, synthesis and accumulation of secondary plant metabolites. Light controls growth and differentiation of plant cell, tissue and organ cultures (Heo *et al*., 2002). The influence of light on secondary metabolite production was quite varied and was being a point of interest for many years. Recently, *Hyoscyamus muticus* cell suspension cultured under light condition showed a better growth pattern than that under dark condition but alkaloid content was increased under dark condition compared with cells cultured under light condition (Aly *et al*., 2010). Similarly, light quality significantly affected the biomass and the total anthraquinone, phenolic, and flavonoid contents in *Morinda citrifolia* induced root (Baque *et al*., 2010).

Another significant factor that could affect product yield was cell density or cell inoculums, due to cell to cell and cell to medium interaction (Aly *et al*., 2010). Establishment of optimum cell biomass was concurrent with optimized inoculum size. Previous report showed that high inoculum size of *Panax notoginseng* cell cultures were needed for the production of saponin (Zhang and Zhong, 1997) and similar results were found for ajmalicine production in *Catharanthus roseus* cells (Lee and Shuler, 2000). High inoculum density of *Morinda citrifolia* adventitious root was favorable to both biomass growth and the production of secondary metabolite (Baque *et al*., 2008). Similarly, high inoculum density cultures increased phenolics and flavonoids concentrations in *Hypericum perforatum* adventitious root when scaling up cultivation (Illg and Faria, 1995).

Nutrient concentration also plays an important role during biomass and metabolites production. Previous report by Yu *et al*. (2000) revealed that full MS strength was optimised for biomass growth and secondary metabolite production of adventitious

roots of *Panax ginseng*. Similarly, full strength of MS was best for root growth of *Panax ginseng* but ¾ strength favourable for ginsenoside production (Hahn *et al*., 2003). In contrast, adventitious roots of *Raphanus sativus* cultured in ½ strength MS medium increased the production of anthosyanin (Betsui *et al*., 2004).

Plant cell cultures are usually grown using simple sugars as carbon source (Ramachandra Rao and Ravishankar, 2002). Generally sucrose, glucose and fructose were the best carbon sources for most plant cell cultures (Abdullah *et al*., 1998). It has been established that, among these carbon sources, sucrose is energetically the most advantages for the cultivation of plant cell cultures (Wu and Zhong, 1999). However catharantine production was doubled in *Catharanthus roseus* cultures when fructose was used as a carbon source (Kim, 2001). It has been reported that initial sucrose concentration also affected the biomass growth and secondary metabolite production. Increased sucrose concentration usually resulted in increased biomass and metabolite production of plant cell cultures as have been observed in *Catharanthus roseus* callus culture (Zhao *et al*., 2001a).

Each plant species has different optimized conditions both for growth of the cells and for production of useful products, so it is necessary to optimize the conditions in each case.

2.3.4 Elicitors

Elicitors were found to be effective in increasing the production of secondary metabolites. For example, methyl jasmonate elicitation increased the production of eleutherosides and chlorogenic acid in the embryogenic suspension cultures *Eleutherococcus sessiliflorus* (Shohael *et al*., 2008).

High yield of secondary metabolites were observed for *Taxus chinensis* and *Panax ginseng* cell cultures by adding the methyl jasmonate and jasmonate derivatives (Qian *et al*., 2004, Thanh *et al*., 2005). In cell suspension cultures of *Capsicum frutescens* addition of a putrescine was found to be effective in enhancing the biomass and secondary metabolite production (Sudha and Ravishankar, 2003).

Hypericin is used in the treatment of depression, and is extracted from herbal plant called *Hypericum perforatum* L. (St. John's wort). A significant increase was observed in accumulation of hypericin in the cell cultures of *H. perforatum* subsequent to the addition of jasmonic acid (Walker *et al*., 2002).

2.3.5 Precursors

Secondary metabolite production was found to be stimulated by addition of suitable precursors or related compounds into the culture media. For example, feeding the shoot cultures with amino acid precursors induced a 3.7-fold increase in the production of adhyperforin of *Hypericum perforatum* (Karppinen *et al*., 2007). Feeding with cinnamyl alcohol enhanced the production of cinnamyl glycosides in compact callus aggregate cultures of *Rhodiola rosea* (György *et al*., 2004).

In order to improve the biomass formation and production of phenylpropanoids p-coumaric acid, ferulic acid and sinapyl alcohol, four precursors (l-phenylalanine, cinnamic acid, ferulic acid, and sinapic acid) were fed to *Larrea divaricata* cell cultures. Feeding phenylalanine resulted in an increase of these compounds production. Addition of cinnamic acid enhanced the cell culture growth, but not the compounds production (Palacio *et al*., 2010). Production of several compounds such as tropane alkaloids, indole alkaloids, and phenolic compounds were also enhanced by addition of appropriate amino acid.

2.3.6 Metabolic engineering

A new approach with high potential to produce abundant important secondary metabolites is metabolic engineering. Eventhough metabolic pathways provide limited knowledge regarding the biosynthetic genes involved, metabolic engineering for the production of alkaloids have been established for *C. roseus* species.

In order to enhance the desired components, numerous metabolic pathways in that particular plant need to be studied and well understood. Metabolic engineering also demand a specific knowledge on the enzymes related to the pathways. Enzymes characterization, regulation and activity should be investigated. Based on the enzymes, targeted gene for cloning and subsequent engineering could be selected. For example, gene cloning can be targeted to enhance the metabolic flux in order to improve the productivity of the targeted metabolite(s) of the plant or plant cell culture system. This purpose can be accomplished either by overexpressing the target pathway or suppressing other pathways. In other words, metabolic engineering is to control related metabolic pathways to acquire greatest outcomes.

Dixon and Steele, (1999) and Forkmann and Martens, (2001), reported successful changes of flower color by modifying a biosynthetic pathway of anthocyanin and flavonoid. This modification also resulted in an increased level for antioxidative flavonol production in tomato (Muir *et al*., 2001). Recently, Quevedo, (2010), revealed that metabolic engineering strategy was successful to enhanced the improve anthraquinone production in plant cell cultures. Transgenic cell lines of *Morinda citrifolia* showed significantly higher levels of anthraquinons (21% and 30% after 3 and 6 days of culture ($p<0.01$) compared control cell lines (Quevedo, 2010).

Nevertheless, up till now, there have been only a small number of successful achievements in revising and modifying pathways to boost up plant derived pharmaceutically important compounds. The first example of successful metabolic engineering in important medicinal plant for production of valuable end product was achieved by Yun *et al*. (1992) by introducing a gene encoding hyoscyamine-6b-hydroxylase extracted from *Hyoscyamus niger* into *Atropa belladonna.* This resulted in high amount of tropane alkaloid scopolamine accumulation as compared to the natural plant.

2.4 BIOREACTOR FOR PLANT CELLS CULTIVATION

Bioreactor is capable for mass production of commercial plant cell propagation. Bioreactor is a self-contained and aseptic environment for cell cultures, often with real-time and online monitoring and controlling of the micro environment conditions such as agitation, aeration, temperature, dissolved oxygen and pH. Bioreactor is normally used in producing biomass of cells where specialized embryogenic cell, shoots or roots are the final products. Bioreactor technology is also widely applied in the production metabolites, enzymes and biotransformation of exogenously added metabolites such as precursors in a metabolic pathway. Plant cells derived compounds e.g. shikonin, ginsenosides and berberine been successfully produced in large scale using bioreactor (Bourgaud *et al*., 2001, Jeong *et al*., 2006, Georgiev *et al*., 2011).

Although mainly undifferentiated cell cultures have been studied, a large interest has been shown in hairy root and other organ cultures. Hairy roots, once established, can be grown at a high growth rate in a medium with low inoculum. Several bioreactor designs have been reported for hairy root cultures taking into consideration their complicated

morphology and shear sensitivity (Giri and Narasu 2000, Eibl *et al*., 2010). The main problem associated with hairy root cultures in bioreactors is the restriction of gaseous oxygen delivery to the central mass of tissue, which may results in a pocket of senescent tissue. Due to branching, the roots form an interlocked matrix that resists fluid flow. The ability to exploit hairy root culture as a source of bioactive compounds depends on development of a novel bioreactor system where several physical and chemical constraints associated with these special cultures must be taken into consideration.

Optimizing the plant cell cultures cultivation conditions in bioreactor system is crucial with respect to the physical parameters such as mixing and shear stress. By lowering the impeller speed of the bioreactor in order to reduce shear stress, the capability of the impeller to distribute the gas bubble in the bioreactor will be affected. This situation has to be seriously considered if large volumes of bioreactor or high density of plant cell cultures were to be cultivated (Zhou *et al*., 2010).

Prakash and Srivastava (2011), showed that an integrated approach has been succesfully adopted by combining the various yield as well as productivity enhancement strategies to increase the production Azadirachtin by suspension culture of *Azadirachta indica.* A pulse-aerated column reactor was designed for high production of verbascoside, one of the highest yields reported to date by suspension culture of *Harpagophytum procumbens* (Georgiev *et al*., 2011).

Various geometrical configuration of bioreactors were available, for example loop-fluidized bed, spin filter, continuously stirred turbine, hollow fiber, membrane stirrer for bubble-free aeration, hybrid reactor with a cell-lift impeller and a sintered steel sparger, as well as a centrifugal impeller bioreactor (Zhao *et al*., 2001b).

Ramakrishnan and Curtis (2004), established a trickle-bed bioreactor for root cultures. Batch, semi-batch, immobilized and continuous cultures have been tested for *C. roseus* cell cultivation in bioreactors with the most common being the batch culture. Various plant species have been studied for biomass or overproduction of secondary metabolites in a number of bioreactor configurations are shown in Table 2.10.

Table 2.10: Laboratory and large-scale plant cell biomass and metabolites production in different bioreactors.

Plant species	**Compounds**	**Bioreactor configuration & volume**	**Researchers**
Azadirachta indica	Azadirachtin	Stirred tank: 3 L	(Prakash and Srivastava, 2008)
Beta vulgaris	Betalain	Bubble column: 3 L	(Savitha *et al*., 2006)
Curcuma zedoaria	Sesquiterpenes	10 L Stir tank : 10 L	(Loc *et al*., 2009)
Echinacea purpurea	Chichoric acid, chlorogenic acid and caftaric acid	Airlift: 20 L Ballon type bubble: 500 L Drum type bubble: 1000 L	(Wu *et al*., 2007)
Eleutherococcus sessiliflorus	Eleutherosides	Ballon type bubble: 3L	(Shohael *et al*., 2005)
Harpagophytum procumbens	Verbascoside	Stir tank: 1 L Column: 1 L	(Georgiev *et al*., 2011)
Hypericum perforatum	Hyperecin	Ballon type bubble: 3L	(Cui *et al*., 2010)
Panax ginseng	Ginsenoside	Ballon type bubble: 5L	(Jeong *et al*., 2006)
Panax ginseng	Ginsenoside	Ballon type: 10 000 L and 20 000 L	Choi *et al*. 2006
Podophyllum hexandrum	Podophyllotoxin	Stir tank: 3 L	(Chattopadhyay *et al*., 2002)
Scopolia parviflora	Alkaloid	Column: 1 L	(Min *et al*., 2007)

CHAPTER 3

ESTABLISHMENT OF CELL SUSPENSION CULTURES

3.1 INTRODUCTION

Plant cell cultures have been considered to be an attractive source of biologically active compounds (Luczkiewicz *et al*., 2002) owing to its ability to transform some organic compounds (Sakui *et al*., 1992). In addition, the establishment of cell culture has considerable potential as an alternative to traditional agriculture for the production of known and new secondary metabolites (Nikam and Shitole, 1998). Cell suspension cultures for instance, have successfully been used for studies on regulation of enzymes related to the metabolism of secondary metabolites, regulation of the biosynthetic capacity for metabolites production, improvement of secondary metabolite production and for the selection of high metabolite producing cell lines (Figueiredo *et al*., 2000).

Looking at the importance of plant cell cultures, therefore, the objective of this study is to establish direct plant regeneration as a source of explant for callus induction and cell suspension cultures of *B. rotunda* for determination of its bioactive compounds. The objective of this chapter is to establish protocols for direct regeneration, callus and cell suspension cultures for *B. rotunda*.

3.2 MATERIALS AND METHODS

3.2.1 Establishment of aseptic explants

Mature rhizomes of *B. rotunda* were purchased from herbal supplier at a local market in Kuala Lumpur, Malaysia. The rhizomes were cleaned and rinsed. The cleaned rhizomes were then placed in an open container to allow shoots sprouting up two to four cm in length. The sprouting buds were collected and washed with 20% (v/v) commercial sodium hypochlorite (Clorox) for 15 min. Under aseptic conditions, shoots were surface sterilized with 0.5% (w/v) aqueous solution of mercuric chloride ($HgCl_2$) for five min and followed by three rinses in sterile distilled water. The shoot buds had their external leaves removed and trimmed down until the size ranging from 0.5 to 0.8 mm and used as explants. They were then inoculated into 350 ml glass jar containing (Murashige and Skoog, 1962) MS medium supplemented with 30.0 g/l sucrose and 2.0 g/l gelrite for 30 days. The pH of the medium was adjusted to 5.7 prior to autoclaving at 121 °C for 21 min. The cultures were maintained in a culture room with 16/8 h photoperiod (light/dark) at 25 ± 2 °C. After 30 days, the aseptic buds of this species were transferred onto MS medium supplemented with 3.0 mg/l 6-benzylaminopurine (BAP) and 0.5 mg/l α-naphthaleneacetic acid (NAA), which is an optimum medium formulated for other Zingiberaceae species by Yusuf *et al.*, (2007), for shoot multiplication for four weeks. The multiple shoots produced were separated and transferred into MS medium devoid of plant growth regulators for four weeks and were used for all experiments.

3.2.1.2 Induction of multiple shoots

Isolated shoot buds were inoculated onto MS medium containing 30.0 g/l sucrose and 2.0 g/l gelrite as solidifying agent supplemented with cytokinins i.e BAP ranging from 0.0 to 5.0 mg/l, and auxin i.e NAA ranging from 0.0 to 2.0 mg/l, for shoot

multiplication. A single shoot was cultured into a 350 ml glass jar and 10 experimental units were used for each combination of media.

3.2.1.3 Scoring of data

All cultures were examined periodically, and the morphological changes were recorded on the basis of visual observations and were made after one to four weeks of culture for four consecutive subcultures. There were 10 cultures per treatment for shoot multiplication and subculturing was carried out at an interval of four weeks. The effect of different treatments and subcultures were quantified on the basis of percentage of cultures showing response for multiple shoot formation. Basic statistical analyses were conducted and values represent means ± SD for 10 cultures per treatment.

3.2.2 Initiation of callus

3.2.2.1 Culture media and conditions

A culture medium containing MS salts supplemented with 30 g/l sucrose and 2.0 g/l (w/v) agar was used in all experiments. The pH of the medium was adjusted to 5.7 before being autoclaved at 121 °C for 20 min. All cultures were incubated in the dark for callus induction and under a photoperiod of 16 h at 25 ± 2 °C for shoot induction study.

3.2.2.2 Callus initiation

Aseptic meristem and shoot base explants were prepared as described in 3.2.1 and the explants (dissected into 2 mm cross section) were placed horizontally in 90 mm diameter petri plates containing 25 ml medium.

The shoot base explants were cultured on MS medium complemented with 1.0 mg/l NAA, 1.0 mg/l indole-3-acetic acid (IAA) and 1.0 mg/l *d*-biotin. Five different

concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D) (1.0, 2.0, 3.0, 5.0 and 10.0 mg/l) and 3, 6-dichloro-*o*-anisic acid (Dicamba) (0.2, 0.5, 0.8, 1.0 and 2.0 mg/l) were also added in the media either in combination or solely. MS medium lacking growth regulators served as a control experiment. The data on callus induction response was collected after eight weeks of culture on callus induction medium. To determine the best time for sub-culture, calluses were removed from the original explants prior to culture on new media and the callus growth was monitored at weekly interval for eight weeks by measuring the fresh weight (FW). Callus growth rate was observed and calculated using the equation described by Yokota (1999).

$$\text{Growth rate} = \frac{\ln \dfrac{\text{FW at } t \text{ measured (g)}}{\text{FW at the initiation of logarithmic growth phase (g)}}}{t \text{ measured (week)}}$$

Where; t = time

After identifying the most suitable medium for callus proliferation, the calluses were sub-cultured when they were at early stationary phase according to the callus growth curve. All of the experiments were conducted with a minimum of 30 replicates per treatment. The cultures were observed periodically and morphological changes were recorded at regular intervals.

3.2.2.3 Shoot induction and multiplication

The calluses growing on induction medium were transferred to the regeneration medium for eight months and sub-cultured at four weeks interval. To study the effect of growth regulators on the regeneration of *B. rotunda* calluses, regeneration medium was supplemented with either kinetin or BAP at five different concentrations 0.5, 1.0, 2.0,

5.0 and 10.0 mg/l individually. The cultures were kept under similar conditions used for callus induction studies. When the color of calluses developed into opaque white, the cultures were kept under 16 h photoperiod to enhance further development. For each medium tested, 30 callus pieces were used. The regeneration capacity was evaluated after eight weeks of culture.

3.2.2.4 Histochemical studies

The established double-staining protocol of Gupta and Durzan (1987), was modified slightly. Adequate 1% (w/v) acetocarmine (Sigma Chemical Co., USA) solution was added to submerge the callus on a microscope slide. The slide was heated above the Bunsen flame for 1 to 2 min and the excess acetocarmine was rinsed off with distilled water. Then, the sample was stained with 0.1% (w/v) Evans Blue (Sigma Chemical Co., USA) solution for 2 to 3 min and then rinsed with distilled water to remove any excess stain. The double-stained sample was allowed to drain dry and then resuspended in distilled water before viewing under light microscope. The colour distribution, especially those of the red acetocarmine, revealed the presence and extent of somatic embryogenesis.

3.2.2.5 Histology studies

Histological sections using resin were done to differentiate between embryogenic and non-embryogenic callus produced throughout this study. The samples were sliced according to the size of the available mold and then fixed for 24 to 48 h at room temperature in a glutaraldehyde–paraformaldehyde–caffeine (GPC) fixative solution (50.0 ml 0.2 M phosphate buffer, pH 7.2; 20.0 ml 10% (v/v) paraformaldehyde; 4.0 ml 25% (v/v) glutaraldehyde; 1.0 g caffeine and topped up to 100.0 ml with distilled water). The samples were then dehydrated in ascending ethanol concentration series

(v/v): 30% (30 min); 50% (45 min); 70% (45 min); 80% (60 min); 90% (60 min); 95% (60 min), and twice in absolute ethanol for 60 min each. The tissues were then prepared for infiltration with basic resin (Leica Historesin Embedding Kit) for 24 to 48 h at 48 °C under slight vacuum. The sinking of a slightly translucent specimen underneath indicated the completion of infiltration. Then the specimens were embedded into molds and the resin was allowed to be fully polymerized, holders were attached and 3 µm sections were sliced using a microtome. Good sections were then stained with 1% periodic acid for 5 min, rinsed four times with distilled water at pH 4.5 and then submerged in Schiff's reagent (1.0 g basic fuchsin, 2.0 g disodium metasulfite in 1 M HCl, 0.5 g neutralized activated charcoal) for 20 min in the dark. The slides were rinsed four times with distilled water (pH 4.5). Finally, for counter staining, Naphthol Blue Black (1.0 g Naphthol Blue Black in 100 ml 7% acetic acid) was used at 60 °C for 5 min. After further rinsing under running water, the sections were dried before viewing under a light microscope. All images were photographed using Leica Microsystems (EC3) (Switzerland).

3.2.2.6 Rooting and acclimatization

The regenerated shoots measuring 4 to 5 cm in length were excised from callus clump and cultured on PGR free MS medium to allow single plantlet development. Plantlets were then sub-cultured on MS medium supplemented with 2 g/l activated charcoal to promote rooting. Plantlets with well-developed shoots and roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing garden soil. Potted plantlets were covered with a transparent plastic bag to ensure high humidity and watered every day for two weeks. Transparent plastic bags were opened after two weeks prior to field transplantation. After four weeks, these

plants were transferred to pots containing garden soil: sand (1:1 w/w) and maintained under normal day length conditions.

3.2.2.7 Statistical analysis

Multiple range tests were conducted to determine the significant differences between mean samples.

3.2.3 Establishment of cell suspension cultures

Establishment of cell suspension cultures were carried out using four weeks old meristem-derived callus in the basal MS liquid medium (pH 5.8) supplemented with 3.0% (w/v) sucrose. The medium pH was then adjusted to 5.8 prior autoclaving. Two grams of the callus was added to 25 ml of the liquid medium in 100 ml Erlenmeyer flasks. The cultures were agitated at 80 rpm on an orbital shaker (lateral displacement) and incubated under a photoperiod of 16 h fluorescent light at 25 ± 2 °C. Sieving with a sterile stainless-steel filter (450 µm) was carried out after 14 days in order to separate the cells from the intact callus.

The subculturing and sieving filtration were repeated every two weeks in order to obtain a suitable growth and homogenous cells for the subsequent treatments. Subculturing involved the transfer of 10 ml batch suspension cultures into 40 ml of fresh basal MS liquid medium, pH 5.8 and supplemented with 3% (w/v) sucrose. The cultures were agitated at 80 rpm on the orbital shaker and incubated under continuous cool fluorescent lights at 25 ± 2 °C. For the preparation of each treatment, 5 ml (approximately 0.1 g fresh weight) of the cells from the stock cultures was added into 45 ml of fresh liquid medium and this was repeated thrice in three replicates.

3.2.3.1 Histochemical and histology studies

Histochemical and histology studies were done as described in 3.2.2.4 and 3.2.2.5

3.2.3.2 Growth of the suspension cultures

Growth of the cell suspension cultures was measured by determining the settled cell volume (SCV) in a 3-day interval for 30 days. The maximum growth period observed was used as an indicator point for further studies. The morphological changes of the cells and pH of culture medium were also monitored at three-day intervals using a stereomicroscope and pH meter, respectively.

3.2.3.5 Statistical analysis

Basic statistical analyses were conducted and values represent means ± SD for 10 cultures per treatment.

3.3 RESULTS AND DISCUSSION

3.3.1 Initiation of shoot buds

Establishment of contamination free cultures is a major task due to the fact that the explants originated from underground rhizomes (Hosoki and Sagawa, 1977). In this study, rhizomes were initially sprouted in a soil-free condition until shoot buds appear. These shoot buds were excised from rhizomes and were used as explants. The buds from the rhizomes of *B. rotunda* that were surface sterilized with $HgCl_2$ solution and Clorox® solution for 15 min could established more than 70% aseptic and surviving explants which remained free of contamination after four weeks in MS medium. Chan (2004), also reported that the use of $HgCl_2$ with two-stage surface sterilization using

Clorox® solution was extremely efficient for establishing aseptic buds of other Zingiberaceae species. Similar sterilization process was also used to establish the aseptic buds of *Cymbopogon nardus* (Chan *et al*., 2005). Once the contamination free cultures of the shoot buds were established, these were easily maintained by sub culturing on fresh medium.

3.3.1.2 Shoots growth and multiplication

The aseptic shoots of *B. rotunda* cultured on MS medium supplemented with different concentrations of BAP (0.0 to 5.0 mg/l) or in combination with NAA (0.0 to 2.0 mg/l) resumed their growth, produced shoots and roots simultaneously (Table 3.1).

Table 3.1. Effects of MS medium supplemented with BAP and NAA on multiple shoot formation of *B. rotunda* over a period of four weeks of culture.

BAP (mg/l)	NAA (mg/l)	Mean no. of multiple shoots
0.0	0.0	1.0 ± 0.0
0.5	0.0	2.9 ± 0.2
1.0	0.0	3.3 ± 0.2
2.0	0.0	4.1 ± 0.3
3.0	0.0	3.9 ± 0.3
5.0	0.0	2.7 ± 0.2
0.0	0.5	1.0 ± 0.0
0.5	0.5	3.8 ± 0.1
1.0	0.5	4.2 ± 0.1
2.0	0.5	4.9 ± 0.1
3.0	0.5	4.0 ± 0.3
5.0	0.5	2.8 ± 0.2
0.0	1.0	1.0 ± 0.0
0.5	1.0	2.7 ± 0.3
1.0	1.0	2.9 ± 0.3
2.0	1.0	3.6 ± 0.4
3.0	1.0	3.2 ± 0.4
5.0	1.0	2.4 ± 0.3
0.0	2.0	1.0 ± 0.0
0.5	2.0	2.4 ± 0.3
1.0	2.0	2.5 ± 0.2
2.0	2.0	2.8 ± 0.2
3.0	2.0	2.6 ± 0.3
5.0	2.0	1.8 ± 0.2

Values represent means ± SD for 10 cultures per treatment

The simultaneous production of shoots and roots were also reported in other Zingiberaceae species (Balachandran *et al*., 1990; Chan, 2004). The growth of the shoots and subsequent multiplication could not be achieved in medium without growth regulators. After 4 to 6 weeks, about 90% of the shoots of *B. rotunda* that were cultured on MS medium supplemented with 0.5 to 3 mg/l of BAP and 0.5 mg/l NAA showed different levels of development with high micropropagation frequency (induced 4 to 5 multiple shoots, Table 3.1) and variable number of leaves with 10 to 15 roots including secondary roots per explant. The maximum number of multiple shoots (4.9 ± 0.1) was obtained in the medium containing 2.0 mg/l of BAP and 0.5 mg/l NAA four weeks after culture initiation (Figure 3.1).

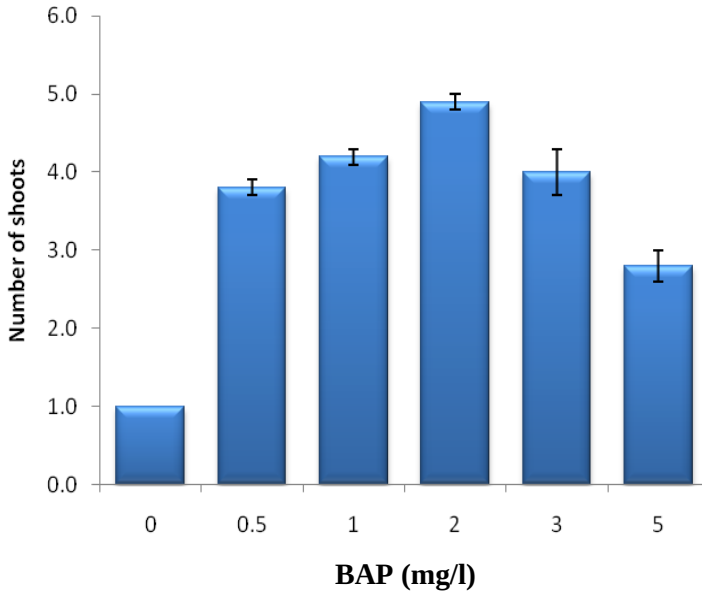


Figure 3.1 : Effects of MS medium supplemented with BAP and low concentration of NAA (0.5, mg/l) on multiple shoot formation of *B. rotunda* over a period of four weeks of culture. Values are mean ± SD for 10 cultures per treatment.

However, the buds cultured on MS medium containing higher concentration of BAP (5.0 mg/l) and NAA (2.0 mg/l) showed low level of development and low micropropagation frequencies (1 to 2 shoots per explant) and also showed abnormalities

viz. even though it produced multiple shoots, the plantlets were stunted and the leaves became yellowish within four weeks of culture. These results indicated that there was an optimal concentration of plant growth regulators required for normal shoot multiplication *in vitro* cultures of *B. rotunda* species. Balachandran *et al*. (1990), also reported that higher concentration of kinetin was not suitable for *Zingiber officinale*. Aseptic cultures in MS supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA induced more roots than shoots. Micropropagation was quantified by the number of shoots per explants. BAP concentration dependent shoots were well formed *albeit* inhibited by NAA. The medium having only NAA had no effect on shoot multiplication or growth. Multiplication frequency was increased until BAP concentration reached 3.0 mg/l.

Since there was no increase in the number of normal shoots formed, low concentration of BAP (2.0 mg/l) and NAA (0.5 mg/l) supplemented into MS medium formulation was chosen as the shoot multiplication medium for *B. rotunda*. Other researchers reported higher concentration of plant growth regulators for the induction of multiple shoot formation for some of the Zingiberaceae species. Loc *et al*. (2005b) reported that MS medium supplemented with 20% (v/v) coconut water, 3 mg/l BAP and 0.5 mg/l IBA could induce the formation of an average of 5.6 shoots per explant for *C. zedoaria*. Bharalee *et al*. (2005) found that MS medium supplemented with 4 mg/l BAP and 1.5 mg/l NAA was the best medium for shoot multiplication of *C. caesia* (average 3.5 shoots per explant) and MS plus 1.0 mg/l BAP and 0.5 mg/l NAA for *C. zedoaria* (average 4.5 shoots per explant). Balachandran *et al*. (1990) reported that *C. domestica, C. caesia* and *C. aeruginosa* could produce an average of 3.4, 2.8 and 2.7 shoots per explant respectively using MS medium supplemented with 3 mg/l BAP. Nayak (2000), reported MS medium supplemented with 5 mg/l BAP was most effective for shoot multiplication of *C. aromatic* producing an average 3.3 shoots per explant.

Similar results indicating cytokinin and auxin effects on shoot multiplication had been reported earlier in ginger (Hu & Wang, 1983; Palai *et al.*, 1997). Hosoki and Sagawa (1977) reported that the requirement of a cytokinin for high-frequency shoot multiplication of ginger was as high as 5.0 mg/l IBA. In addition, Palai *et al*. (1997), indicated that the combination of two cytokinins along with auxin increased the rate of shoot multplication (16-fold) within four weeks of culture. The present findings suggested that a high frequency of shoot production from a single shoot meristem could be achieved by manipulating the plant growth regulators.

The number of shoots generated from *B. rotunda* shoot explants cultured on the proliferation medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA remained unchanged after either four or eight weeks of subculture. Thus, the *in vitro* plantlets could be subcultured every eight weeks in terms of culture preservation. In addition, the *in vitro* plantlets could be maintained on solid basic MS medium for two months without any subculturing required and the plants remained healthy. Hence MS medium without the addition of plant growth regulator(s) could be potentially used for the *in vitro* germplasm conservation of *B. rotunda*.

The effect of subculture on shoot multiplication was tested in MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA for 16 weeks. The results indicated that the number of multiple shoots were not significantly different upon subcultures at $p < 0.05$ (Figure 3.2).

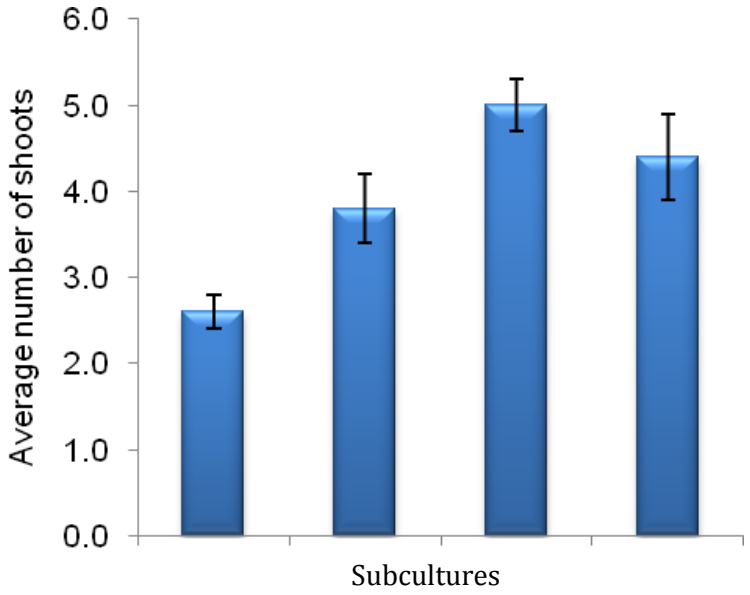


Figure 3.2 : Effects of subculture on multiple shoots formations of *B. rotunda* over a period of four subcultures in MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA. Values are mean ± SD for 10 cultures per treatment.

Micropropagation stages involving shoot bud initiation until rooting are shown in Figure 3.3. Shoot multiplication occurred after four to six weeks. Plantlets formed were then placed in rooting medium in order to enable plantlets to form complete roots. Plantlets were formed in MS without plant growth regulators (PGR) supplemented with 1.0 mg/l activated charcoal after four to six weeks. Well rooted plants were then acclimatized for three weeks followed by field planting.

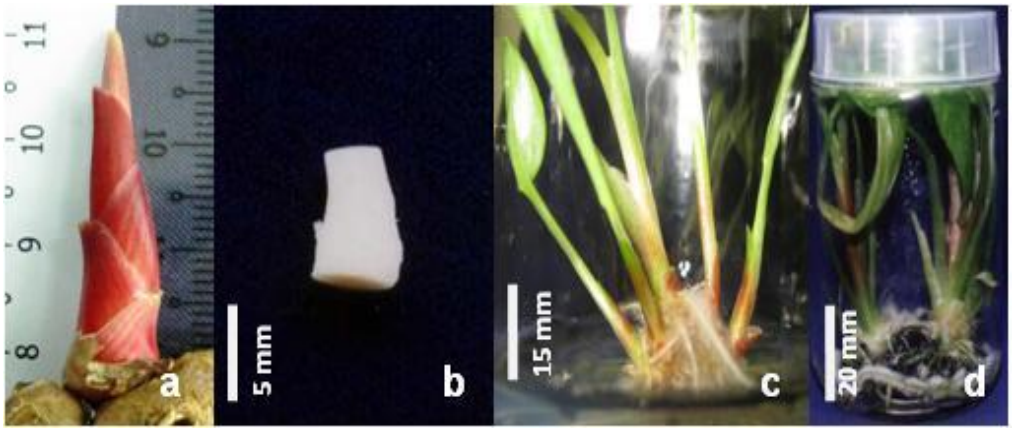


Figure 3.3: *In vitro* regeneration and plant establishment of *B. rotunda*. a) Shoots sprouted from mature rhizomes of *B. rotunda* (30 to 50 mm). b) Shoots after surface sterilization under aseptic conditions with external leaves removed and trimmed down (5 to 8 mm) as explants. c) Induction of multiple shoots of *B. rotunda* on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA after four weeks of culture. d) Formation of roots on acclimatized semi-solid MS medium devoid of plant growth regulators with 2.0% (w/v) activated charcoal after four weeks of culture.

3.3.2 Initiation of callus

Shoot base explants cultured on MS medium supplemented with dicamba or 2,4-D showed callusing after four weeks of inoculation. Swelling of the explants was observed within 1 to 2 weeks, followed by emergence of callus after another two weeks. Callusing commenced at the cut surface of the explants and finally covered the surface of the explants completely. Incubation of explants in auxin-free MS medium (control) produced non-embryogenic (NE) calluses with 6.7% of callusing response. On the other hand, the response was higher in cultures complemented with auxin than control. The explants gave 6.7% to 30% callusing response on medium supplemented with different concentrations of dicamba. The response fraction decreased gradually from 30, 26.7, 13.4 and finally 6.7% as the dicamba concentration increased from 0.2, 0.5, 0.8 and 2.0 mg/l respectively (Table 3.2).

Table 3.2 : Effect of dicamba and 2,4-D on callus induction from shoot base explants of *B. rotunda* cultured on MS medium after four weeks. (*n*)=30.

Auxin mg/l	Percentage of explants giving response (%)	Percentage of cultures forming embryogenic callus (%)	Percentage of cultures forming NE callus (%)
Dicamba			
0	6.7	-	6.7
0.2	30.0	-	30.0
0.5	26.7	-	26.7
0.8	13.4	-	13.4
2.0	6.7	-	6.7
2-4 D			
1.0	30.0	13.3	16.7
2.0	53.4	36.7	16.7
3.0	33.3	16.7	16.7
5.0	6.7	-	6.7
10.0	3.3	-	3.3

All cultures obtained from dicamba augmented medium produced NE callus as observed through morphological and histological studies. NE callus showed large, spongy, soft and wet structures (Figure 3.4a). Histological section illustrated that these cells were vacuolated and did not comprise of embryogenic cells (Figure 3.4b). The NE callus showed blue coloration when double-stained using established method of Gupta and Durzan, (1987). These results showed that MS medium supplemented with dicamba at different concentrations failed to provide favorable conditions for the production of embryogenic callus for *B. rotunda*.

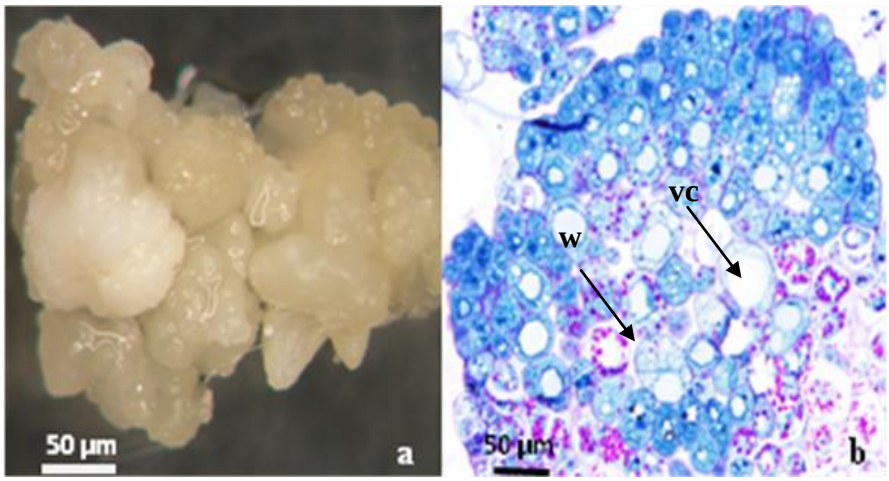


Figure 3.4 : Morphology (a) and histology (b) of NE callus of *B. rotunda*. Symbols; vc: vacuolated cell; w: wall.

Callus induction was facilitated in MS media supplemented with 2,4-D at all concentrations tested. The results were summarized in Table 3.2. The cultures produced better response of callusing at lower concentration of 2,4-D compared to higher ones. The highest frequency (53.4%) of explants producing callus was achieved on medium containing 2.0 mg/l 2,4-D. Callus induced from media supplemented with low concentration of 2,4-D (1.0 to 3.0 mg/l) was observed to be embryogenic but contrary observation was made at higher 2,4-D concentrations (5.0 and 10.0 mg/l). The best response in terms of percentage of explants forming embryogenic calluses was on MS medium supplemented with 2.0 mg/l 2,4-D (36.7%). The percentage of response, growth and morphology of callus were influenced by the type of growth regulators used and their concentrations. Morphological observation of embryogenic callus under the stereo microscope showed yellow and friable structures (Figure 3.5a). The embryogenic callus revealed dense cytoplasm with prominent nuclei and laden with protein giving rise to blue-black stained cells (Figure 3.5b).

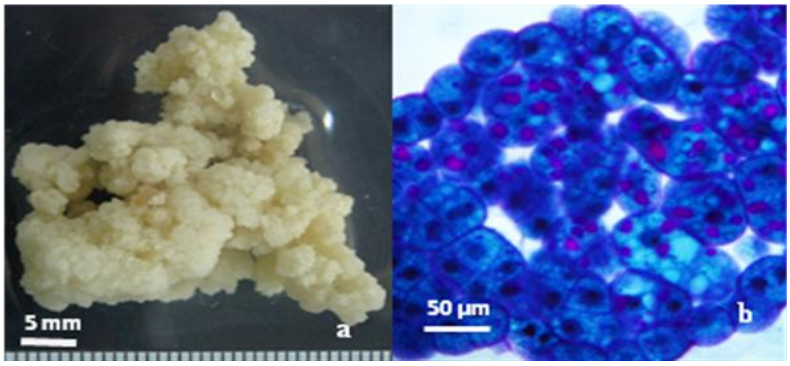


Figure 3.5: Morphology (a) and histology (b) of friable callus

This embryogenic callus characteristic was in accordance with the intense absorption of red acetocarmine when double-stained with 1% (w/v) acetocarmine and Evan's blue method (Tan *et al*., 2005). The differences in the percentages of cultures forming embryogenic calluses were negligible between 1.0 mg/l and 3.0 mg/l 2,4-D. Upon increasing the concentration of 2,4-D from 3.0 to 5.0 mg/l, a gradual decrease in percentage of explants forming callus was observed whilst 10.0 mg/l 2,4-D resulted in no callusing response. The outcome showed that 2,4-D was essential for *B. rotunda* to generate embryogenic callus. From previous reports on Zingiberaceae species, the highest amount of callus formation was in 0.5 mg/l 2,4-D in combination with 1.0 mg/l BAP supplemented media for ginger meristem tips (Malamug *et al*., 1991). Dicamba and picloram (2.0 mg/l) or 5.0 mg/l NAA in combination with 0.5 mg/l BAP were used to produce callus from turmeric leaf base (Salvi *et al*., 2001). For ginger (*Z. officinale*), leaf derived callus was obtained at 58% and 52 % using 1.0 mg/l and 1.5 mg/l 2,4-D, respectively but the percentage reduced to 21% at 3.0 mg/l 2,4-D (Kackar *et al*., 1993). Similar results were reported in this study where a declined callus development was a function of higher concentration of 2,4-D. For proliferation of callus, sub-culturing was

carried out in MS medium supplemented with different concentrations of dicamba or 2,4-D without other auxins (Table 3.3).

Table 3.3: Callus growth and regeneration rate on proliferation media.

PGR (mg/l)	Growth rate (G/week, mean ± S.D.)*	Regeneration rate (%, mean ± S.D.)*
Dicamba		
0.2	0.36 ± 0.007	-
0.5	0.36 ± 0.008	-
0.8	0.37 ± 0.004	-
2.0	0.37 ± 0.006	-
2-4 D		
1.0	0.35 ± 0.005	-
2.0	0.36 ± 0.008	-
3.0	0.38 ± 0.004	42 ± 0.004

*Multiple range test was conducted for test of significance (*n*=10).

Callus growth rate was calculated and found to be within similar range for all treatments i.e. 0.35 to 0.38 g per week. To obtain high growth rate (g/week), the callus cultures could be grown in MS media supplemented with either Dicamba (0.5-0.8 mg/l) or 2,4-D (1-3 mg/l). The result showed that the concentration of these PGR significantly ($p < 0.5$) affected the growth rate of callus cultures. Based on these results, all further sub-culturing was carried out in MS medium supplemented with 3.0 mg/l 2,4-D which showed maximum rate of proliferation (0.38 ± 0.004 g per week). Similar findings were reported by Tan *et al*. (2005), which showed that the fastest callus proliferation was observed in MS medium supplemented with 3.0 mg/l 2,4-D. The growth rates were comparable for the second and third sub-cultures which subsequently decreased gradually (data not shown). Malamug *et al*. (1991) and Tan *et al*. (2005) showed similar pattern of growth proliferation of ginger and *B. rotunda* respectively.

After identifying the most suitable medium for callus proliferation, growth curve of callus culture was determined. Biomass profile based on fresh weight in callus cultures revealed that the highest yield was obtained after third week of culture (Figure 3.6).

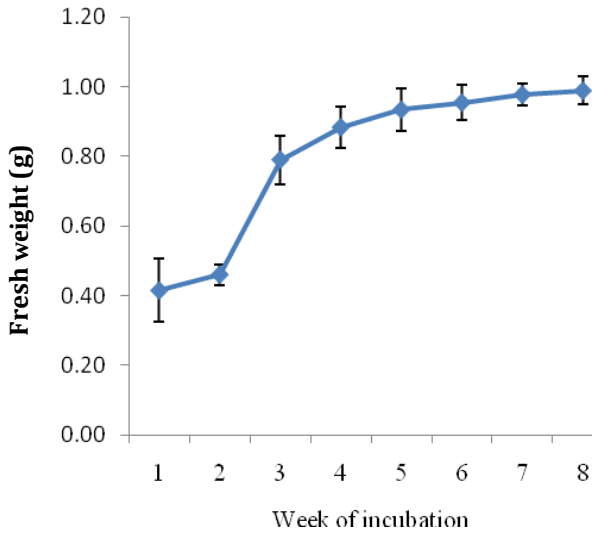


Figure 3.6: Growth curve of *B. rotunda* after eight weeks of culture on MS medium supplemented with 3.0 mg/l 2,4-D.

During the second and third week, calluses were found to be in exponential phase. However, after the third week, the growth of the callus started to enter early stationary phase. The callus growth was negligible as the culture period progressed into eighth week. After three months, the callus started to turn brown and eventually became necrotic possibly due to depletion of nutrients. Accumulation of toxic products and other limiting factors might have led to cell death and eventually a decline in biomass after several weeks of culture. Based on these results, the appropriate period for subculture was approximately on the fourth week of culture, which was between the end of exponential and early stationary phases.

Plantlets were regenerated directly from the original calluses cultured on 3.0 mg/l 2,4-D (42 ± 0.004%) supplemented MS medium. These plantlets were produced only from

nodular callus which showed the best morphology of callus to ensure further development and plant regeneration. The initial callus stage was crucial in tissue culture which determined a successful percentage of plantlet regeneration (Çirak *et al*., 2007). In order to improve regeneration capacity of calluses, the embryogenic callus was sub-cultured on MS medium containing different concentration of BAP or kinetin. The callus developed into somatic embryos during first sub-culture under a 16 h photoperiod. Subsequently, the callus color turned to opaque white (Figure 3.7a and 3.7b), and later became green (Figure 3.7c) forming plantlet (Figure 3.7d).

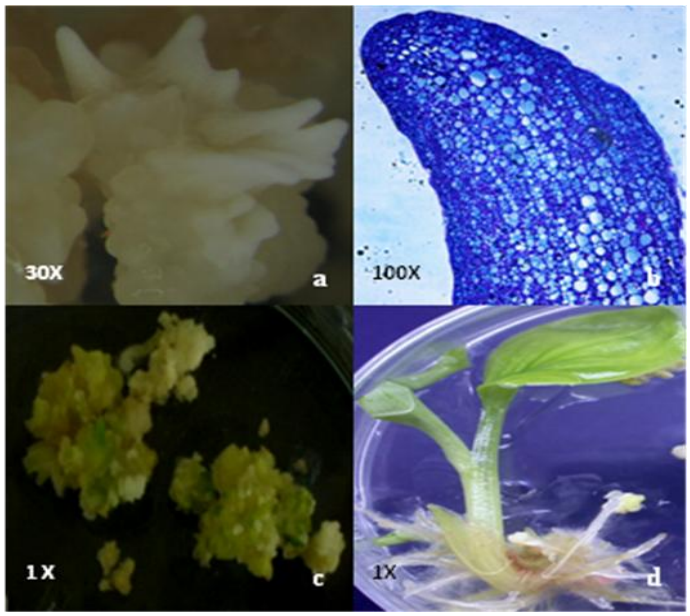


Figure 3.7: Shoot regeneration from *B. rotunda* callus. a) Differentiation of callus into white shoot primordial. b) Histology of white primordial shoot. c) Shoot proliferation in MS + 2.0 mg/l BAP. d) Shoot development on MS + 2 mg/l BAP.

In the present study, complete plantlets were regenerated only in MS medium supplemented with 3.0 mg/l 2,4-D (Table 3.3). Adventitious shoot buds were initially observed from the surface of callus within four weeks of culture and subsequently formed leaves. Shoot elongation took place after another month. The eventual

developmental period from callus initiation to complete plant formation was within two months (Figure 3.7d).

The callus culture inoculated on MS medium responded differently to various concentrations of cytokinins (BAP and kinetin) (Table 3.4).

Table 3.4: Effect of BAP and kinetin on shoot regeneration from shoot base segment of *B. rotunda* in MS medium after eight weeks of culture.

Hormone	Regeneration percentage (%, mean ± S.D.)*
BAP	
0.5	31.2 ± 0.5
1.0	45.6 ± 0.4
2.0	49.8 ± 0.5
5.0	10.2 ± 0.1
10	0.0
Kinetin	
0.5	15.5 ± 0.2
1.0	28.6 ± 0.4
2.0	22.8 ± 0.3
5.0	26.7 ± 0.4
10.0	0.0

*Multiple range test was conducted for test of significance (n = 10).

The highest regeneration frequency (49.8 ± 0.5%) was achieved with 2.0 mg/l BAP supplemented MS medium. When the concentration of BAP was increased up to 5.0 mg/l, a decrease in regeneration ability was observed. On MS medium supplemented with 0.5 to 10.0 mg/l kinetin, the highest regeneration frequency (28.6 ± 0.4%) was recorded at 1.0 mg/l kinetin. Developments of somatic embryogenesis into complete plantlets have been reported previously for some Zingiberaceae species. Malamug *et al.* (1991) revealed that low concentrations (1.0 to 3.0 mg/l) of BAP resulted in successful shoot induction from ginger rhizome derived calluses. The findings in this study agrees with that of Kackar *et al*. (1993) who described prominent regeneration at 2.0 mg/l BAP

from ginger leaf derived callus. Nevertheless, higher level of BAP (5.0 mg/l) was used to induce green shoot primordia of turmeric callus while half strength MS medium supplemented with 1.0 mg/l kinetin was employed for further development of shoots (Salvi *et al*., 2001). Instead of MS medium supplemented with cytokinin, Tan *et al*., (2005) reported that the regeneration of *B. rotunda* on MS medium with 3.0 mg/l 2,4-D only was sufficient for regeneration process from callus culture. However from this study, callus regenerated in MS medium supplemented with cytokinin (2.0 mg/l BAP) gave higher rate of regeneration of complete plantlets of *B. rotunda* compared to those in MS medium supplemented with 2, 4-D only.

Plant growth regulators, particularly cytokinins and auxins, play an extremely significant task in the process of callus induction and its proliferation. However, the optimum concentration for inducing callus differs according to the type of auxin and genotype (Varshney and Johnson, 2010). The present study revealed that different types and concentrations of cytokinin significantly affected the regeneration rate of *B. rotunda* callus cultures with the highest regeneration rate observed in MS medium supplemented with 2.0 mg/l BAP ($p < 0.05$).

In addition, in this study, the regeneration percentage reduced gradually upon sub-culture. According to previous work (Malamug *et al*., 1991; Tan *et al*., 2005), shoot-formation competency from ginger callus was reduced on the third sub-culture and regeneration which commenced during the first sub-culture produced better plantlets. Current investigation revealed that kinetin generally induced lower percentage of regeneration compared to BAP, with the highest percentage of plant regeneration at 49.8% (Table 3.4). Different responses of cytokinins in this study, i.e. BAP and kinetin was possibly due to dissimilarity in nutrient uptake, levels of endogenous growth

regulators, and recognition by cells (Varshney and Johnson, 2010). Regeneration stage is highly reliant and controlled by growth regulators.

Meristem explants produced more abundant proliferating yellow nodular callus on MS medium containing 2.0 mg/l 2,4-D than shoot base explants (Table 3.5). The results in Table 3.5 indicated that these explants showed different callus growth response on similar media formulation.

Table 3.5: Effect of explants on embryogenic callus formation of *B. rotunda* on MS medium supplemented with 1.0 mg/l NAA, IAA, *d*-Biotin and 2.0 mg/l 2,4-D.

Explants	Number of explants	Number of explants forming embryogenic callus	Percentage of cultures forming embryogenic callus	Number of explants forming NE callus	Percentage of cultures forming NE callus
Shoot base	30	9	30%	8	26.7%
Meristem	30	17	56.6%	-	-

By culturing the meristem of *B. rotunda*, it could induce more callus formation with an average of 56.6% of explants inducing embryogenic callus after eight weeks cultivation than shoot base explants. None of the meristem explants formed neither NE callus nor organized structure such as roots and leaves. Whilst 30% of the shoot base explants produced callus and 26.7% of these explants formed NE callus (Table 3.5). Some of the responses observed were either root or leaf formation from these explants. On the contrary, the meristem explants showed an increase in the number of explants forming embryogenic callus. Through observation, embryogenic callus derived from both explants remained normal with similar morphological characteristics. The present results showed the efficiency of the meristem explants in the induction of callus and concomitantly plant regeneration. This might be due to the presence of high auxins in young meristematic cells compared to mixture of young and mature cells in the shoot base explants.

To accomplish the establishment of plantlets in field conditions relied on the formation of vigorous root formation. In this study, plantlets were readily rooted and cultured individually on MS medium supplemented with activated charcoal (Figure 3.8). The occurrence of root development on auxin-free medium was attributed to the likely presence of endogenous auxin in *in vitro* plantlets (Minocha, 1987). Hardening and acclimatization of *in vitro* plantlets were 100% successful. The *in vitro* raised plants did not show any observable phenotypic variation.

Figure 3.8: Developed plantlet obtained from MS medium supplemented with activated charcoal

3.3.3 Establishment of cell suspension cultures

The cell suspension cultures were light yellow in color and contained homogenous small cells within 3 months of initiation (Figure 3.9a-c). Similar morphology observation was reported as fine dispersed yellowish cell suspension of *M. acuminata* cv. Mas (AA) was revealed after 3-4 months of commencement in liquid medium (Jalil *et al*., 2003). Under an inverted microscope, the suspension cultures showed vigorous growth and cell proliferation. The suspension characteristics indicated the embryogenic condition. Heterogeneous suspension cells with abundant accumulation

of vigorously dividing spherical cells could be a signal of the embryogenic condition of cell suspensions (Williams and Maheswaran, 1986).

On the 14th day after sieving and subculture, cell suspensions comprised of a large portion of cytoplasmic rich, spherical cells (Figure 3.9 a-b). In this study, the findings were comparable with previous reports by Jalil *et al*. (2008) and Côte *et al*. (1996). Apart from that, elongated and vacuolated cells were also observed as shown in Figure 3.9 d. This vacuolated feature suggested that this group or aggregated cell suspension were non-embryogenic (Jalil *et al*., 2008).

The morphological characteristics showed by *B. rotunda* cell suspension suggested an initial signal of embryogenic cells. Further tests such as histology and double staining were carried out to validate the morphological characteristics.

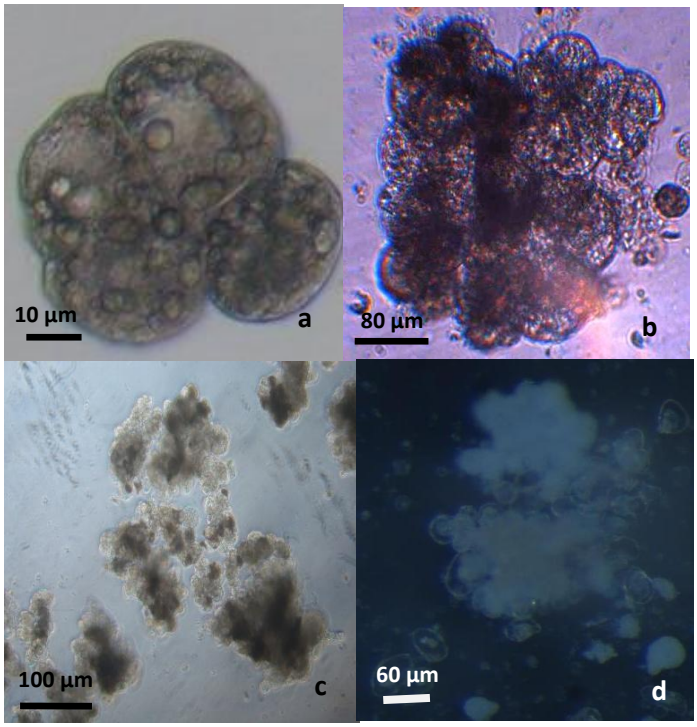


Figure 3.9: Multiplication and proliferation of dense cells of the suspension cultures. a) Proliferating cell suspension cultures after sieving using 450 µm stainless steel filter under inverted microscope. b) Aggregated cell suspension at day 14th of culture. c) Aggregated cell cultures without debris representing good and stable cell suspension cultures to be used for further experiments. d) Aggregated cell suspension with elongated and vacuolated cells under light microscope.

Plant cell suspension cultures usually have an aggregating tendency to form cohesive cell aggregates or larger callus clusters during culture because some polysaccharides excreted by plant cells increase the viscosity of the culture system at later stages of the culture (Zhao *et al*., 2001b). Unlike in *Morinda elliptica* (Abdullah *et al*., 1998), *B. rotunda* cells started to develop the aggregate cells clump, indicating that the cells colony started to mature. The clumpy aggregate has been reported for *Capsicum frutescens* (Williams *et al*., 1988) and *Pilocarpus pennatifolius* cell suspension cultures (Abdullah *et al*., 1998). Cytoplasmic cells indicated vigourous growth due to the active cytoplasmic streaming present in the cells. In some cases, this could be used to calculate cell viability.

3.3.3.1 Histology of cell suspension

Histology profile of cell suspension cultures were viewed under light microscope. Figure 3.10 shows cell suspensions with complete structures of cells including the cell wall, membrane, nucleus and dense cytoplasm which were stained blue black. An embryogenic cell showed by high nucleoplasmic and dense cytoplasm cells with substantial soluble protein and no starch reserve characteristics (Domergue *et al*. 2000). This study revealed *B. rotunda* cell suspension characteristic which was in agreement with earlier observations that was carried out by (Côte *et al*., 1996; Khalil *et al*., 2002; Jalil *et al*., 2008).

Periodic acid Schiff (red stain) specifically stains polysaccharide (starch reserves and walls) while Blue black naphthol stains soluble or reserve protein (blue black stain). This indicated that polysaccharide reserves were depleted in the cells and high content of protein was present in preparation of further cell developmental stage (Domergue *et*

al. 2000). Histological study of the NE cell suspension displayed strong evidence of NE character with lack of blue-black stained cells.

Investigation through histological profile was adequate for suspension cultures studies to facilitate the embryogenic characteristics of the cells. Another technique to prove this characteristic was histochemical study which was carried out in this study.

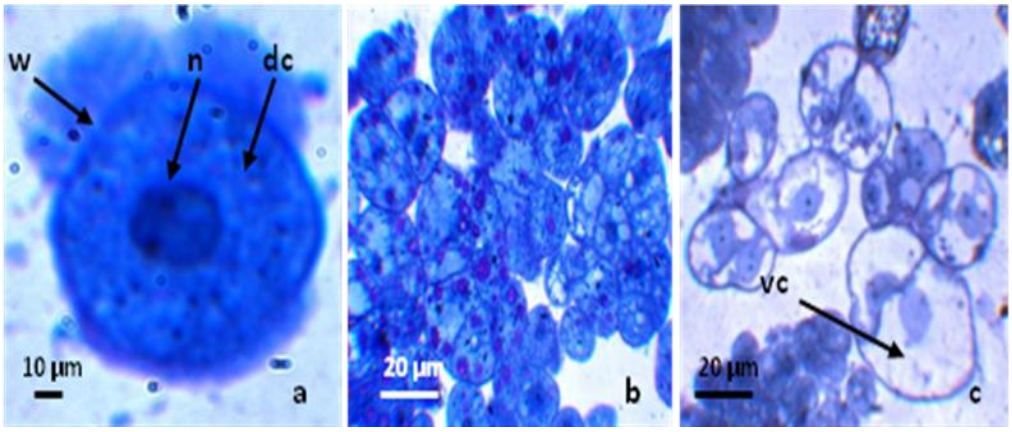


Figure 3.10: a) Histology of embryogenic cell suspension after sieving with 450 µm stainless steel filter. b) Histology of embryogenic cell suspension at day 14. c) Histology of vacuolated cell suspension. Symbols; dc: dense cytoplasm; n: nucleus; vc: vacuolated cell; w: wall.

3.3.3.2 Histochemical studies of embryogenic cell suspension

Embryogenic characteristics of the cell suspension cultures were reconfirmed through histochemical staining as proposed by Gupta and Durzan, (1987). The used of acetocarmine stain was to detect glycoproteins, chromatins and DNA in cytochemical studies. Absorption of Evan's blue determined the non-viability of cells where less viable cells and nuclei allowed cells to absorb more dye. This study revealed that embryogenic cell suspension cultures of *B. rotunda* showed absorption of acetocarmine and were stained intensely red (Figure 3.11) while NE cells were stained blue *via* Evan's blue. Similar observation was reported in NE callus where the nuclei were

difficult to stain using the same procedure (Gupta and Durzan, 1987). The result from double staining was found to be consistent with histological evidence where cell suspensions were expected to have dense cytoplasm with prominent nuclei and rich in protein (Figure 3.10). The same observations on embryogenic cells were made by other researchers (Côte *et al*., 1996; Khalil *et al*., 2002; Jalil *et al*., 2008).

The observation from histology and histochemical study used to validate the morphological findings in order to determine the embryogenic characteristics of the cells. Subsequently, the embryogenic and NE cell suspensions of *B. rotunda* could be differentiated. The NE cells were discarded and only embryogenic cells were used in further studies.

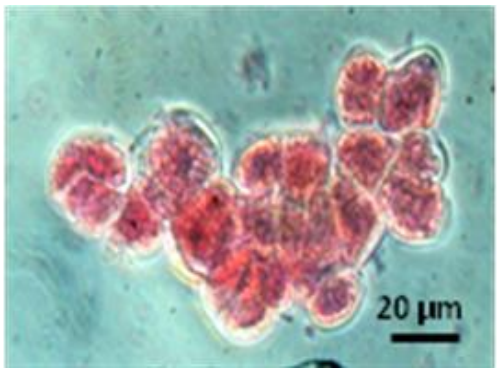


Figure 3.11: Cell clusters from suspension culture stained red showed embryogenic characteristic by double-staining method.

3.3.3.3 Growth curve of cell suspension cultures

Study of the biomass growth based on settled cell volume (SCV) had been carried out on the cell suspension cultures where the cell volume was seen to increase and the volume doubled every three days until day 15 (19 folds over initial SCV). It was found that the cell suspension showed a sigmoidal growth pattern by achieving maximum growth in 24 days (21 folds over initial SCV). From day 18 to 24, the cells started to

enter stationary phase. Decrease of cell growth was observed after 24 days of culture (Figure 3.12).

The sigmoid growth trend obtained in this study is peculiar to plant culture in batch mode cultivation such as for *Morinda elliptica* cell cultures (Abdullah *et al*., 1998). This characteristic is a result of low saturation constant relative to initial sugar level in the medium which results in the majority of growth taking place during exponential phase. The high cell density at the end of exponential phase rapidly depleted the remaining carbon substrate, bringing the specific growth to zero (Omar, 2003).

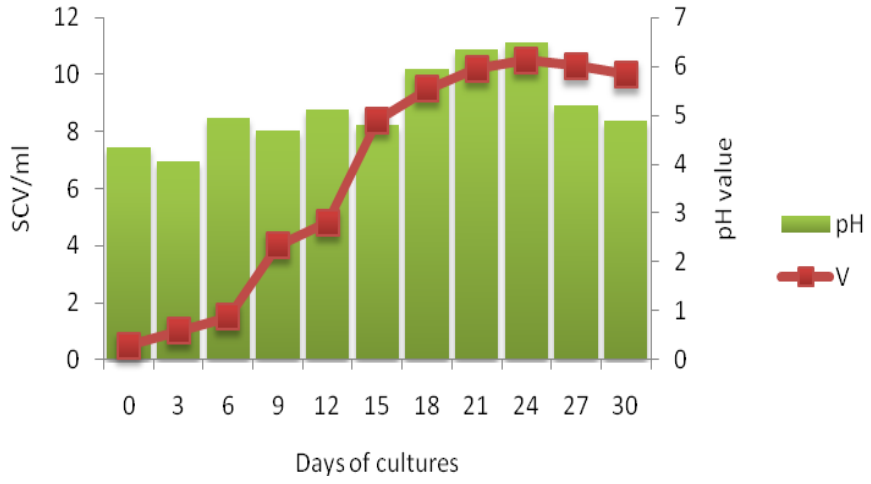


Figure 3.12: Growth of *B. rotunda* cell suspension culture. Symbol; v=volume.

Growth of cell suspension and pH exhibited an interesting trend. Lag phase for cell suspension cultures of *B. rotunda* occurred in the first 3 days of culture. Following autoclaving, pH of the culture medium decreased from 5.80 to 4.05, resulted in gradual increased in SCV. During this stage, cell suspension cultures adapted to the environment and nutrients in their new culture media, allowed only competent cells to survive. Non competent cells will be necrotic and produced phenolics. As a result the pH will drop.

Survival competent cell suspension cultures will divide actively and produce less phenolic exudates leading to increase in pH value. When the pH rose to 4.95 from 4.05 between 6 to 15 days, exponential increase in SCV was observed. More efficient uptake of nutrients took place during this log phase encouraged the vigorously dividing cells. The cultures start multiplying exponentially, doubling in number every 3 days (Figure 3.12). For *B. rotunda* cell suspension cultures, this state remained for 9 days. This period was restricted duration caused by depletion of some limiting resource such as nutrient and space.

As more and more cells were competing for depleting nutrients, active growth gradually ceased. During the stationary phase, the cells were becoming necrotic and the pH increased from 4.95 to 6.5 (Figure 3.12). Initially, this stage showed browning of cells which turned black through prolonged culture. In this experiment, pH increased from 4.05 at the start of the experiment to about 6.5 when the culture had reached 30 days (Figure 3.12). In all the cases, the pH had a dramatic effect on the biomass of *B. rotunda* cells that had become necrotic after 21 days of culture.

In this study, the growth pattern was similar to previous reports on growth curve of suspension cultures which revealed slow growth in first 3 days (lag phase) following a boosted growth started on day 6 and extensive accumulation of biomass over a period of 15 days (log phase). Subsequently about 5-6 fold over initial culture was accumulated on day 21 which was maximum biomass (Nakano *et al*., 2000, Mudalige and Longstreth, 2006, Pathirana and Eason, 2006). Likewise, a fivefold increase in cell cultures of *Bouteloua gracillis* was reported by Aguado-Santacruz *et al*. (2001). However, Pathirana and Eason, (2006) showed the dry mass of cells peaked at day 7 and then declined.

CHAPTER 4

PROFILES OF SELECTED FLAVONOIDS IN *BOESENBERGIA ROTUNDA* RHIZOMES AND *IN VITRO* CULTURES

4.1 INTRODUCTION

4.1.1 Flavonoids

Flavonoid compounds, derived from either 2-phenylbenzopyrone or 3-phenylbenzopyrone, can be categorize into few groups such as chalcones, flavanones, flavones, flavonols, anthocyanidins (flavylium cations), flavan 3-ols (catechins), flavan 3,4-diols (proanthocyanidins), biflavonoids and oligomeric flavonoids, isoflavonoids, and the aurones.

More than 1300 different flavonoid compounds have been isolated from plants. Individual flavonoids in a group differ from each other by the number and position of the hydroxy, methoxy, and sugar substituents. As a rule, flavonoid compounds occur in plants as glycosides, with hexoses such as glucose, galactose, and rhamnose, and pentoses such as arabinose and xylose as the most commonly found sugars. The sugars can be attached singly or in combination with each other.

Flavonoids are secondary metabolites, meaning they are organic compounds that have no direct involvement with the growth or development of plants. Flavonoid is widely distributed throughout plants and contributes to the vibrant colors of flowers and fruits of many plants. They also play a role in protecting and attracting pollinators to the plants. More importantly, the consumption of foods containing flavonoids has been linked to numerous health benefits.

The objective of this chapter is to study the distribution of 5 flavonoids in rhizomes, callus and cell suspension cultures.

4.1.2 Bioactive compounds in *Boesenbergia rotunda*

Boesenbergia rotunda had been reported to produce a range of chalcones (Trakoontivakorn *et al*. 2001), flavonoids (Jaipetch *et al*. 1982) and flavones (Jaipetch *et al*. 1983). Pinostrobin (5-hydroxy-7-methoxy-flavanone) and alpinetin (5-methoxy-7-hydroxyflavanone) were extracted from a diethyl ether extract of dried rhizomes by Mongkolsuk and Dean, (1964). Jaipetch *et al*. (1982) successfully retrieved 4 other compounds from *B. rotunda* black rhizome extract: pinocembrin (5,7-dihydroxy-flavanone), cardamonin (2',4'-dihydroxy-6'-methoxychalcone), 2',6'-dihyroxy-4'-methoxychalcone, and a new chalcone boesenbergin A (±)-(E)-1-{7'-hydroxy-5'-methoxy-2'-methyl-2'-(4"-methylpent-3"-enyl)-2'H-chromen-8'-yl]-3-phenylprop-2-enone. A year later, Jaipetch *et al*., (1983) reported to have isolated 9 flavones such as 5-hydroxy-7-methoxyflavone, 5-hydroxy-7,4'-dimethoxyflavone, 5,7-dimethoxyflavone, 5,7,4'-trimethoxyflavone, 5,7,3',4'-tetramethoxyflavone, 5-hydroxy-3,7-diemthoxyflavone, 5-hydroxy-3,7,4'trimethoxyflavone, 3,5,7-trimethoxyflavone and 5-hydroxy-3,7,3',4'-tetramethoxyflavone from the black rhizome variety. Subsequently boesenbergia B (±)-(E)-1-{5'-hydroxy-7'-methoxy-2'-methyl-2'-(4"-methylpent-3"-enyl)-2'H-1-benzopyrano-6'-yl]-3-phenylprop-2-en-1-one was isolated by Mahidol *et al*., (1984). In 1984, Mahidol *et al*. (1984) and Tuntiwachwuttikul *et al*. (1984) also isolated a cyclohexenyl chalcone derivative (±)-panduratin A, (±)-2,6-dihyroxy-4-methoxyphenyl)-[3'-methyl-2'-(3"-methylbut-2"-enyl)-6'-phenylcyclohex-3'-enyl]-methanone. Another cyclohexenyl chalcone derivative, (±)-4-hydroxypanduratin A, (±)-2,4,6-trihyroxyphenyl)-[3'-methyl-2'-(3"-methylbut-2"-enyl)-6'-phenylcyclohex-3'-enyl]-methanone was also reported (Trakoontivakorn *et al*.,

2001; Tuchina *et al.*, 2001). In the same report, Tuchinda *et al.* (2001) also claimed to have isolated sakuranetin (5,4'-dihydroxy-7-methoxyflavanone) and dihydro-5,6-dehydrokawain.

4.1.3 High pressure liquid chromatography (HPLC)

High performance liquid chromatography is basically a highly improved form of column chromatography. Instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures of up to 400 atmospheres.

HPLC has various applications such as chemical separations, purification, identification and quantification. Preparative HPLC refers to the process of isolation and purification. Analytical HPLC focuses on obtaining information on identification, quantification and resolution of the samples.

In this study, HPLC is mainly used for quantification of the bioactive compounds. The process of determining the unknown concentration of a compound in a solution is called quantification HPLC.

4.2 MATERIALS AND METHODS

4.2.1 HPLC instrument setup and solvent gradient configuration

The Waters HPLC system consisted of a W600E multisolvent delivery system, W2489 UV/visible detector, W2707 auto sampler and in-line de-gasser. The system was controlled by Empower 2 software. The guard and reverse columns (Chromolith RP-18 encapped, 100-4.6 mm) used were from Merck. HPLC grade methanol (Merck Ltd.), HPLC grade acetonitrile (Fischer Ltd.) and HPLC grade phosphoric acid (BDH Ltd.)

were used. Water used for preparation of the phosphoric acid solution for elution was purified using Millipore filter. The injection volume was 20 µL and the eluate was observed at 285 nm and 330 nm. The solvents used for elution were 0.1% (v/v) phosphoric acid (A) and acetonitrile (B). Table 4.1 summarized the solvent gradient employed and each run was 18 minutes. The configuration described in table 4.1 was found to be the best for separation of 5 *B. rotunda* flavonoids after many attempts with different combinations and configurations. The guard column and column were flushed with pure HLPC grade acetonitrile before and after use.

Table 4.1: HPLC solvent gradient arrangement

Step	Time	Flow	%C	%D	Curve
1	0.01	1.50	80.0	20.0	6
2	0.50	1.50	80.0	20.0	6
3	1.00	1.50	65.0	35.0	6
4	5.00	1.50	65.0	35.0	6
5	5.50	1.50	40.0	60.0	6
6	10.00	1.50	40.0	60.0	6
7	10.50	1.50	0.0	100.0	6
8	11.00	1.50	0.0	100.0	6

4.2.2 Plant material

Four different sources of plant materials were used in this study i.e. rhizomes, callus, cell suspension cultures and liquid medium from cell suspension cultures. The rhizomes were from intact plant while the rest were from *in vitro* cultures. All materials used were from the same source as those described in Chapter 3.

4.2.3 Establishment of standard calibration of compounds using HPLC

Four pure flavonoid compounds obtained from the Universiti Teknologi Malaysia (UTM) Skudai, Johor and Panduratin A obtained from Plant Biotechnology Research Laboratory (Mr. Foo Gen Teck), UM were used as standards. The retention time of the

five compounds was obtained from individual HPLC chromatograms of each compound. The solvent used to prepare all solutions was methanol. Standard calibration comprising the 5 *B. rotunda* compounds were developed at λ = 285 nm and 330 nm. A methanolic stock solution comprising all the five standards was diluted as shown in Table 4.2. From the HPLC chromatograms, the peak area of each dilution for each standard compound was determined and a standard calibration was obtained for every compound.

Table 4.2: Compounds and concentrations used for standard solution

Components	**Concentration (mg/ml) (Stock)**	**Value diluted (150X)**	**Value diluted (300X)**	**Value diluted (600X)**	**Value diluted (1200X)**	**Value diluted (4800X)**	**Value diluted (9600X)**	**Value diluted (38400X)**
Alpinetin	100.0	0.6667	0.3333	0.1667	0.0833	0.0208	0.0104	0.0026
Pinocembrin	140.0	0.9333	0.4667	0.2333	0.1167	0.0292	0.0146	0.0036
Cardamonin	100.0	0.6667	0.3333	0.1667	0.0833	0.0208	0.0104	0.0026
Pinostrobin	60.0	0.4000	0.2000	0.1000	0.0500	0.0125	0.0063	0.0016
Panduratin A	130.0	0.8667	0.4333	0.2167	0.1083	0.0271	0.0135	0.0034

4.2.4 Preparation of extract for analysis of flavonoid content

4.2.4.1 Rhizome

B. rotunda rhizomes were cleaned, sliced and oven dried at 38 °C. The oven-dried samples were pulverized to powder and then sieved. A weighed amount of the powder was soaked in methanol for 3 days and the filtered solution was evaporated using rotary evaporator (BÜCHI Rotavapor R-114). The slurry residue was then partitioned against equal volume of ethyl acetate and water, and the ethyl acetate fraction was again evaporated. The mass of the partitioned ethyl acetate extract was recorded and re-dissolved in methanol at ratio of 1 mg of extract to 200 µL methanol. This methanolic

solution of the extract was filtered through 0.45 µm PTFE filter (Sartorius 13 CR) prior to HPLC injection.

4.2.4.2 *In vitro* cultures

Four weeks old harvested calluses and two weeks old cell suspension cultures were oven-dried for 48 hours at 38 °C until constant dry weight was achieved prior to grinding using mortar and pestle. One grams of powder was extracted according to section 4.2.4.1 and contents of flavonoids were analyzed using HPLC.

Liquid medium from cell suspension cultures were filtered using Whatman no 1 and 30 ml of medium were used for extraction and flavonoids content were analyzed according to section 4.2.4.1.

Each analysis in 4.2.4 was repeated three times. Figure 4.1 summarizes the preparation of extract for HPLC analysis.

4.2.4.3 Statistical analysis

Results were subjected to analysis of variance (ANOVA) and central tendency analysis. Differences were considered statistically significant at a level of $P < 0.05$.

Sample preparation for HPLC analysis

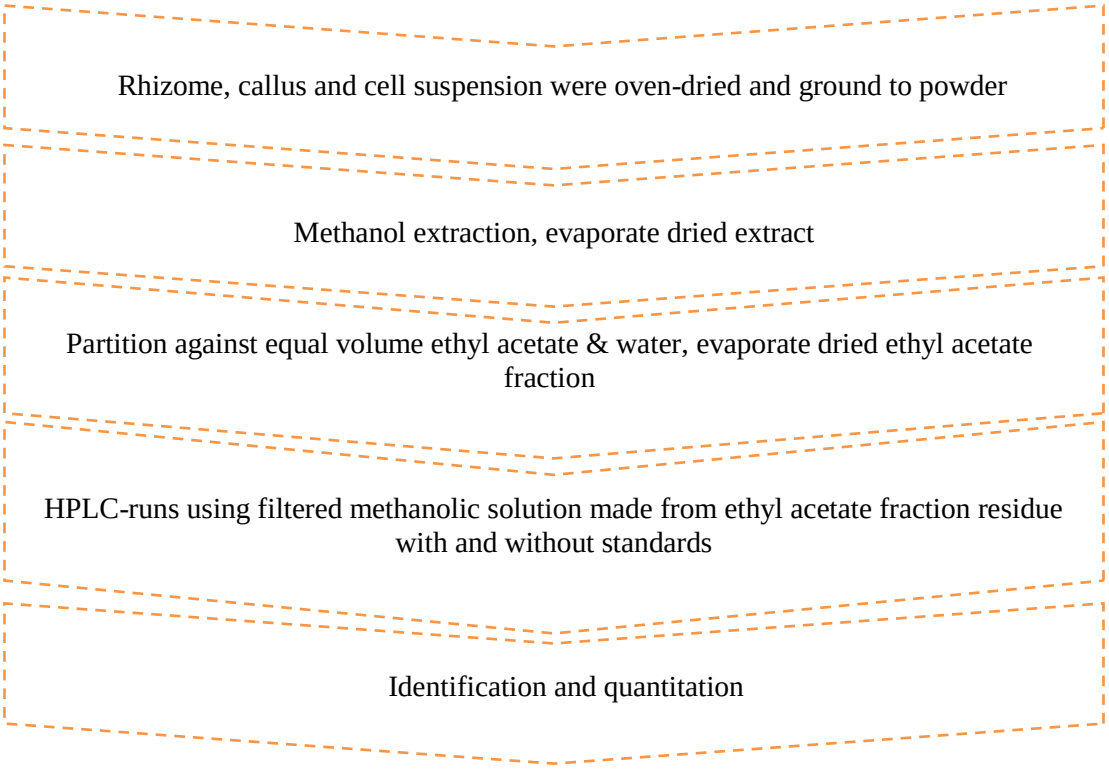


Figure 4.1: Summary of sample preparation for HPLC quantitative determination

4.2.5 Identification of *Boesenbergia rotunda* compounds

The *B. rotunda* compounds in the extract were identified by matching their retention times and spectral characteristics to those of the known standards. When there was uncertainty, the extract was spiked with a mixture of standards. The retention times, spectral characteristics and intensities of the peaks of the resulting chromatograms with and without spiking were compared, and the compounds were identified. The concentrations of compounds were calculated as follows:

$$\text{Flavonoids } (\mu g/g) = \frac{\text{Peak area of samples}}{\text{Peak area of Standard area}} \times \text{Standard concentration } (\mu g/ml) \times \frac{\text{Extract volume (ml)}}{\text{Dry weight (g)}}$$

4.3 RESULTS AND DISCUSSION

4.3.1 Establishment of standard calibration

Preliminary work which involved finding a solvent system and elution gradient that could elute all 5 *B. rotunda* compounds was carried out to optimize resolution and accomplish good peak profiles. Adjustments to concentration for every standard compound were also made to improve resolution results.

Standard curves comprising of five *B. rotunda* compounds were developed at λ = 285 nm and 330 nm. These two wavelengths were chosen because earlier studies indicated that all the flavanones and cyclohexenyl chalcone derivatives obtained from this study showed maximum absorption at wavelength 285 nm but less at λ = 330 nm, whilst maximum absorption of all the chalcones was at 330 nm (Tan, 2005). In addition, most phenolics were least absorbed at wavelength ranging from 285 to 360 nm.

Similar to previous report, initial trials illustrated that pinostrobin, pinocembrin, alpinetin and panduratin A could be detected at λ = 285 nm but their signals were less strong at λ =330 nm. Conversely cardamonin gave strong signals at λ = 330 nm and contrary at λ = 285 nm. This necessitates the use of two wavelengths to detect these compounds' signals. The overlay chromatograms for both wavelengths of mixture standard are shown in Figure 4.2.

HPLC analysis showed that the retention times of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A were at 6.102, 9.066, 9.885, 10.578 and 14.330 minutes, respectively (Figure 4.2 and Table 4.3).

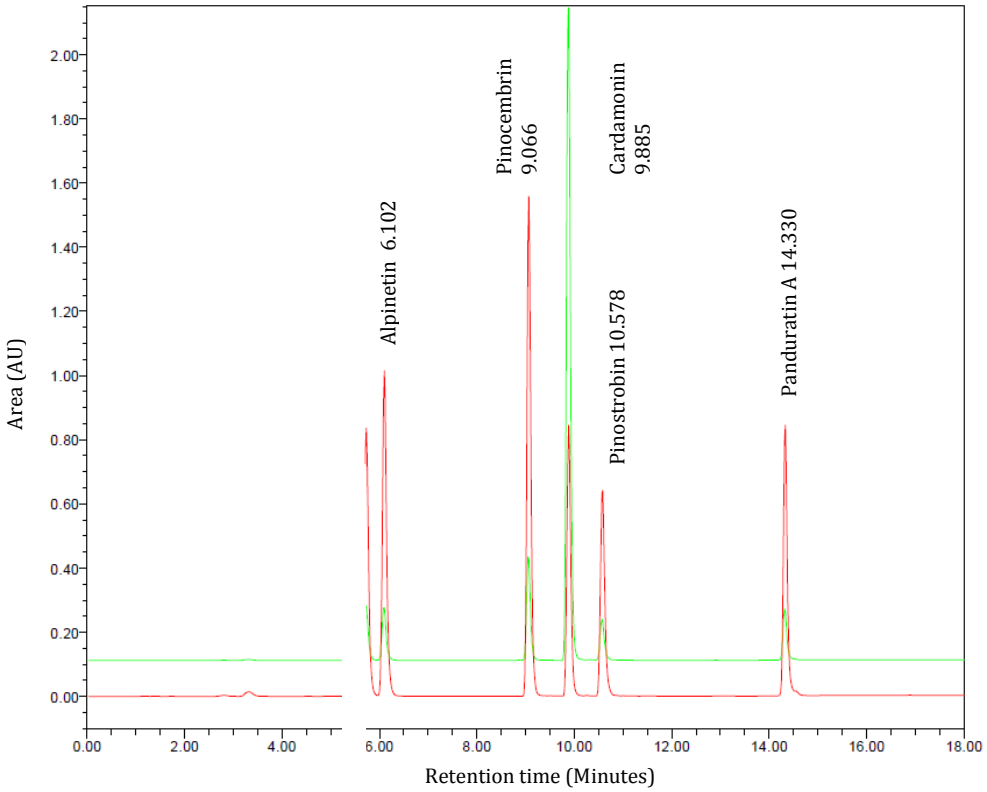


Figure 4.2: Overlay of HPLC chromatogram for *B. rotunda* flavonoids mixture standards detected at λ = 285 (———) and 330 nm (———) showed only cardamonin was absorbed strongly at 330 nm.

Table 4.3: Retention time, concentration and amount of standard mixture used in this study

Compound	Retention time (RT)/min	Concentration µg/ml
Alpinetin	6.102	100.0
Pinocembrin	9.066	140.0
Cardamonin	9.885	100.0
Pinostrobin	10.578	60.0
Panduratin A	14.330	130.0

All the flavonoid compounds in standard solution were identified by matching their retention times and spectral characteristics to individual run of each of the five BR compounds. With the HPLC data collected, individual standard calibrations were drawn for all compounds (Figure 4.3-4.7).

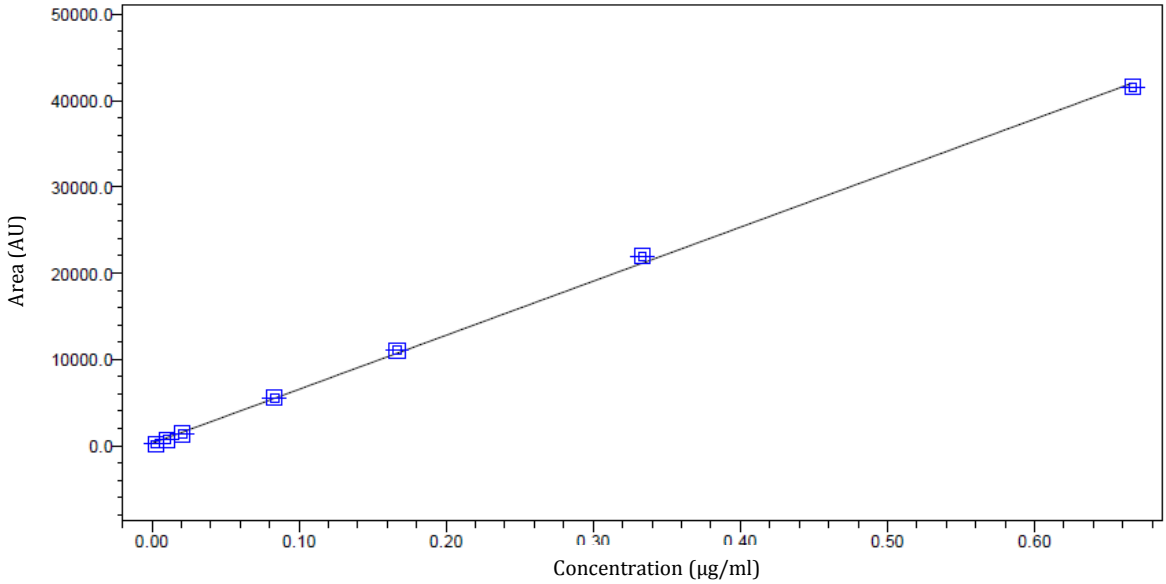


Figure 4.3: Alpinetin standard calibrations. RT 6.102, equation, Y=62600X + 272. Intercept, 0.012957, R^2 = 0.999164.

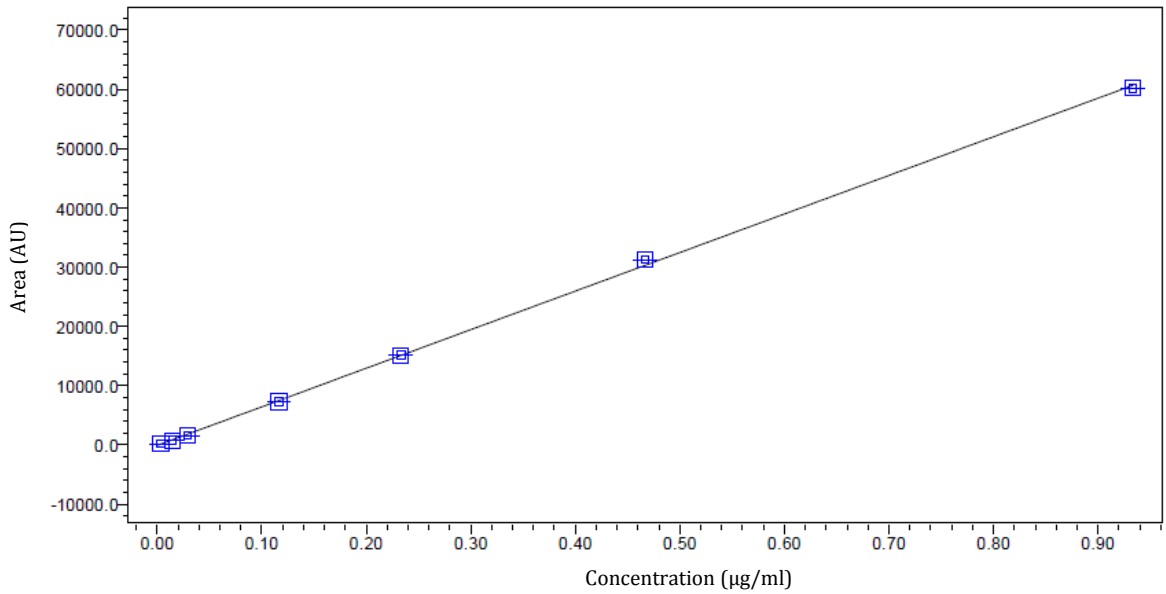


Figure 4.4: Pinocembrin standard calibrations. RT 9.066, equation, Y= 61500X + 69.3. Intercept, 0.002275, R^2 = 0.999565.

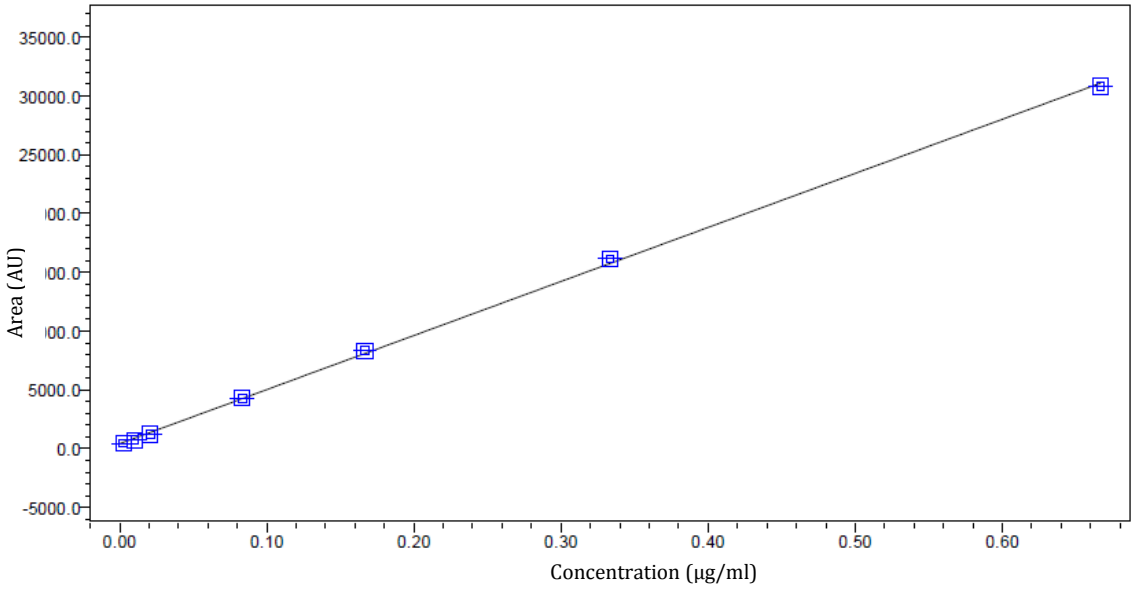


Figure 4.5: Cardamonin standard calibrations. RT 9.886, equation, Y=46000X + 425. Intercept, 0.027589, R^2 = 0.999537.

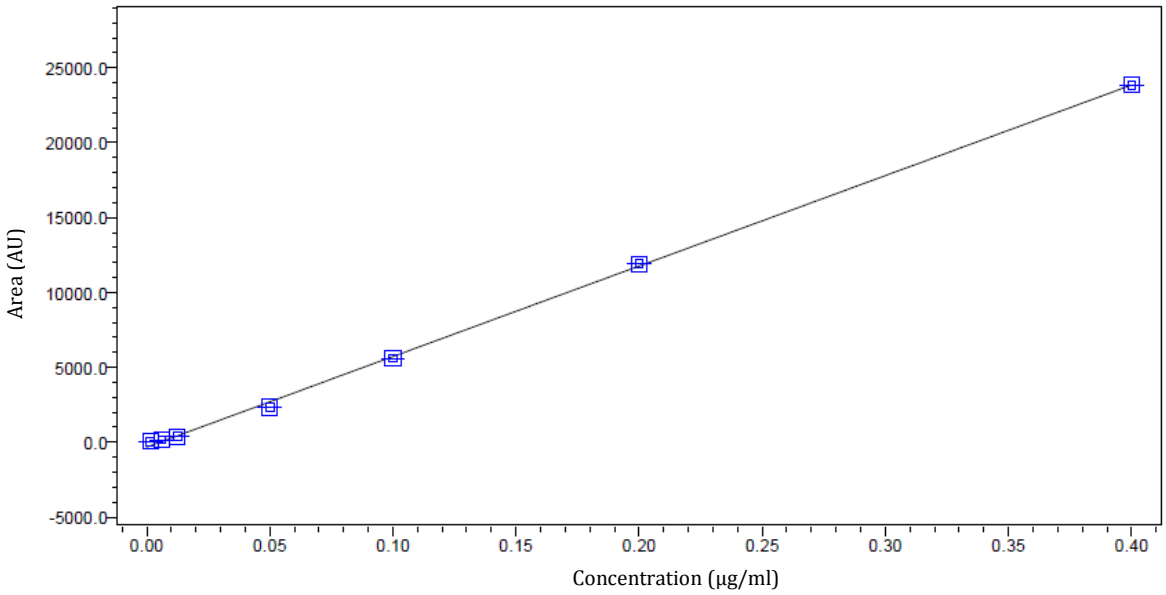


Figure 4.6: Pinostrobin standard calibrations. Equation, Y=60400X - 302000, intercept, 0.026393, R^2 = 0.999466.

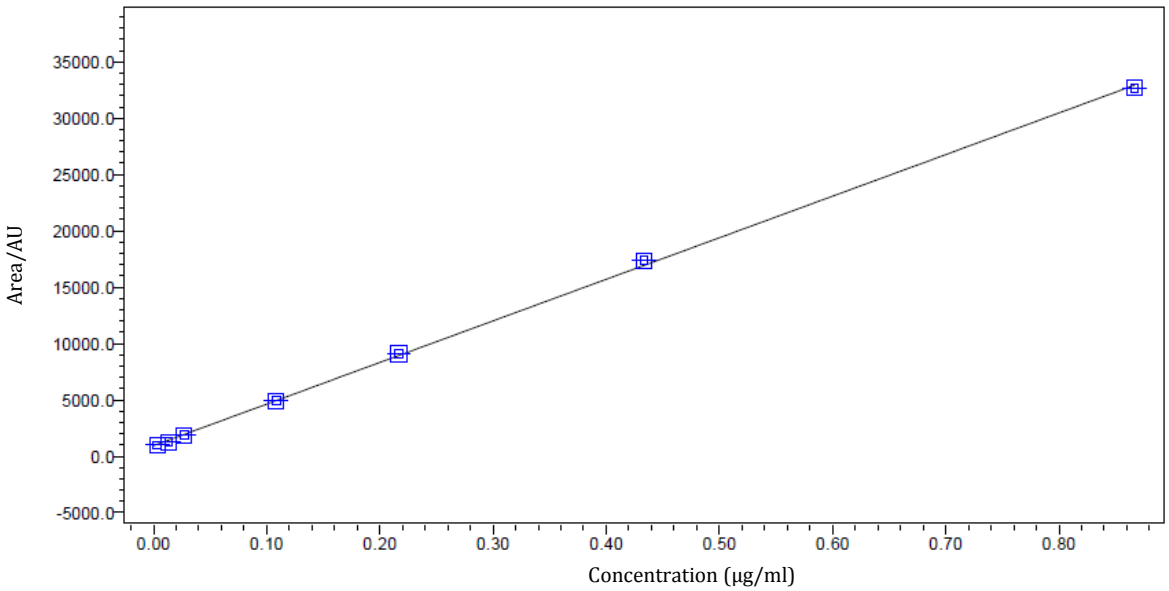


Figure 4.7: Panduratin A standard calibrations. RT, 14.330, Equation, Y=36900X + 937. Intercept, 0.058350, R^2 = 0.999596.

4.3.2 Flavonoid profile and quantitation in rhizomes and *in vitro* cultures of *Boesenbergia rotunda*

4.3.2.1 Flavonoids production in rhizome

Five flavonoids formed major peaks as indicated in Figure 4.8. Identification was based on retention time, peak characteristics and overlay of HPLC chromatogram. The HPLC chromatograms of rhizome extract with and without spiking and overlay with the standard chromatography to identify and confirm the individual flavonoid peaks in the sample are shown in Figure 4.8 to 4.10. Similarly the identification of flavonoid compounds in the other samples was confirmed using the respective identification stages.

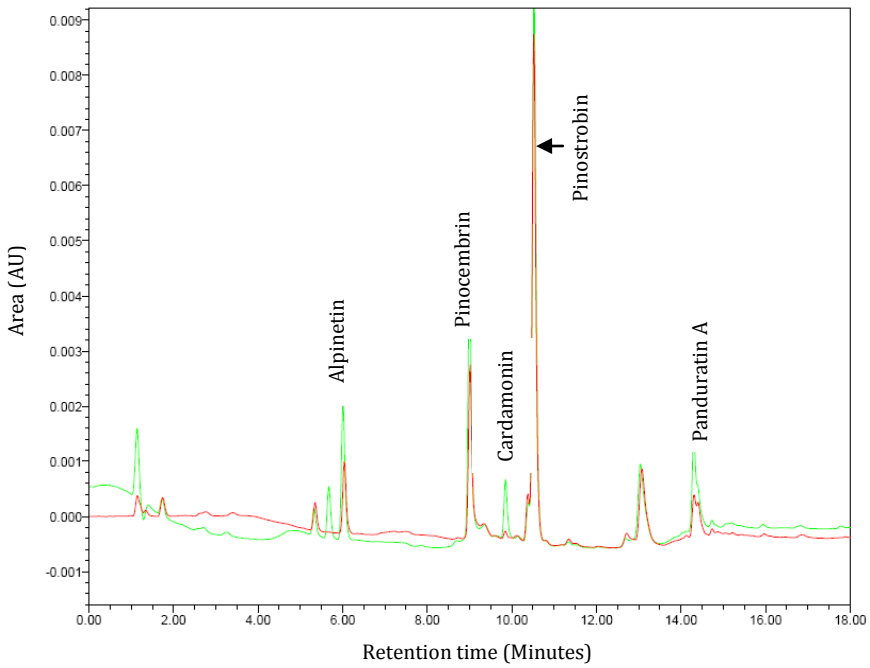


Figure 4.8: HPLC chromatogram of rhizome extract with and without spiked with standard mixture. Symbols; ——— (Rhizome extract) ——— (Rhizome extract spike with standard mixture).

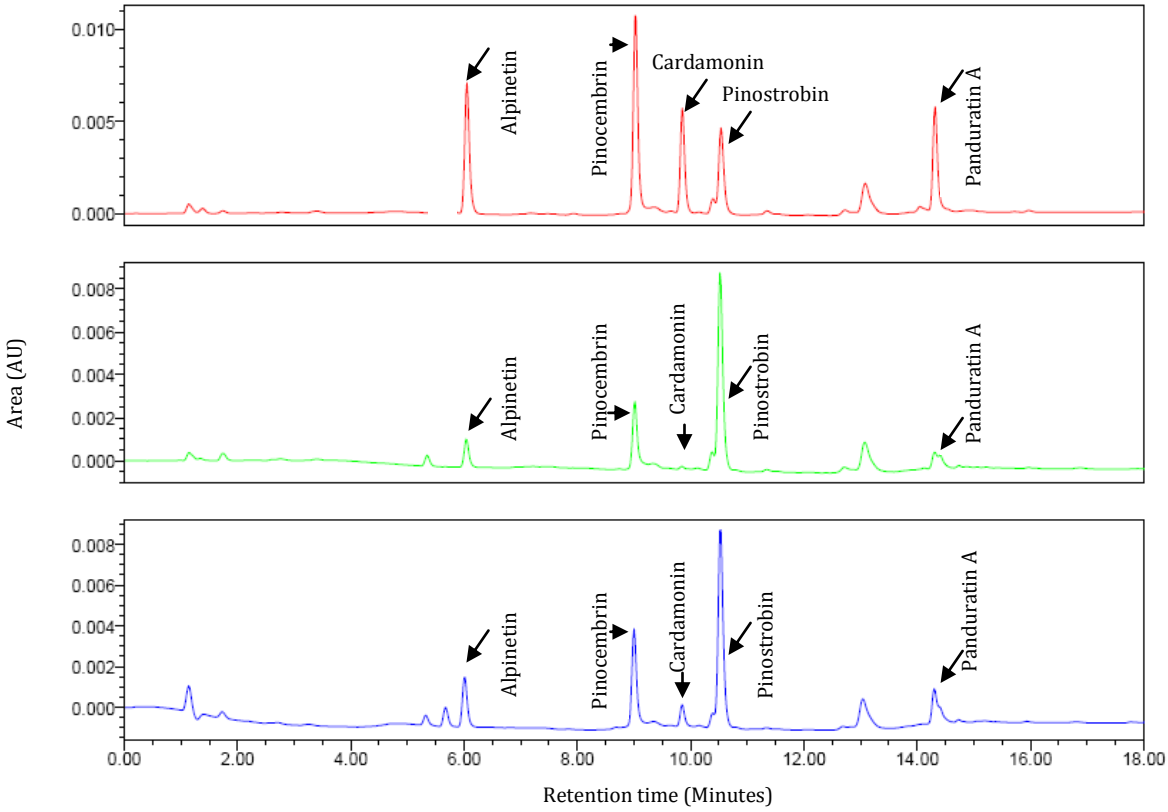


Figure 4.9: HPLC chromatogram showed same migration time for rhizome extract, extract spike with standard mixture solution and standard mixture in order to identify the peaks of flavonoids. Symbols; ——— (Standard mixture) ——— (Rhizome extract) ——— (Rhizome extract spiked with standard mixture).

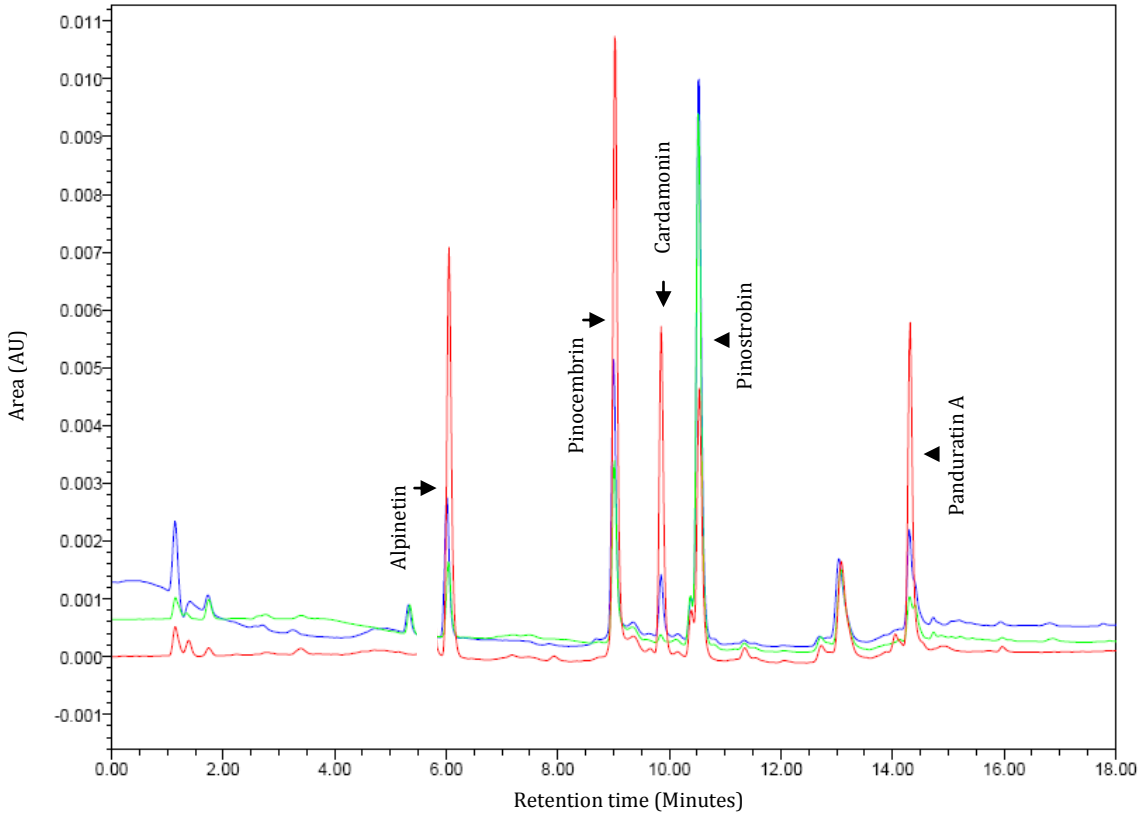


Figure 4.10: HPLC chromatograms showed same migration time for rhizome extract, extract spiked with standard mixture and standard mixture in order to identify the flavonoids peaks. Symbols; ——— (Rhizome extract) ——— (Rhizome extract spiked with standard mixture) ——— (Standard mixture)

Quantification using HPLC showed that all five flavonoids, alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A were present in rhizome extract of *B. rotunda* (Figure 4.11). However, the amount of each compound (dry weight) detected differed from one metabolite to another. The yields obtained in rhizome extract were 1139.24 ± 6.2, 2613.78 ± 14.24, 57.20 ± 0.03, 8220.73 ± 45.66 and 944.58 ± 5.31 µg/g dry weight of alpinetin, pinocembrin, cardamonin, pinostrobin and Panduratin A respectively.

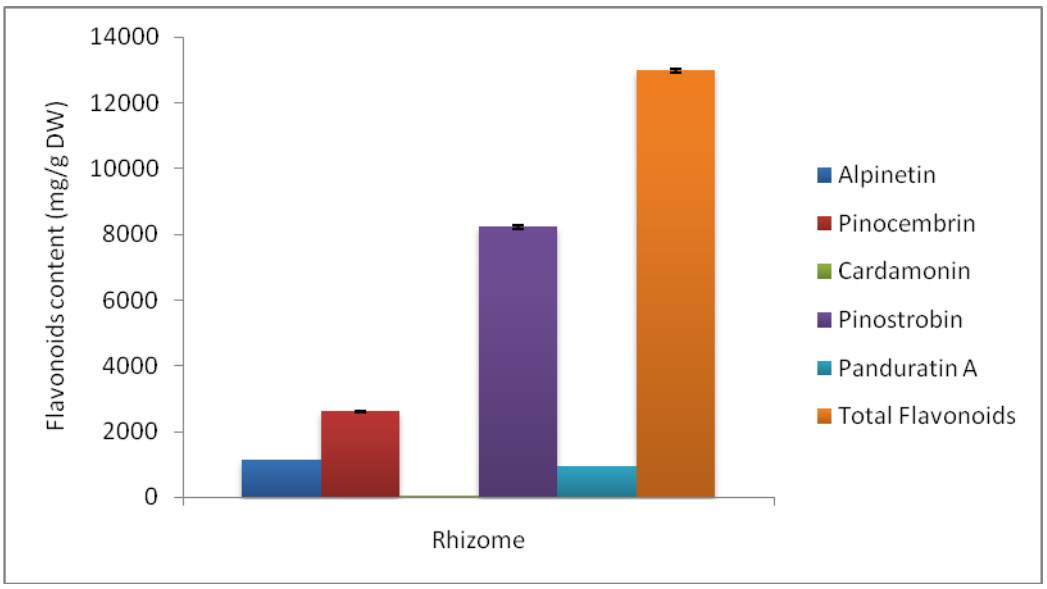


Figure 4.11: Concentrations of 5 *B. rotunda* compounds and total flavonoid compound in rhizome extract. Bar indicates the standard deviation of mean (*n*=3).

Chemical studies of the rhizome of *B. rotunda* have resulted in the isolation and identification of several chalcones such as boesenbergin A, boesenbergin B, cardamonin, panduratin A, dihydromethoxychalcone, and flavanones such as pinocembrin, pinostrobin, alpinetin and 5-hydroxy-7-methoxyflavanone (Jaipetch, 1982 and Mahidol, 1984; Tuchnida *et al*., 2002).

This study revealed that pinostrobin is the major compound in rhizome, followed by pinocembrin, alpinetin, panduratin A and finally cardamonin. Similar observation has been reported by Tuchnida *et al*. (2002) who isolated abundant of pinostrobin amount, followed by pinocembrin and finally panduratin A. Different compounds and amount were extracted in this study compared to previous reports may due to the plant material which grow in different location and using difference of solvent and extraction method. In this study, solvent and method were adopted according to Kiat *et al*. (2006) because of the identical plant material.

Pinostrobin which was found in abundance in the rhizomes is a natural flavanone with important anti-helicobacter pylori as well as anti-tumor activity (Le Bail *et al*., 2000; Bhamarapravati *et al*., 2006). Pinostrobin may also mediate anti-inflammatory effects by inhibition of cycloxygenases (Wu *et al*. 2002).

Besides *B. rotunda*, pinostrobin has also been purified from the leaves of *Cajanus cajan* (Wu *et al*. 2009), buds of *Populus balsamifiera* L. (Yamovoi, 2001) and shoots of *Alnus firma* (Yoshinori, 1970).

4.3.2.2 Profile in callus culture

HPLC quantification showed that all 5 flavonoid compounds were present in callus culture of *B. rotunda* (Figure 4.12). The yield of the compounds in callus cultures were 4.50 ± 0.00, 17.76 ± 0.02, 1.63 ± 0.00, 96.30 ± 0.00 and 0.42 ± 0.01 µg/g dry weight of alpinetin, pinocembrin, cardamonin, pinostrobin and Panduratin A respectively. Successful production of secondary metabolite has also been reported in callus cultures of *Hypericum rumeliacum* (Pasqua *et al*., 2003), *mentha longifolia* (Lucchesini *et al*., 2009), *Cissus sicyoides* (Danova *et al*., 2010), *Echinacea angustifolia,* (Rodrigues and Almeida, 2010), *Hypericum perforatum* (Shekhawat and Shekhawat, 2010), *Arnebia hispidissima* (Krzyzanowska *et al*., 2011) and *Centella asiatica* (Bonfill *et al*., 2011). Results obtained in this study revealed that callus cultures of *B. rotunda* contained high amount of pinostrobin followed by pinocembrin, alpinetin, cardamonin and panduratin A after four weeks in culture.

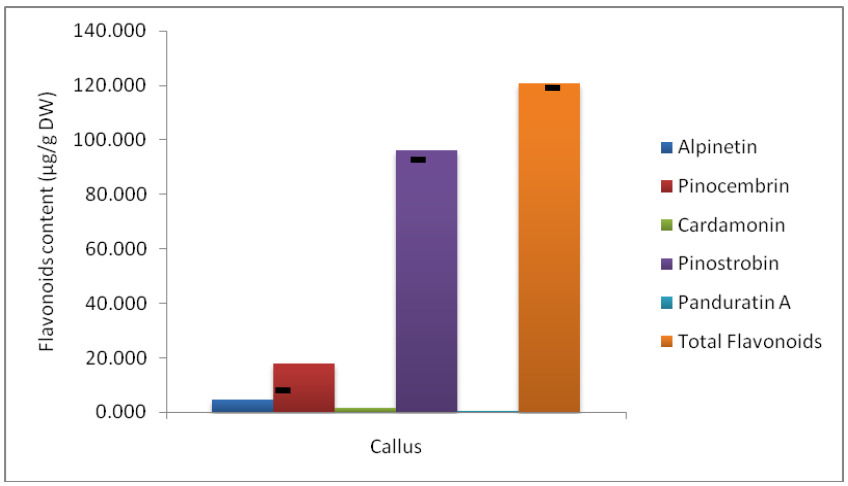


Figure 4.12: Concentrations of 5 *B. rotunda* compounds and total flavonoid compounds in callus extract.

4.3.2.3 Flavonoids production in cell suspension culture and its medium.

Studies were also carried out to determine the content of flavonoid compounds in cell suspension cultures and its liquid medium. Unlike in the cell suspension cultures of *Digitalis lanata*, which is unable to synthesize cardenolides, either in dark or light condition (Haussmann *et al.*, 1997), the cell suspension culture and liquid medium of *B. rotunda* produced all 5 flavonoid compounds. The yields obtained in cell cultures were 0.30 ± 0.04, 0.56 ± 0.08, 0.10 ± 0.01, 2.36 ± 0.34 µg/g dry weight of alpinetin, pinocembrin, cardamonin, pinostrobin respectively. Based on the chromatogram, the yield of the 5 compounds in liquid medium were lower than cell suspension cultures where 0.13 ± 0.10, 0.31 ± 0.20, 0.31 ± 0.01 and 0.90 ± 0.47 µg/l medium of the name your compounds respectively were produced. Panduratin A was found in both cell suspensions and medium extracts which were found in trace amounts and were unable to be quantified by HPLC (Figure 4.13).

In contrast, terpenoid compound namely limonene were found only in the media of celery (*Apium graveolens*), (Watts *et al.*, 1984) and essential oils of *M. piperita* (Kim *et al.*, 1996).

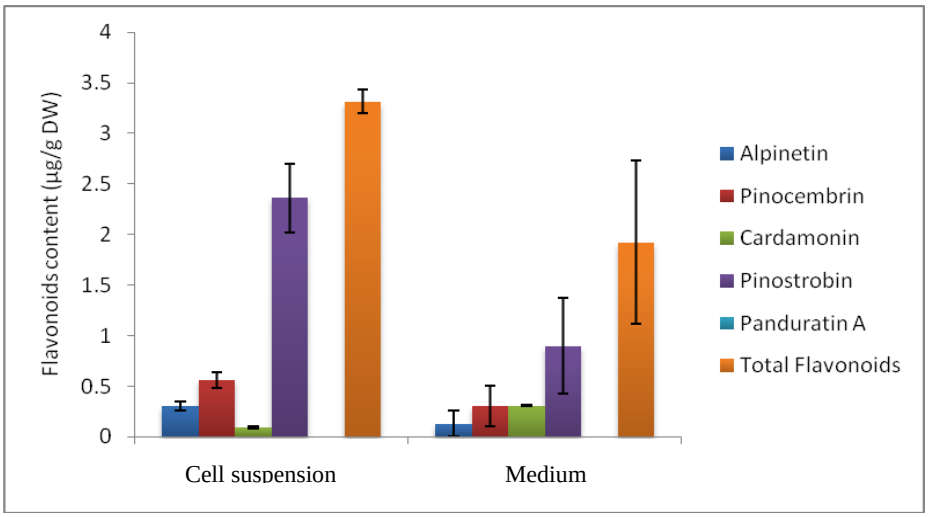


Figure 4.13: Concentrations of 5 *B. rotunda* compounds and total flavonoid compounds in cell suspension and medium extract. Bar indicates the standard deviation of mean (*n*=3).

4.3.2.3 Comparison between flavonoids compound profile in rhizome and in *in vitro* cultures

The comparison between the flavonoid content produced in rhizome of mother plants and in *in vitro* cultures of *B. rotunda* was made in Table 4.4.

Table 4.4: Comparison between flavonoids content in rhizome of mother plants and in *in vitro* cultures

	Amount of flavonoids from different sources			
Compounds*	**Rhizome (µg/g DW)**	**Callus (µg/g DW)**	**Cell (µg/g DW)**	**Medium (µg/l)**
Alpinetin	1139.24 ± 6.27	4.50 ± 0.00	0.30 ± 0.04	0.13 ± 0.12
Pinocembrin	2613.77 ± 14.24	17.76 ± 0.02	0.56 ± 0.08	0.31 ± 0.20
Cardamonin	57.20 ± 0.30	1.63 ± 0.00	0.10 ± 0.01	0.31 ± 0.01
Pinostrobin	8220.72 ± 45.66	96.30 ± 0.00	2.359 ± 0.34	0.90 ± 0.5
Panduratin A	944.58 ± 5.31	0.421 ± 0.00	Trace	Trace
Total 5 Flavonoids	12975.52 ± 71.78	120.61 ± 0.01	3.32 ± 0.10	1.92 ± 0.81

* At p=0.05, significant differences were observed for each flavonoid contents from different sources.

Among the four different types of samples, rhizome was found to produce the highest amount of flavonoids. The total flavonoids decreased in the order of rhizome > callus > cell suspension and liquid medium. The most abundant flavonoid in rhizomes was pinostrobin, followed by pinocembrin, alpinetin, panduratin A and cardamonin. While callus accumulated the highest biomass of pinostrobin followed by pinocembrin, alpinetin, cardamonin and panduratin A. Compounds produced from both rhizomes of mother plants and callus cultures were similar although differed in the quantity.

In most secondary metabolites studies through *in vitro* system, the compound showed lower amount in the callus than in the mother plant. From the study, the total flavonoids produced in callus were almost 100 times lower than the intact rhizome. Similar to our results, secondary metabolite production were found low in callus cultures compared to the naturally grown sources in *Datura innoxia* and *Hypericum perforatum* L., (Santarém and Astarita, 2003; Zayed *et al*., 2006). Kumari *et al,* (2007) reported that secondary metabolites like inulin and esculin were found in low level when extracted from callus culture. This could be due to cells which were initiated from callus is undifferentiated entities (Kim *et al,* 1996; Pande *et al*, 2002; Hiraoka *et al*, 2004; Ma *et al*, 2006; Zayed *et al,* 2006).

Lack of tissues and organ differentiations in most calluses caused the lower yield of secondary metabolites (de Klerk, 2004). Low production of secondary metabolites could also be due to high hormone concentration (1 or 2 mg/l), which promotes non-productive callus since all the cells are mitotic rather than producing secondary metabolites (Rateb *et al*. 2007).

Contrast to the present study, alkaloids corybulbine, corydaline and cavidine which produced in the intact tubers, were absent in the callus of *Corydalisambigua* (Hiraoka *et*

al. 2004), while, azadirachtin was high in the callus cultures than the differentiated tissues of *Azadirachta indica* (Wewetzer, 1998). The production of antihypertensive alkaloid production was equal in the callus and leaf tissue of *Catharanthus roseus* (Namdeo *et al*. 2006).

The unexpected accumulation of secondary metabolite in the *in vitro* tissue culture system may be due of many factors such as PGR, the nutrient medium, the carbohydrate source employed and the cell line. Once the desired compound were detected in the systems, feasible approaches to enhance the product are possible.

The most abundant flavonoid in cell suspension cultures was pinostrobin, followed by pinocembrin, alpinetin and cardamonin. Paduratin A which was accumulated in rhizome was only in trace amount in cell suspension cultures. The study revealed that the cell culture of *B. rotunda* also managed to produced phytochemicals but at a much lower level than in intact rhizome similar to the study by Briskin *et al*., (2001) on the production of kavapyrones by *Piper methysticum* cells. This production was similar to cell suspension cultures of *Camptotheca acuminate*, which produced camtothecin and 10-hydroxycamptothecin 100-times lower than in the original plant material (Wiedenfeld *et al*., 1997).

The low secondary metabolite production in *B. rotunda* cell cultures could be due to undifferentiated stage used for the extraction study. Synthesis of secondary metabolites has been linked to the degree of differentiation of individual cells. The lack of single specialized cell, cell compartments and tissues or specialized part of organs that serve as the synthesis and storing sites for secondary metabolites (Endress, 1994).

Among the *in vitro* cultures, callus was found to produce the highest amount of flavonoids with pinostrobin was the most abundant while liquid medium from cell

suspension culture of *B. rotunda* produced the lowest flavonoid. Pinostrobin remain the highest compound in liquid medium.

In this study, total flavonoids obtained in cell suspension and liquid medium was 37 and 63 times lower than the callus cultures, respectively. Callus cultures are usually more productive than cell suspension of the same origin, cultivated on the same medium composition (Endress, 1994). Other than differentiation stages, static media may be could accumulate higher flavonoids than in liquid medium.

Possible secretion of flavonoids into the surrounding medium as well as the degradation of the product could be the contributing factors for low flavonoids yield in cell suspension cultures of *B. rotunda*. This has been shown in *Alpinia officinalis* culture where 29% of oleanolic acid glycosides were transported out of the leaf protoplasts through cell membrane (Omar, 2003). Secretion of terpenoids such as essential oil was reported by Evert (2006). It could possibly be due to cells lyses causing the flavonoid to be present in the liquid medium.

The concentration of flavonoids in the liquid medium was low and was not comparable to the amount found in the callus culture and rhizomes. In many cases plant cell suspension yield of secondary metabolites are quite low.

By working with cell suspension cultures system, reproducible abundance quantity of calluses in a rapid phase is possible. Moreover the accumulation of flavonoids could be produced in higher yields in the cells because the best way to propagate meristematics cells is through liquid culture. It is crucial to increase and accumulate maximum active biomass producing metabolite by cell suspension cultures in order to achieve the higher possible production of bioactive compound because compound production from undifferentiated cells is only feasible if have enough biomass. The balance between

rapid cell growth and high compound production is crucial. Additionally, cell suspension cultures system is preferable form for up scaling purposes where the best part of this system is the reproducibility of consistent and constant source of desired compounds.

Increasing industrial significance of plant secondary metabolites resulted in great interest towards enhancing the product via biotechnology approaches. *In vitro* plant cell cultures offer the possibility of obtaining desirable medicinal compounds. Different strategies using cell suspension cultures were extensively studied in the next chapter with the objective of improving the production of *B. rotunda*'s secondary metabolite production in the *in vitro* system.

CHAPTER 5

EFFECTS OF DIFFERENT CULTURE CONDITIONS AND MEDIUM VARIATIONS ON THE BIOMASS AND PRODUCTION OF SELECTED FLAVONOIDS IN *IN VITRO* CULTURES

5.1 INTRODUCTION

Numerous secondary metabolites which cannot be acquired by chemical processes could be potentially produced *via* plant cell cultures. Plant cell cultures have been manipulated for the production of secondary metabolites which have high value for food and pharmaceuticals industries (Makunga *et al*., 1997). Exploitation of the culture media components and conditions has been used to effectively boost the accumulation of secondary metabolites in various plant cell cultures. Nevertheless, it was frequently described that the initiated plant cell cultures accumulated lesser amounts of a desired compound in contrast to the intact plant (Trejo-Tapia *et al*., 2003).

For enhancement of biomass development and flavonoids production, rational modification of the medium composition is essential. Plant growth regulators are among of the culture conditions that regularly have controlling effects on the cell growth and compounds accumulation (Wu and Zhong, 1999). However, since culture medium consists of more than 20 components, it is not feasible to manipulate all the components. Hence, this study focused on the effects of several key components such as initial pH, initial inoculation volume, temperature, agitation, plant growth regulators and source of carbon supplied on the biomass and flavonoids production in cell suspension cultures of *B. rotunda.* The objective of this chapter is to study the effects of

various culture conditions and PGR on the biomass growth and accumulations of selected flavonoids in cell suspension cultures.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

Cell suspension cultures of *B. rotunda* was maintained in MS medium (pH 5.8) with 3% (w/v) sucrose. Establishment and maintenance of the cell suspension cultures were carried out according to section 3.2.3, Chapter 3. For each treatment, 0.5 ml SCV (approximately 0.1 g fresh weights (FW) of the cells from the stock cultures was added into 50 ml of fresh liquid medium and this was repeated in 9 replicates. Fourteen days old cells were harvested for the measurement of the biomass growth and determination of flavonoids content.

5.2.2 Culture conditions

5.2.2.1 MS strength

The effect of MS basal medium strength on growth and flavonoids production from *B. rotunda* cells was also examined. The strengths of MS basal medium were ¼, ½, normal and doubled the standard concentrations prepared at normal formulation.

5.2.2.2 Initial volume

Effects of initial inoculation volumes were observed at different volumes of inoculants (0.25, 0.5, 1.0 and 1.5 ml of SCV). The SCV of the cells were measured by letting the cells to settle for 20 minutes prior to inoculation in Falcon tubes.

5.2.2.3 Initial pH medium

Effects of initial pH values were examined within the range of 4.8 to 6.8. The pH of the medium was adjusted to 4.8, 5.3, 5.8, 6.3 and 6.8 using 0.1N sodium hydroxide (NaOH) and 0.1N hydrochloric acid (HCl).

5.2.2.4 Temperature

Effects of temperature on biomass and flavonoids production were investigated at 15, 20, 25 and 30 °C. Temperatures were programmed by using incubator shaker (Zhicheng, China).

5.2.2.5 Agitation

Effects of agitation on biomass and flavonoids accumulation were investigated at 80, 100 and 120 revolutions per minute (rpm). The speed is automatically controlled by the orbital shaker (New Brunswick Scientific, USA).

5.2.2.6 Plant growth regulators (PGRs)

Two types of auxins, NAA and 2,4-D along with cytokinins, BAP and kinetin, all acquired from Sigma (Sigma Chemical Co. USA) were used in the range of 1.0 to 5.0 mg/l for all media tested. The most responsive auxin and cytokinin combinations were further tested for optimal concentrations. Medium without PGR served as control.

5.2.2.7 Carbon sources and concentrations

Glucose, fructose, mannitol, sorbitol and sucrose at 3% (w/v) were screened to study the effect of carbon sources on the response of cell growth. The best carbon source obtained was then further studied using concentrations of 1 to 5% (w/v) with 0% as the control experiment to determine the optimal concentration.

5.2.2.8 Effects of combined optimized culture conditions of the biomass growth and flavonoids production

Cell suspension cultures of *B. rotunda* were cultured in optimized condition. The optimum culture conditions for growth biomass were employed for flavonoids production using normal MS medium 2% (w/v) sucrose, 1 mg/l NAA and 1 mg/l BAP cultured under 100 rpm at 25 °C and pH 5.8 with 1.0 ml SCV of initial inoculation volume.

5.2.3 Determination of cell biomass

The cells were harvested on day 14th of cultivation. They were separated from the liquid media by passing them through a Whatman No. 1 filter paper. The cells were washed 3 times with distilled water, followed by drying at 38 °C until a constant weight was achieved. The growth data were presented as g dry weight (DW) per flask.

5.2.4 Extraction and HPLC analysis

Fourteen days old cells were dried at 38 °C until constant weight was achieved prior to grinding using mortar and pestle. One gram of powder was extracted for flavonoids and analyzed as described in section 4.2.4 and section 4.2.5, Chapter 4. Analysis was performed in triplicates and data presented as µg compound(s)/g DW.

5.2.5 Statistical analysis

Data were subjected to analysis of variance (ANOVA) and Tukey's multiple range test (TMRT) at 5% probability level. Variability in data was expressed as the mean ± standard deviation.

5.3 RESULTS AND DISCUSSION

5.3.1 Effect of MS strength

Basal MS media showed that the highest final yields (14th day) was gained using normal strength. The biomass obtained was 0.24 ± 0.01 g and was significantly different compared to other treatment tested ($P < 0.05$). It was then followed by double strength (0.16 ± 0.03 g), half strength (0.12 ± 0.01 g) and quarter strength of MS basal media (0.09 ± 0.02 g). For accumulation of total flavonoids (TF), the results showed that all extract from cells cultivated in media with different strengths were not significantly different ($P < 0.05$). The highest TF was obtained in normal strength MS (3.93 ± 0.30 µg/g), followed by half strength (3.76 ± 0.68 µg/g), double strength (3.22 ± 2.29 µg/g) and finally quarter strength (1.96 ± 1.25 µg/g) (Figure 5.1).

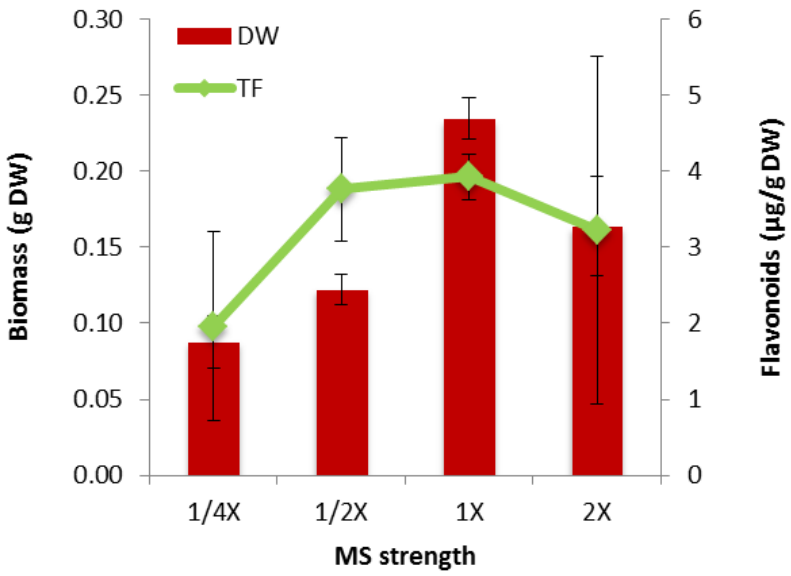


Figure 5.1: Effects of media strength on biomass and TF production in cell suspension cultures of *B. rotunda* after 14 days culture. Error bars indicates the standard deviation of the mean values.

Detailed examination revealed that different strength of MS significantly affected the production of specific flavonoids such as alpinetin and panduratin A ($P < 0.05$). Highest accumulation of alpinetin and panduratin A were achieved at double strength MS salts with 0.33 ± 0.01 and 1.83 ± 0.85 µg/g, respectively. However, maximum pinocembrin and pinostrobin production were obtained in full strength MS at 3.39 ± 0.3 and 0.44 ± 0.02 µg/g, respectively. Half strength MS produced cardamonin at highest level with 0.61 ± 0.11 µg/g. Quarter MS strength produced neither high biomass growth nor secondary metabolites in this study (Figure 5.2).

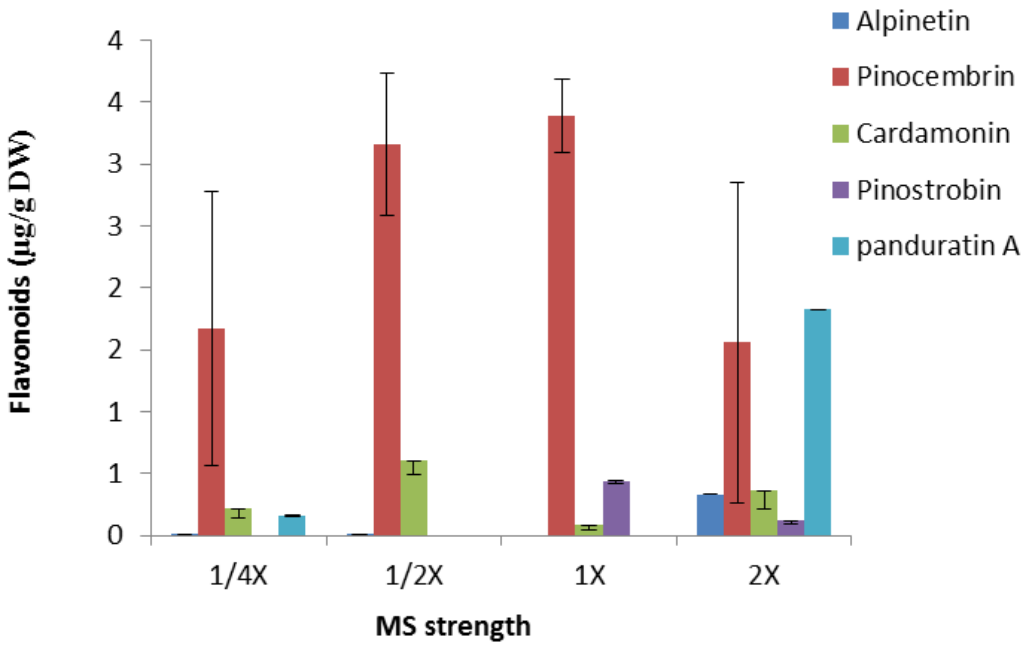


Figure 5.2: Effects of media strength on flavonoids production in cell suspension cultures of *B. rotunda* after cultured for fourteen days. Error bars indicates the standard deviation of the mean values.

From the biomass profile, normal strength MS medium was shown to be producing maximum biomass and flavonoids accumulation in cell suspension cultures of *B. rotunda*. Normal and half strength were found to have similar effects on the accumulation of *B. rotunda* flavonoids as opposed to biomass growth. Quarter strength

of MS showed limited effects on both biomass and secondary metabolites production. This observation agrees with the earlier report by Nagella and Murthy, (2010) where normal strength MS medium favored the accumulation of biomass and withanolide A production in cell suspension cultures of *Withania somnifera*.

In contrast to our results, Lian *et al*., (2002) revealed that half and normal strength medium were equally comparable for biomass growth and ginsenoside production in cell suspension cultures of *Panax ginseng* whereas, the double salt strength inhibited both the cell growth and ginsenoside productivity. Hyoscyamine and scopolamine production in hairy root culture of *Dature metel* was also observed in half strength of medium (Cusido *et al*., 1999). Similar observation has also been described for cultures of adventitious roots from mountain ginseng (Yu *et al*., 2000).

Nutrient requirements for growing plant cells in suspension cultures have been studied extensively. In plant cell cultures, suitable nutrient strength is crucial in order to produce the good growth and production degree of secondary metabolites (Ramachandra Rao and Ravishankar, 2002). The capability of different media strength in supporting plant cell growth and the synthesis of plant secondary metabolites has been linked to the ionic balance in the medium (Drewes and Staden, 1995). Therefore, transfer of cells to differing ionic strengths might affect the secondary metabolites production (Endress, 1994). At low nutrient concentration, insufficient nutrients may be experienced by the cells and at high nutrient concentration an osmotic stress for the cell cultures might occur (Lipavská and Vreugdenhil, 1996). Thus, it is important to find suitable level of basal medium strength in order to obtain the best production of biomass and flavonoids in a specific plant species used.

5.3.2 Initial volume

Effects of initial inoculation volume were also observed at different level of inoculants (0.25, 0.5, 1.0 and 1.5 ml of SCV). The final dry weights were 0.11 ± 0.01, 0.20 ± 0.04, 0.25 ± 0.04 and 0.21 ± 0.06 g and the production of TF were 3.70 ± 0.03, 4.38 ± 0.58, 15.11 ± 4.83 and 16.78 ± 13.87 µg/g when the initial inoculum were 0.25, 0.50, 1.00 and 1.50 ml of SCV, respectively (Figure 5.3).

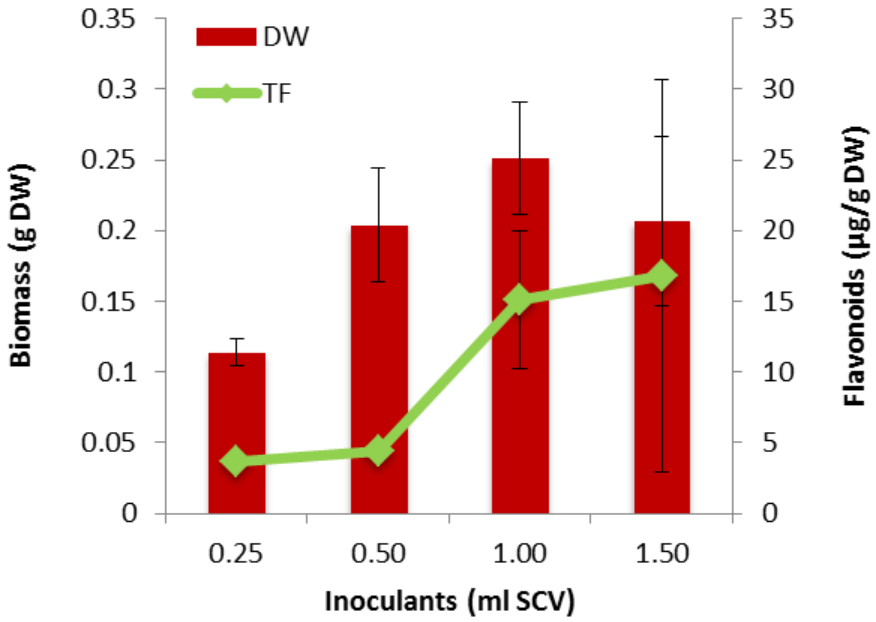


Figure 5.3: Effects of initial inoculation volumes on biomass growth and five flavonoids production in cell suspension cultures of *B. rotunda* after fourteen days. Error bars indicates the standard deviation of the mean values.

In this study low initial inoculation volume (0.25 ml SCV) resulted in poor biomass and flavonoids yield (Figure 5.3). This may be due to the inoculum size which was insufficient to survive in the liquid medium. Nutrients abundance in the liquid medium might gave negative effect to the cell growth if the inoculum level is too low. The study

showed that changing the inoculum size of *B. rotunda* cell suspension cultures significantly affect its biomass accumulation ($P < 0.05$).

The biomass and total flavonoids profiles also revealed that by using greater initial inoculation volume, the final dried weights and accumulations of compounds were increased. Initial inoculum volume of 1.00 ml SCV was found suitable and significantly different at $p < 0.05$, as this yielded highest production of TF (15.11 ± 4.83). Further increase in the inoculum volume to 1.5 ml SCV were not significantly different ($p < 0.05$) on the production of final biomass and the amount of TF (Figure 5.3). Contin *et al*. (1998) suggested that higher density cultures were subjected to either nutrient or oxygen limitation.

The results also showed that the highest accumulation of alpinetin, pinocembrin, cardamonin and panduratin A were obtained when 1.00 ml SCV inoculum was used. This study showed that the increase of the inoculum size from 0.25 to 1.0 ml of SCV per 50 ml of medium significantly favored the accumulation of alpinetin, pinocembrin and cardamonin with maximum yields of 0.82 ± 0.25, 8.57 ± 2.63, 0.36 ± 0.11 µg/g, respectively ($P < 0.05$). Meanwhile the production of pinostrobin was not significantly affected by inoculum volume with highest accumulation at 9.46 ± 7.90 µg/g when 1.50 ml inoculum was used ($P < 0.05$) (Figure 5.4).

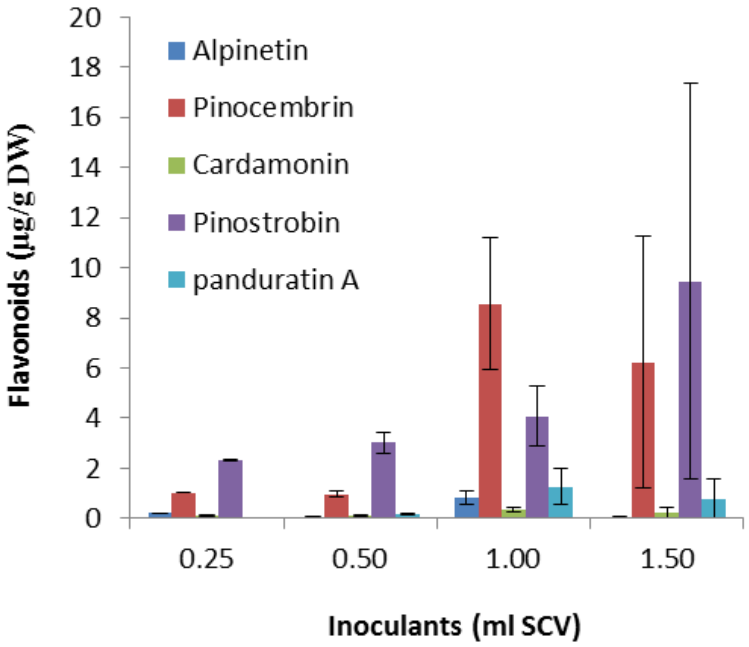


Figure 5.4: Effects of initial inoculation volumes on five flavonoids production in cell suspension cultures of *B. rotunda*. Error bars indicates the standard deviation of the mean values.

The results agreed with previous studies where the intracellular paclitaxel production was increased in *Taxus yunnanensis* cell suspension cultures when a moderately high inoculum size was used (Zhang *et al*., 2002a).

The development of *B. rotunda* cell suspensions was tremendous using 2% (1 ml of SCV) initial inoculation volume which provided about 5-fold final biomass increased (Figure 5.3). Studies have also been conducted for initial inoculation volume above 3% (1.5 ml SCV) but the cell suspension turned brown within 12 days of culture (data not documented). These may be due to the rapid growth of cell suspension of this species. According to the observation, increasing the initial inoculation volumes above 3% could decrease the culture age and frequent subcultures needed which not time and economically feasible.

Intracellular production of metabolites is depending on the needs of the cells and the availability of the substrate. The observed effects could be due to many factors. As observed in the present study, an intracellular flavonoids accumulation was improved in cell suspension cultures of *B. rotunda* when a moderately high inoculum size was used. Inoculums size is an important factor for cultured cells and subsequently triggered the biomass and secondary metabolites production. Two percent of initial inoculation volume of the medium was optimum for production of biomass and flavonoids content in cell suspension cultures of *B. rotunda*.

5.3.3 Initial pH values

The effects of initial pH medium (4.8, 5.3, 5.8, 6.3 and 6.8) on biomass accumulation and TF production from cell suspension cultures are presented in Figure 5.5. The pH of the culture medium was not adjusted with culture growth.

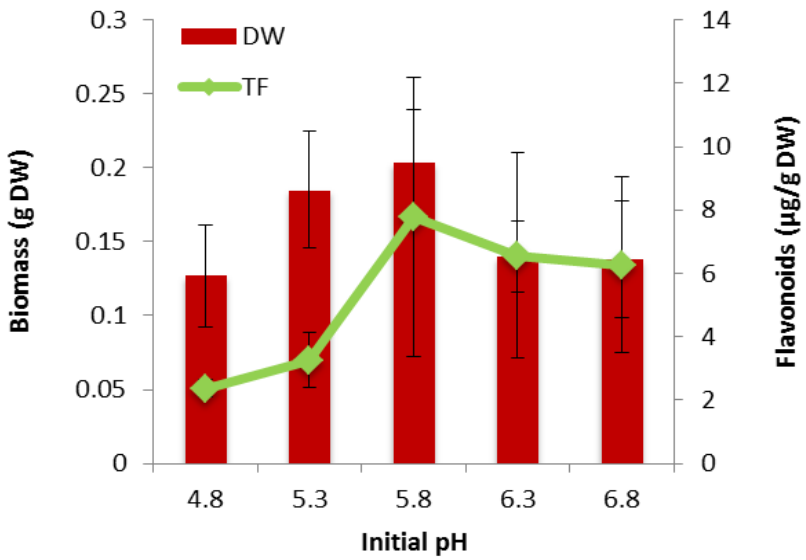


Figure 5.5: Effects of medium's initial pH on biomass growth and TF accumulation after 14 days culture. Both final biomass and TF content were maximum at pH 5.8. Error bars indicates the standard deviation of the mean values.

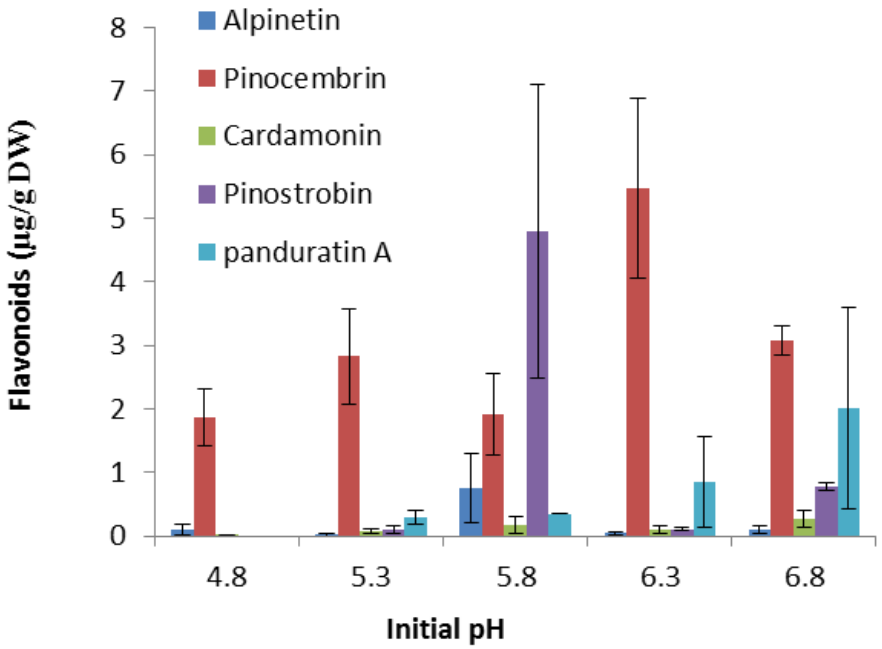
Highest accumulation of biomass was observed when the medium's initial pH was set at 5.8 (0.20 ± 0.04 g) followed by pH 5.3 (0.19 ± 0.04 g), pH 4.8 (0.13 ± 0.03 g), pH 6.3 (0.14 ± 0.04 g) and finally pH 6.8 (0.14 ± 0.02 g). Among the initial pH value, pH 5.3 and 5.8 showed significant effect on the final yield of cell suspension cultures of *B. rotunda*. Initial pH medium lower than 5.3 and above 5.8 did not favor the biomass accumulation of *B. rotunda* cell suspension cultures ($P < 0.05$) (Figure 5.5). This study also showed that cells in suspension cultures at low initial pH (4.8) and high initial pH (6.8) displayed different morphology compared to other treatment. These cultures exhibited pale yellow in color, small and stunted morphology in contrast to the other cultures which was light yellow and vigorously multiplying.

In contrast to the biomass production, all initial pH tested did not significantly affect the accumulation of TF. Highest TF produced were 7.77 ± 4.42, followed by 6.56 ± 3.24, 6.26 ± 2.77, 3.26 ± 0.87 and finally 2.37 ± 0.27 µg/g at initial pH 5.8, 6.3, 6.8, 5.3 and 4.8 respectively ($P < 0.05$). In this study, low initial pH (acidic) enhanced the hydrolysis process due to the presence of acid where energy was released too early in the media. Hydrolysis breaks the glycosidic bond in sucrose which provides a quick source of energy. In high initial pH medium, hydrolysis process was too slow due to alkaline environment. As a result, insufficient energy was supplied to the cell suspension cultures and this explained why the cell cultures in low and high pH medium was stunted in morphology and not vigorously growing.

This finding was also in agreement with (Hedrich *et al*., 1989), where growth and nutrient assimilation in cell cultures were sensitive to pH of media. It was also described that the availability of many compounds was pH dependent (Arnon *et al*., 1942, Van Winkle *et al*., 2003). However, a change of medium's pH may cause various effects that

may or may not encourage performance and development of cells according to (Hall and De Klerk, 2008).

Nevertheless, medium's initial pH set at 6.3 was found favorable for pinocembrin accumulation as compared to other initial values ($P < 0.05$). Alpinetin and pinostrobin were found to be in maximum production at pH 5.8 with 0.75 ± 0.55 and 4.79 ± 2.32 µg/g DW, respectively. Acidic pH medium (pH 4.8) resulted in trace amount of pinostrobin and panduratin A accumulation (Figure 5.6).



Figures 5.6: Effects of medium's initial pH on the accumulation of five flavonoids of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of the mean values.

This finding was in agreement with previous research by Ho and Shanks, (1992), who revealed that at low (pH 4.2) and high (pH 7.2) initial pH, hairy root cultures of *Catharanthus roseus* exhibited low growth. Similar result was also observed by Luthfi (2004), who revealed that initial pH medium at 5.75 exhibited the highest cell biomass

and total alkaloids in cell suspension cultures of *Eurycoma longifolia.* In contrast to our study, Moreno *et al*. (1995) showed that alkaloid productivity was influenced by pH values of medium. Asada and Shuler, (1989) showed that by shifting the pH of the medium between low and high values can change the permeability of cell membrane and released intracellular alkaloids into the culture medium.

Our study showed shifting the initial pH medium at range of 4.8 to 6.8, did not significantly ($P < 0.05$) affect the metabolite production of *B. rotunda* compounds suggesting further trials at pH value lower than 4.8 or higher than 6.8 to confirm whether accumulation of flavonoids compounds were pH dependent.

5.3.4 Temperature

From this study carried out, cell suspension cultures of *B. rotunda* showed that temperature had a major effect on cell biomass production. The cell suspension cultures incubated at 15 and 20 °C (low temperatures) revealed poor growth in biomass with dried weight of 0.09 ± 0.01 and 0.11 ± 0.08 g, respectively. It was observed that the cells grew very slowly at these temperatures. Eventhough the growth was slowed down in low temperature, cells were neither lysed nor died. Low temperature treatment also resulted in low accumulation of flavonoids (Figure 5.7). The suspension cultures grew rapidly if this culture were shifted to optimal temperature (25 °C) suggesting that low temperature could also be used to preserve the cell suspension cultures of *B. Rotunda* (data not shown). Cell growth at 25 °C was more vigorous than at other temperatures followed by cells at 30 °C with 0.23 ± 0.02 and 0.19 ± 0.05 g, respectively. At these temperatures (15-25 °C), it was observed that the cells multiplied were healthier (light yellow) than those at 30 °C (pale yellow). The study showed that the final dry weight of

B. rotunda biomass was significantly different when treated at 25 °C and 30 °C ($P < 0.05$).

An increase from 15 to 20 °C did not result in a ($P < 0.05$) increase of the flavonoids production. Further temperature increased to 25 °C reduced the flavonoids production, and increased slightly at 30 °C but not significantly ($P < 0.05$). Generally, the study found that the production of TF was similar (5-6 µg/g DW) at all temperatures tested (Figure 5.7).

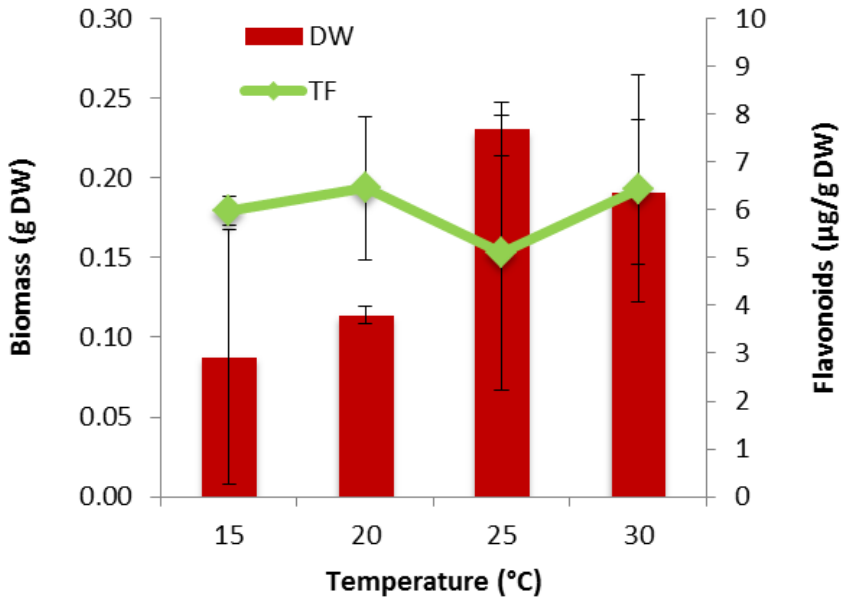


Figure 5.7: Effects of temperature on biomass and TF production in cell suspension cultures of *B. rotunda* after culturing for fourteen days. Error bars indicates the standard deviation of the mean values.

Although the temperature treatment did not show significant effect on TF, it significantly affected pinocembrin and cardamonin accumulation at 20 °C where 5.04 ± 1.26 and 1.21 ± 0.31 µg/g DW were produced, respectively ($P < 0.05$). The study also

showed that the panduratin A accumulation was significantly different at 30 °C with 1.79 ± 0.00 µg/g DW ($P < 0.05$) (Figure 5.8).

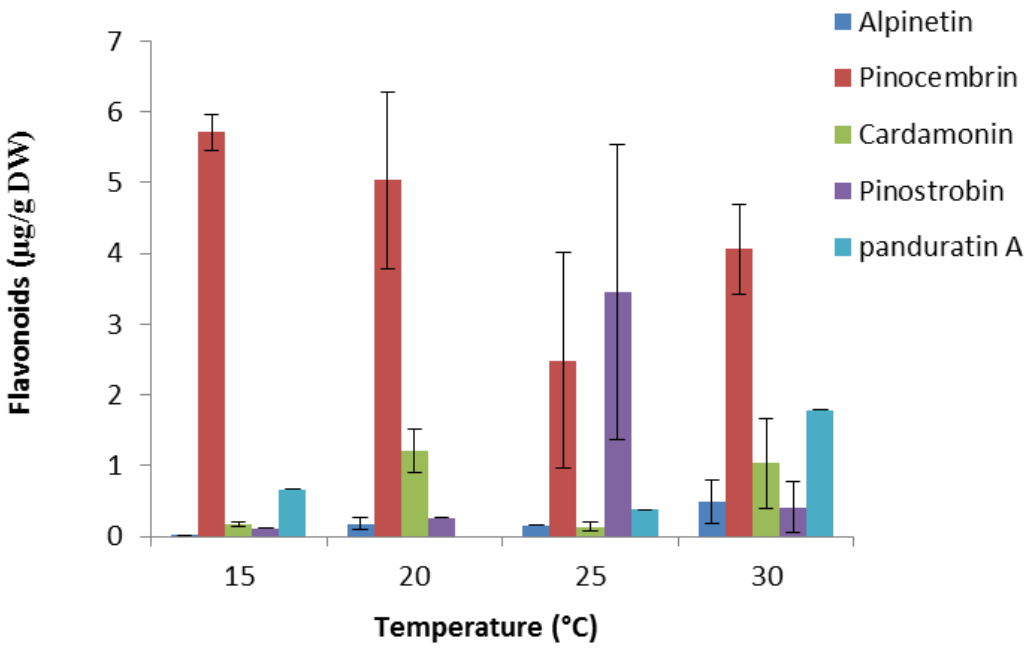


Figure 5.8: Effects of temperature on individual flavonoids production in cell suspension cultures of *B. rotunda* after 14 days culture. Error bars indicates the standard deviation of the mean values.

This study revealed similar result with Chan *et al*., (2010) where biomass production of *Melastomata malabatricum* cell suspension cultures were influenced by temperature treatment. Our finding was also in agreement with Matsumoto *et al*., (1972) who found that a higher growth rate of cell suspension cultures of *Populus* hybrids and *Nicotiana glutinosa* were observed at 24 °C to 30 °C than 15 °C. Similarly, Zhong and Yoshida, (1993) exhibited that temperatures in a range of 22 to 28 °C supported a high growth rate for suspension cultures of *Perilla frutescens.* In cell cultures of *Catharanthus roseus*, it was reported that the growth rate was maximal at 35 °C (Morris, 1986). It is

important to cultivate cells at or close to the optimum temperature and that cell growth at 30 °C is much better than that at 25 °C in cell suspension cultures of *Haplopappus gracillus* (Wellmann *et al*., 1976).

In contrast, temperature treatment by cell cultures of *C. wilfordii* had no effect on biomass accumulation and secondary metabolite synthesis (Shin *et al*., 2003). Cell suspension cultures of *Perilla frutescens* produced low anthocyanin at high temperature (28 °C) and showed maximum production at 25 °C (Zhong and Yoshida, 1993). However, cell suspension cultures of strawberry showed the highest anthocyanin content at low temperature (15 °C) compared to 35 °C treatment (Zhang *et al*., 1997).

Temperature played an important role on the rate of intracellular reactions in plant cell cultures, nutritional needs, mechanisms of metabolic regulation as well permeability (Zhang and Zhong, 1997a). Temperature range between 17 to 25 °C is used for the induction of callus tissues and growth of cultured cells (Ramachandra Rao and Ravishankar, 2002). Nevertheless, every plant species may prefer a different temperature. In this study, the cells were cultured in the range of 15 to 30 °C where temperature has significant effect on cell biomass accumulation and production of pinocembrin, cardamonin and panduratin A ($P < 0.05$). The results suggested that temperature change in plant cell culture might be due to the change of physiology and metabolism of cultured cells and subsequently influenced biomass and secondary metabolites production. An optimal temperature for cell growth and accumulation of secondary metabolites appear to be dependent on the species cultured and native condition of the plant.

5.3.5 Agitation

To investigate the effect of agitation on cell growth and flavonoids production, experiments were performed by varying the speed of agitation of the shaker. The effect of agitation on biomass and metabolites accumulation is shown in Figure 5.9.

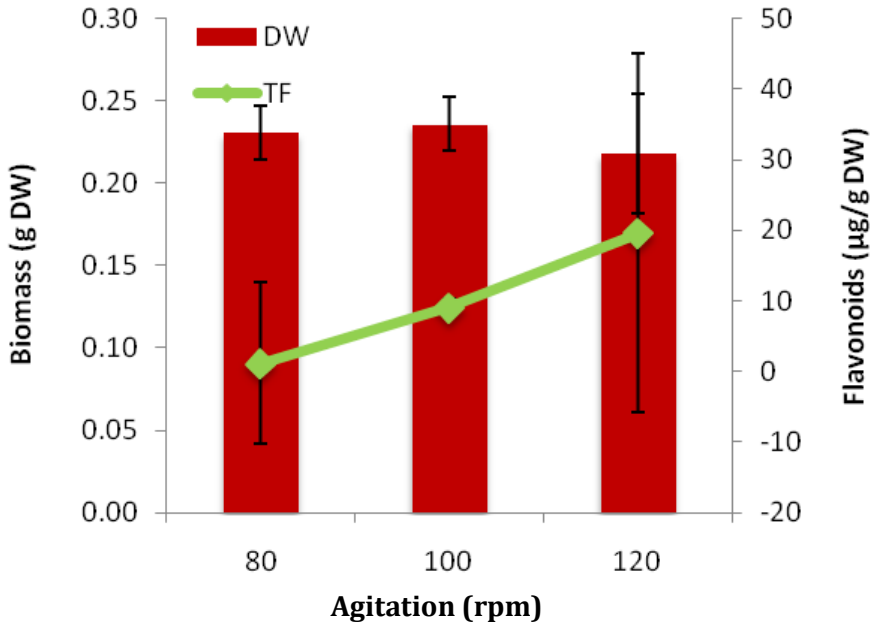


Figure 5.9: Effects of agitation on biomass and TF production in cell suspension cultures of *B. rotunda* after 14 days culture. Error bars indicates the standard deviation of the mean values.

Cell suspension cultures at 80 and 100 rpm exhibited characteristic of healthy light yellow cultures and were actively multiplying with final dried weights at 0.23 ± 0.02 and 0.26 ± 0.04 g, respectively after 2 weeks in culture. The lowest yield of final dried weight was from cultures agitated at 120 rpm (0.22 ± 0.02 g). In addition, these cells were vacuolated and light brown in colour.

It was observed that the total flavonoids seemed increased as the speed of agitation increased but not statistically significant ($P < 0.05$). TF accumulation was high in culture agitated at 100 rpm (19.63 ± 15.35 µg/g DW). However, individual flavonoids production that is pinocembrin, pinostrobin and panduratin A displayed significantly different level of yields, 8.69 ± 3.52, 8.76 ± 2.65 and 2.15 ± 1.52 µg/g DW respectively ($P < 0.05$) (Figure 5.10).

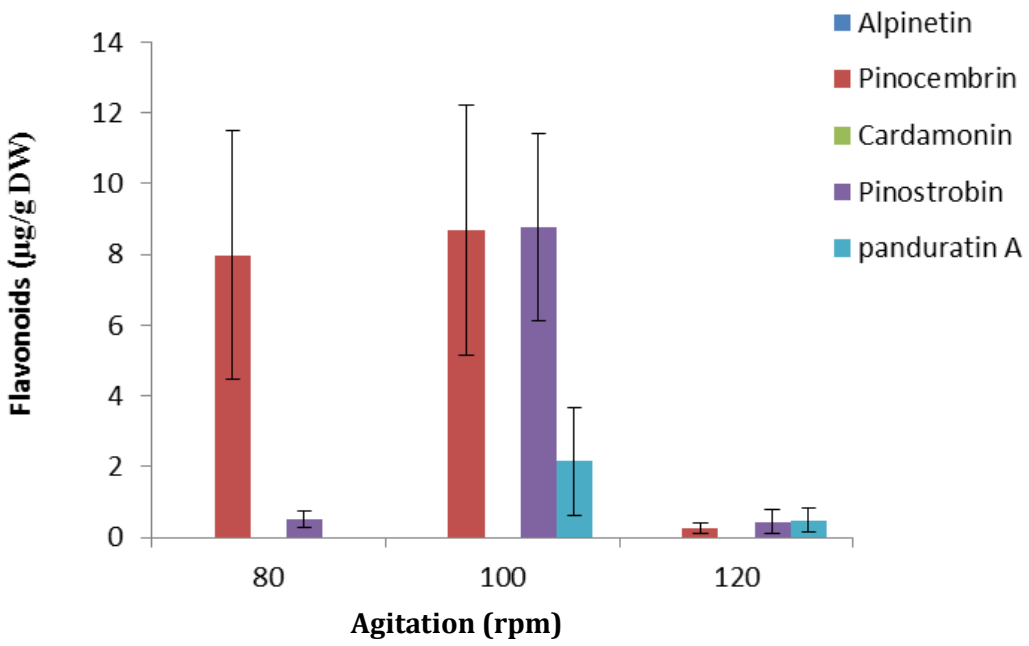


Figure 5.10: Effects of agitation on biomass and flavonoids production in cell suspension cultures of *B. rotunda* after 14 days of culture. Error bars indicates the standard deviation of the mean values.

Similar results was reported by Srivastava *et al*. (2011) where agitation was important variable influencing the growth and viability of cells. The viability profile showed that at low speed (60 rpm) cells died due to aggregation and clumping whereas at high speed (240 rpm), the cells died due to rupture. Likewise, *Podophyllum hexandrum* cells

propagated at moderate agitation (125 to 150 rpm) exhibited low cell injury. However, the increasing agitation speeds up to 200 rpm significantly decreased the cell viability (Chattoppadhyay *et al*., 2002). On the other hand, Zhao and Verpoorte (2007) indicated that some plant cells were not remarkably sensitive to shear forces and some cell lines were resistant to it.

In cell cultures system, agitation produced shear forces that can cause many physiological and morphological variations including cell size, shape, aggregation, cell wall composition, integrity, viability, and might eventually affect biomass accumulation and secondary metabolism (Zhao and Verpoorte, 2007). This study showed that shaking conditions might be one of the controlling factors for biomass accumulation, since over shaking inhibited cell growth. It was also revealed that increased in agitation also improved the productions of flavonoids in *B. rotunda* cell suspension cultures. Shaking might cause cell injury due to shaking, even though intense shaking could bring rapid oxygen supply. Agitation of cell culture systems helped mix cells with liquid medium and subsequently facilitate the nutrient uptake. Thus, the optimal agitation speed plays an important factor to produce good biomass growth and secondary metabolite production.

5.3.6 Plant growth regulators (PGRs)

5.3.6.1 Effects of auxins

Studies were carried out using two different auxins, NAA and 2,4-D at concentrations 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l. These two auxins induced different growth performances in *B. rotunda* cultures. For NAA, the highest growth 0.23 ± 0.02 g DW/culture was obtained in 1 mg/l while for 2,4-D, the highest growth of 0.25 ± 0.03 g DW/culture was achieved in 5.0 mg/l (Figure 5.11). It is also shown that the growth of the suspension

cultures decreased significantly at NAA concentrations higher than 2.0 mg/l ($P < 0.05$). On the other hand, for 2,4-D, the cell growth was proportionately increased with increasing concentrations.

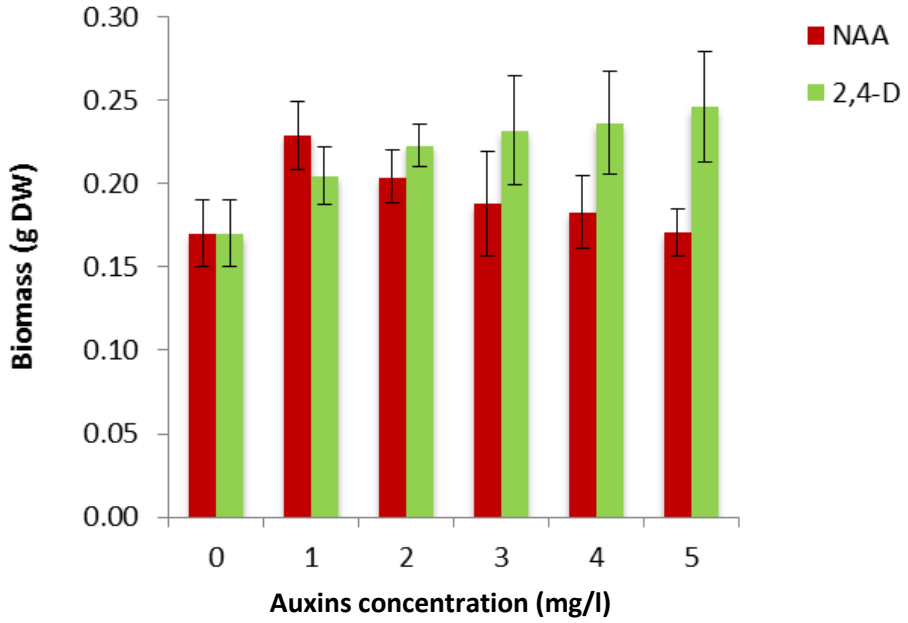


Figure 5.11: Effects of auxins on biomass production in cell suspension cultures of *B. rotunda* after 14 days cultivation. Error bars indicates the standard deviation of the mean values.

Even though NAA is a common auxin which ensured the growth and viability of cell cultures (Endress, 1994) it was not necessary for every species. Therefore, level of auxins that ensured the *B. rotunda* cells growth was investigated. Among all the concentrations and auxins tested in this study, 5.0 mg/l of 2,4-D was found to be the best in producing high growth suspension cultures after two weeks of culture. However, the study revealed that amount of biomass produced in 1 mg/l NAA was not significantly different compared to 5 mg/l 2,4-D, suggesting the usage of 1 mg/l NAA is

economically feasible for the maintenance of *B. rotunda* cells growth ($P < 0.05$) (Figure 5.11).

Cells grown in hormone-free medium showed normal growth morphology but did not enhance the growth rate. The cell growth reduced gradually following four subcultures. *Morinda elliptica* cells grown in hormone-free medium turned necrotic after three subcultures (Abdullah *et al*., 1998). Wai-Leng and Lai-Keng, (2004) also showed that cell suspension cultures of *Orthosiphon stamineus* turned necrotic within 24 days of the culture period in PGR free medium.

Even though the control was not supplemented with auxins, the suspension cultures could still grow but slower than media with auxins. This may be possible due to the presence of endogenous hormones produced by the plant cells for growth maintenance. The presence of endogenous hormones was previously demonstrated in carrot cultures (Jiménez *et al*., 2005). However, production of endogenous hormones alone was not sufficient in promoting long-term cell growth.

This study along with previous reports confirmed the importance of incorporating auxins in the medium for continuous growth of cells (Plas *et al*., 1995). Auxins are a group of plant hormones capable of promoting several aspects of plant growth and development, such as cell division, cell extension, vascular differentiation, adventitious root formation and apical dominance (Centeno *et al*., 1999). However, individual cultures differ in their sensitivity to these hormones.

In view of TF production, among the two auxins tested, it was found that increased concentration of NAA resulted in lowered production of TF. The highest amount of TF was found in extract from cells grown in media supplemented with 1 mg/l NAA at 4.18 ± 0.23 µg/g DW. However, contrasting results were found in the extracts of cells grown

with 2,4-D where the concentration of 2,4-D up to 4 mg/l enhanced the total flavonoid compounds significantly ($P < 0.05$). Further increase in the concentration of 2,4-D decreased the total production of 5 flavonoids (Figure 5.12).

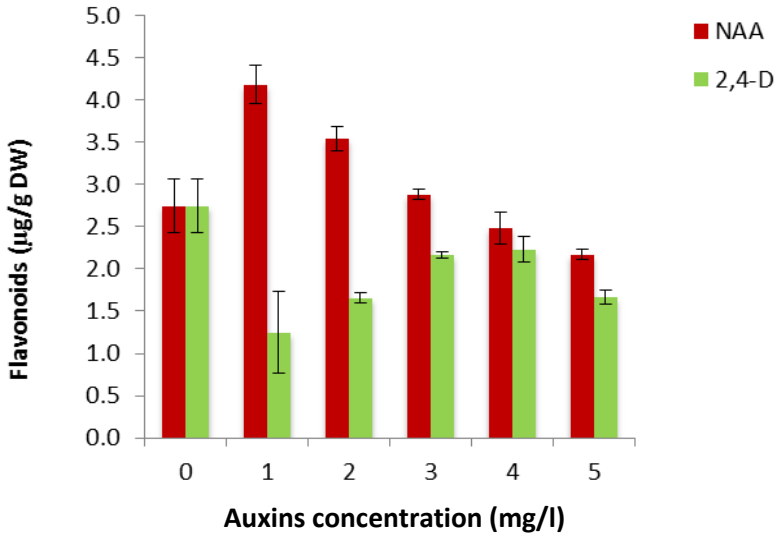


Figure 5.12: Effects of auxins on total 5 flavonoids production in cell suspension cultures of *B. rotunda* after 14 days cultivation. Error bars indicates the standard deviation of the mean values.

Individual production of pinocembrin, cardamonin and panduratin A were found highest in cells grown in 1 mg/l NAA at 4.05 ± 0.06, 0.12 ± 0.02, 0.002 ± 0.001 µg/g DW respectively. Meanwhile, maximum accumulation of alpinetin and pinostrobin were found in media containing 2 mg/l NAA with 0.44 ± 0.001 and 0.13 ± 0.001 µg/g DW correspondingly (Figure 5.13). The differences in the contents of the compound for different treatments were significant at 5% level of significance.

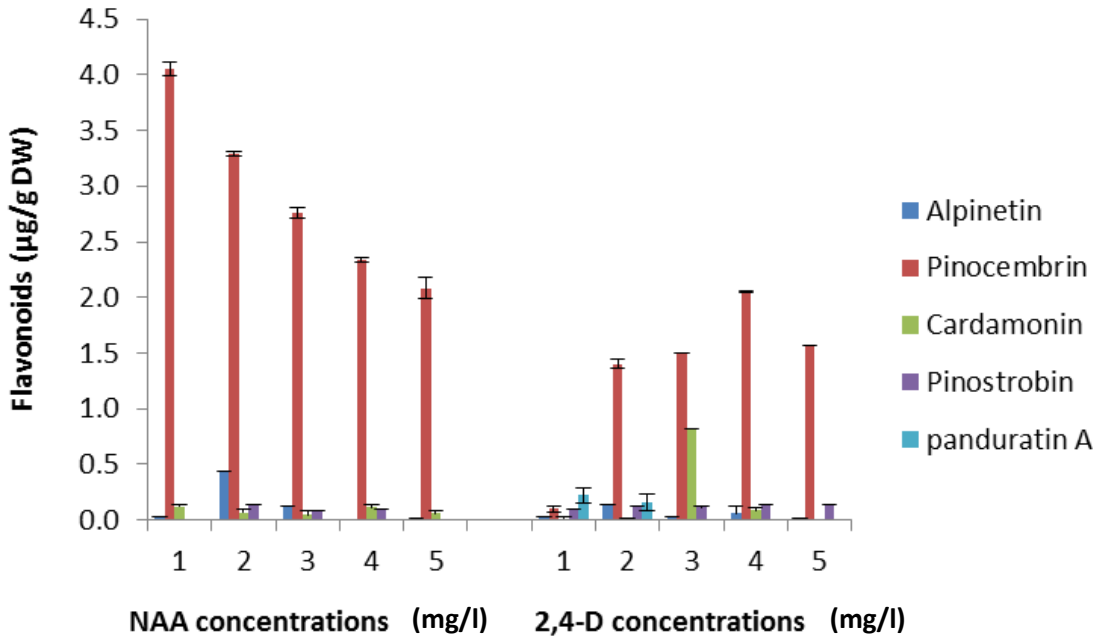


Figure 5.13: Effects of auxins on production of 5 flavonoids in cell suspension cultures of *B. rotunda* after 14 days cultivation. Error bars indicates the standard deviation of the mean values.

Our results were similar to those of *Rubia cordifolia* cell cultures where low concentration of NAA (0–0.6 mg/l) was ideal for cell biomass and anthraquinone accumulations (Suzuki *et al*., 1982). Further increase in NAA concentration in the species studied also displayed similar result with *R. cordifolia* cell cultures, which inhibited the cell growth and ineffective for accumulation of secondary metabolites. The present results were also in agreement with Narayan *et al*., (2005) where 2,4-D has been shown to inhibit the production of secondary metabolites. The flavonoids content was lower in hormone-free medium than with hormones incorporated (Figure 5.12).

Contrary to our findings, stimulations by 2,4-D had been observed in anthocyanin production and essential to support biomass of *Cleome rosea* callus cultures (Simões *et al*., 2009). Similar results were reported for cell suspension cultures of *Ipomoea batatas* L. (Konczak-Islam *et al*., 2000) as well as in formononetin accumulation in suspension

cultures of *Glycyrrhiza glabra* (Asiras-Castro *et al*., 1993). Misawa (1994) also reported that the production of L-DOPA in *Mucuna pruriens*, ubiquionone-10 in *Nicotiana tabacum* and diosgenin in *Dioscorea deltoidea* were stimulated by increasing levels of 2,4-D.

Our results suggested that secondary product accumulation was enhanced with incorporation of low amount of auxins. However, the production of secondary metabolites were suppressed by the presence of high auxin levels (in this study, NAA and 2,4-D). An increase in the auxin level often leads to a dedifferentiation of cell division and consequently diminishes the level of secondary metabolites (Charlwood and Rhodes, 1990). This is in agreement with indole alkaloid and anthocyanin production from *Catharanthus roseus* (Zhao *et al*., 2001b) and *Oxalis reclinata* (Makunga *et al*., 1997), respectively.

Between both types of auxin and their concentrations, 1 mg/l NAA enhanced the accumulation of biomass and flavonoids accumulation in *B. rotunda* cell suspension cultures. 2,4-D at all concentrations were not favorable to produce flavonoids in contrast to biomass growth in *B. rotunda* cell cultures.

5.3.6.2 Effects of cytokinins

Studies were also carried out with two different cytokinins, BAP and kinetin, with concentrations of 1.0, 2.0, 3.0, 4.0 and 5.0 mg/l. BAP and kinetin, produced similar growth pattern in *B. rotunda* cultures. For BAP, the highest growth 0.21 ± 0.03 g DW/culture was recorded in 1 mg/l meanwhile for kinetin, the highest growth 0.24 ± 0.01 g DW/culture was achieved in 1.0 mg/l (Figure 5.14). Growth response of cell suspension cultures towards BAP are not significantly different at all concentration

tested ($P < 0.05$), while the values were significantly different at 5 mg/l kinetin for each concentration of BAP and kinetin.

Data obtained also showed that the growth of the suspension cultures decreased significantly ($P < 0.05$) with concentrations higher than 2.0 mg/l for both BAP and kinetin. Comparable biomass yield in cytokinins treated cells was observed to those subjected to auxins treatments in the cell suspension cultures of *Glycyrrhiza glabra* (Arias-Castro *et al*., 1993). However, the effects of cytokinin were dependent on cell line and its concentration (Moreno *et al*., 1995). As for the present study, incorporation of auxins and cytokinins to the media gave similar results where the concentration of 2,4-D at 5 mg/l also produced 0.25 ± 0.03 g DW/culture, while for cytokinins, 1 mg/l kinetin yield the highest biomass at 0.24 ± 0.01 g DW/culture (Figure 5.14).

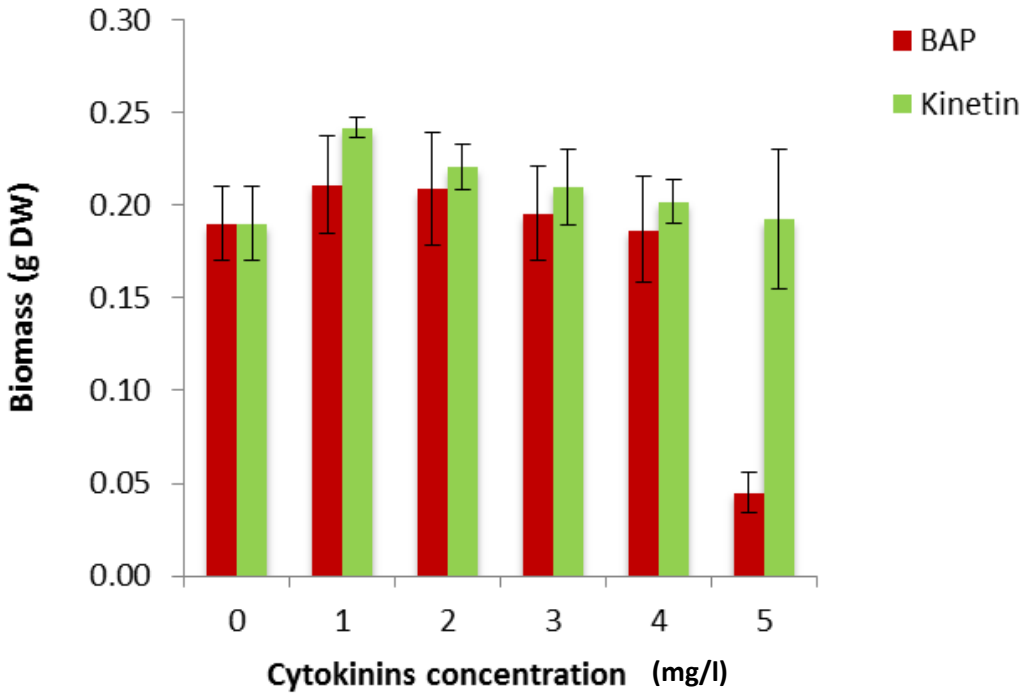


Figure 5.14: Effects of cytokinins on biomass production in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

Production of flavonoids was quantified in the above cell cultures and maintain in the experiments (Figure 5.15).

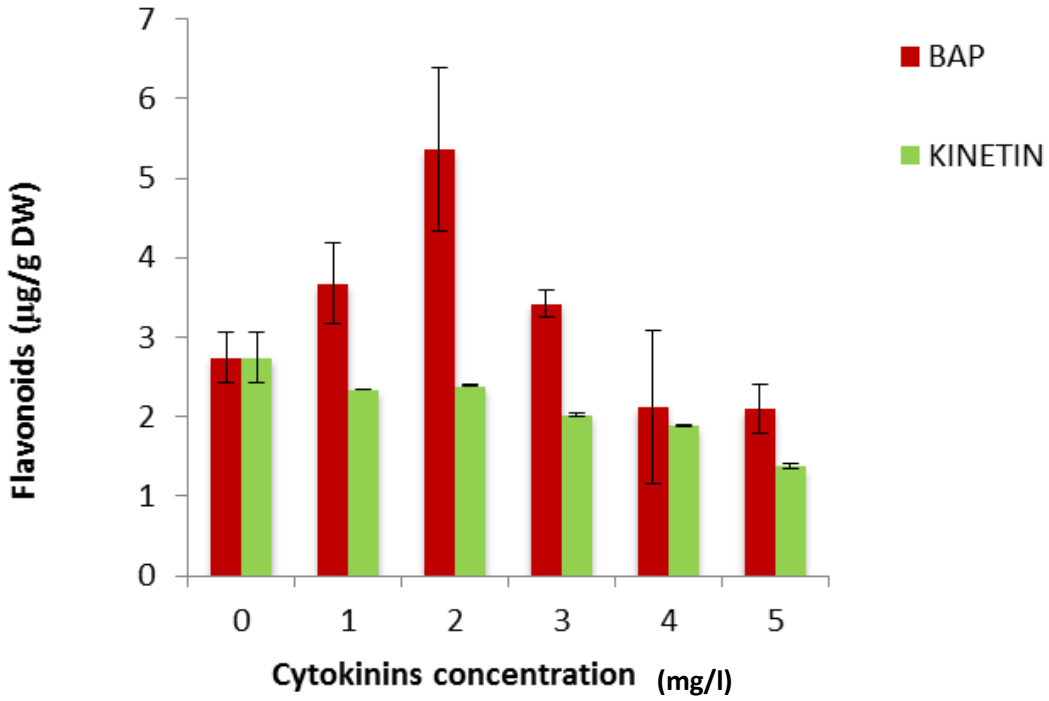


Figure 5.15: Effects of cytokinins on flavonoids production in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

Among the two cytokinins studied, BAP exhibited better accumulation of flavonoids than kinetin at all concentration tested. The increase in the concentration of BAP from 1 to 2 mg/l significantly increased the accumulation of flavonoids in the cell cultures ($P < 0.05$). However, further increase in concentration resulted in decreasing amount of flavonoids. There were no significant differences ($P < 0.05$) in the concentration of flavonoids accumulated when the cell cultures were treated with different concentration of kinetin. Different concentration of kinetin tested did not significantly affect the flavonoids content ($P < 0.05$). Highest flavonoids content of 5.36 ± 1.03 µg/g DW was found in cells treated with 2 mg/l BAP which was 2.25 folds higher than cells treated

with 2 mg/l kinetin and 1.95 folds higher than control. Maximum yield of flavonoids in cells treated with kinetin was also at 2 mg/l concentration (2.39 ± 0.01 µg/g DW) (Figure 5.15).

For individual flavonoid content, alpinetin was found highest at 4 mg/l BAP with 0.52 ± 0.31 µg/g DW but the content was not significantly different among the BAP concentrations tested ($P < 0.05$). The accumulation of cardamonin and pinostrobin were greatest when cultured in 2 mg/l BAP at 0.29 ± 0.02 and 3.44 ± 0.80 µg/g DW, respectively. Meanwhile, yield of panduratin A was highest in 3 mg/l BAP which produced 2.31 ± 1.01 µg/g DW compared to other BAP concentrations tested ($p < 0.05$). In contrast to pinocembrin, the highest content was produced when cultured in 1 mg/l kinetin at 2.18 ± 0.10 µg/g DW where the value was not significantly different among the BAP concentrations tested ($P < 0.05$). Individual flavonoids data were presented in Figure 5.16.

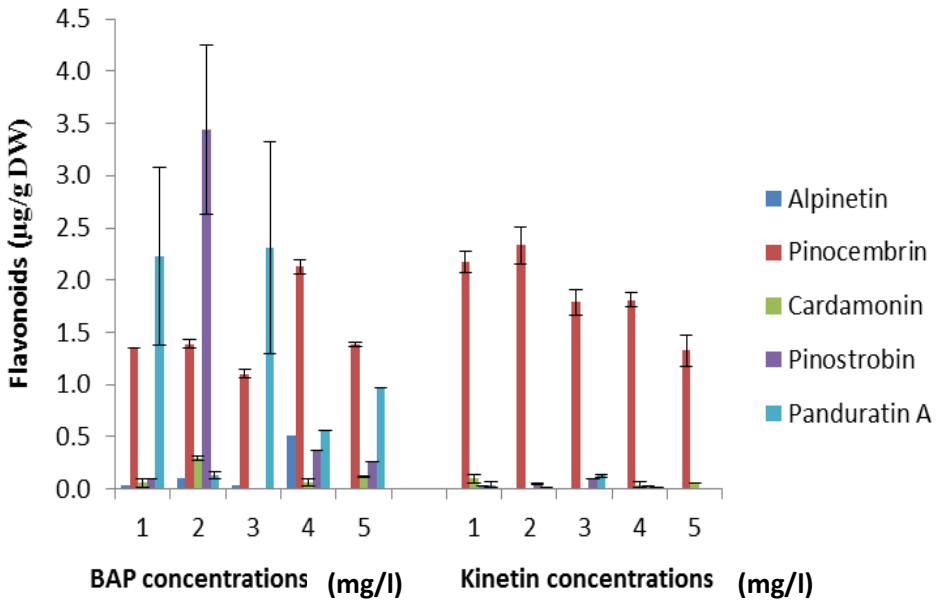


Figure 5.16: Effects of cytokinins on production of 5 flavonoids in cell suspension cultures of *B. rotunda* after 14 days cultivation. Error bars indicates the standard deviation of mean values.

Cytokinins have different effects on the type of metabolite produced and species used. Cytokinins were found to stimulate alkaloid biosynthesis in some tumorous cell lines of *Catharanthus roseus* (Moreno *et al*., 1995). Kinetin stimulated the production of anthocyanin in *Haplopappus gracius* but inhibited the formation of anthocyanins in *Populus* cell cultures (Ramachandra Rao and Ravishankar, 2002). It was also reported that only cytokinin stimulate the production of an isoflavonoid formation from *Glycyrrhiza glabra* cells (Arias-Castro *et al*., 1993). BAP was also observed to enhance callus proliferation and isoflavonoid production of the *Glycine max* (Tuominen and Musgrave, 2006). These results are similar to our findings where cells cultured in cytokinin at 2 mg/l BAP produced significantly higher flavonoids than cells cultured in auxin ($P < 0.05$) (1 mg/l NAA).

From the present study, it was revealed that low level of PGR was ideal for accumulation of biomass and flavonoids. Similar results were previously reported where optimum concentrations for a production medium with low levels of PGR were used in species *Morinda elliptica* (0.5 mg/l NAA and 0.5 mg/l kinetin) (Collin, 2001). Each plant species requires different kinds and levels of phytohormones for callus induction, growth and metabolite production, thus, it is important to select the most appropriate growth regulators and to determine their optimal concentrations (Endress, 1994).

5.3.6.3 Combination of optimal auxin and cytokinin

Further investigations were also carried out on the effects of biomass and flavonoids production using cell suspension cultures of *B. rotunda* using combinations of auxin and cytokinin in media. Data were presented in Figure 5.17 and 5.18, respectively.

From the present study, it was observed that combination of optimized auxins and cytokinins concentration in the liquid medium further enhanced both biomass and flavonoid accumulations. Among the combinations tested, 1 mg/l NAA plus 1 mg/l BAP yield cell biomass at 0.25 ± 0.2 g DW/culture. However this yield was not significant ($P < 0.05$) from those obtained in cultures supplemented with 5 mg/l 2,4-D and 1 mg/l BAP (0.25 ± 0.01 g DW/culture) and also 1 mg/l NAA with 1 mg/l kinetin (0.24 ± 0.2 g DW/culture). However, biomass obtained from 5 mg/l 2,4-D plus 1 mg/l kinetin (0.17 ± 0.01 g DW/culture) was significantly lower ($P < 0.05$) (Figure 5.17).

This study revealed that different biomass yield obtained from combined PGR was not significantly different compared to cells cultured in optimal media consisting of either auxin (5 mg/l 2,4-D) or cytokinin (1 mg/l kinetin) used singly but were found significant when compared to NAA or BAP supplemented media at all concentrations tested ($P < 0.05$) (Figure 5.17).

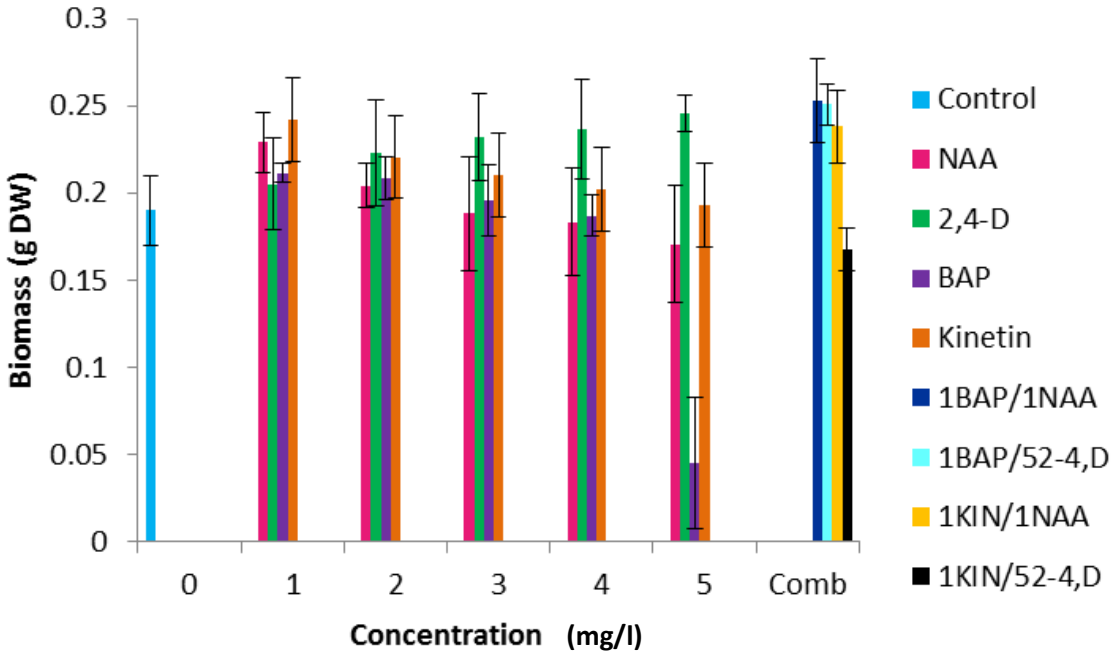


Figure 5.17: Effects of PGR on biomass production in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

For flavonoids accumulations, best combination of auxin and cytokinin was found in 5 mg/l 2,4-D plus 1 mg/l BAP (5.07 ± 0.3 µg/g DW) and was significantly different compared to other combinations tested ($P < 0.05$). However, the value was not significantly different compared to cells treated only with 2 mg/l BAP ($P < 0.05$). Nevertheless, combinations of optimum BAP and 2,4-D produced the highest flavonoids content among all the PGR tested alone or in combinations (Figure 5.18).

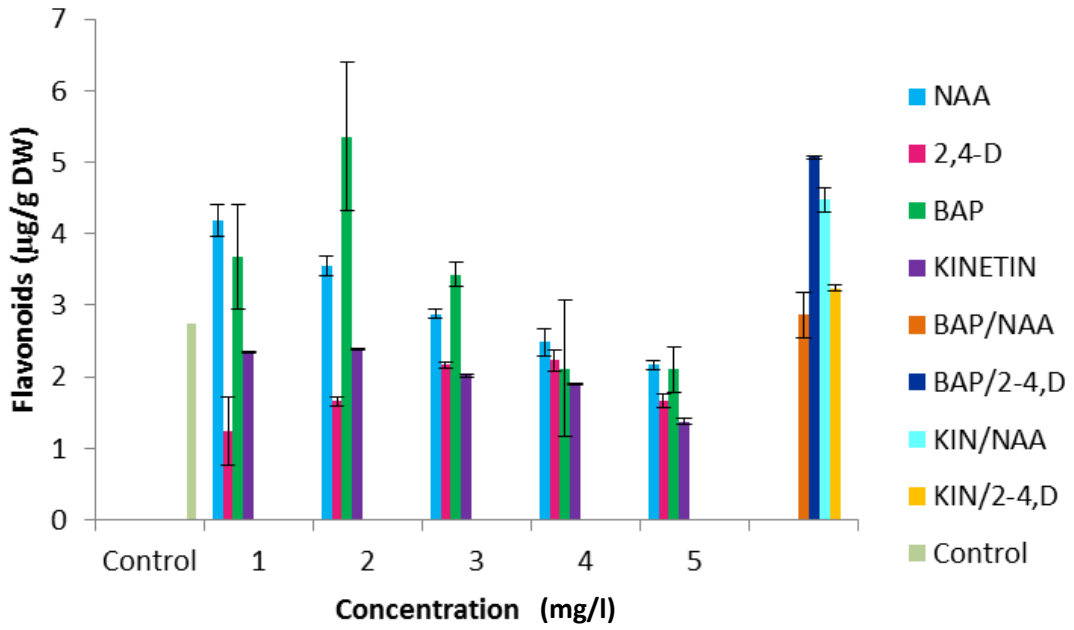


Figure 5.18: Effects of PGR on flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean.

Similar to this study, Wu and Zhong, (1999) demonstrated that not only individual phytohormone level but also their combination had a significant influence on cell growth and ginsenoside accumulation in *Panax notoginseng*. Comparable observations

were also made in the production of camptothecin by *Nothapodytes foetida* (Thengane *et al*., 2003), anthraquinone by *Morinda elliptica* (Abdullah *et al*., 1998) and *Rheum ribes* (Farzami Sepehr and Ghorbanli, 2002), Jaceosidin by *Saussurea medusa* as well as indole alkaloid by *Catharanthus roseus* (Zhao *et al*., 2001b).

This study revealed that the ratio of concentration of auxin and cytokinin was critical in determining the rate of cell differentiation in *B. rotunda* cell cultures. Appropriate PGR combinations influenced the composition and concentration of biomass and secondary compounds produced in cell cultures. In addition, the importance of incorporating hormones in the medium to enhance biomass and flavonoids production in species studied was shown.

5.3.7 Carbon sources and concentrations

Several carbon sources such as glucose, fructose, mannitol, sorbitol and sucrose at 3% (w/v) were used to study the effect on cell growth and flavonoids accumulation. The results are shown in Figures 5.19 and 5.20, respectively. Growth was comparable with sucrose and glucose as the carbon sources. Nevertheless, the culture medium that was supplemented with 30 g/l sucrose showed enhanced biomass yield (0.20 ± 0.03 µg/g DW). Fructose did not support growth as high as that achieved in sucrose while mannitol and sorbitol inhibited the cell growth. The results exhibited that carbon sources produced significant effect on growth of cell suspension cultures of *B. rotunda* ($P < 0.05$).

A similar trend has also been shown with *Psoralea corylifolia* cell suspension cultures where sucrose improved the cell growth while fructose inhibited the growth of the suspension cultures (Shinde *et al*., 2009). A similar glucose preference rather than fructose has also been reported in other cell lines such as *Marchantia polymorpha*

(Chiou *et al*., 2001), *Coleus blumei* and *Catharanthus roseus* (Zhang *et al*., 1996). This could be due to the fact that glucose uptake rate was higher than fructose as observed in the cell culture of *Lavandula vera* MM (Ilieva and Pavlov, 1997). Meanwhile, mannitol and sorbitol were found unable to promote the growth of the *B. rotunda* cell suspension cultures. Mannitol and sorbitol also exhibited growth inhibition effects on *Marchantia polymorpha* cell cultures (Chiou *et al*., 2001).

In contrast, addition of sorbitol has been observed to enhance *in vitro* culture growth and morphogenesis in certain rice genotypes (Al-Khayri and Al-Bahrany, 2002). In *Morinda elliptica*, higher biomass was yielded in 5% glucose compared to 5% sucrose (Abdullah *et al*., 1998).

The fact that carbon source is an essential element for any cell growth in plant tissue culture system was proven in suspension cultures of *B. rotunda*. Suspension cultures grown in media without sucrose that was used as the control for this experiment was found to be not suitable in maintaining the suspension cultures growth as most of the cells died within one week of culture.

Plant cell cultures generally depend on simple sugars as carbon sources for growth (Ramachandra Rao and Ravishankar, 2002). Usually sucrose, glucose or fructose at 2 to 4% was the best carbon sources for the growth of various plant cell cultures (Abdullah *et al*., 1998). Among these carbon sources, sucrose is the most beneficial for the development of plant cell cultures and biosynthesis of secondary metabolites. It was been established that the cell cultures differ in utilizing the products of sucrose breakdown (Wu and Zhong, 1999). The disaccharide sucrose may be hydrolyzed to glucose and fructose during high temperature sterilization or can be hydrolyzed in the cell wall (Zhong *et al*., 1996).

In terms of the flavonoids production in different carbon sources treated cells, the accumulation was highest when medium was supplemented with mannitol, followed by sorbitol, fructose, sucrose and finally glucose at 9.05 ± 0.18, 5.24 ± 0.82, 4.54 ± 0.18, 3.23 ± 1.14 and 2.34 ± 0.21 µg/g DW respectively. All values were significantly different ($P < 0.05$) (Figure 5.19).

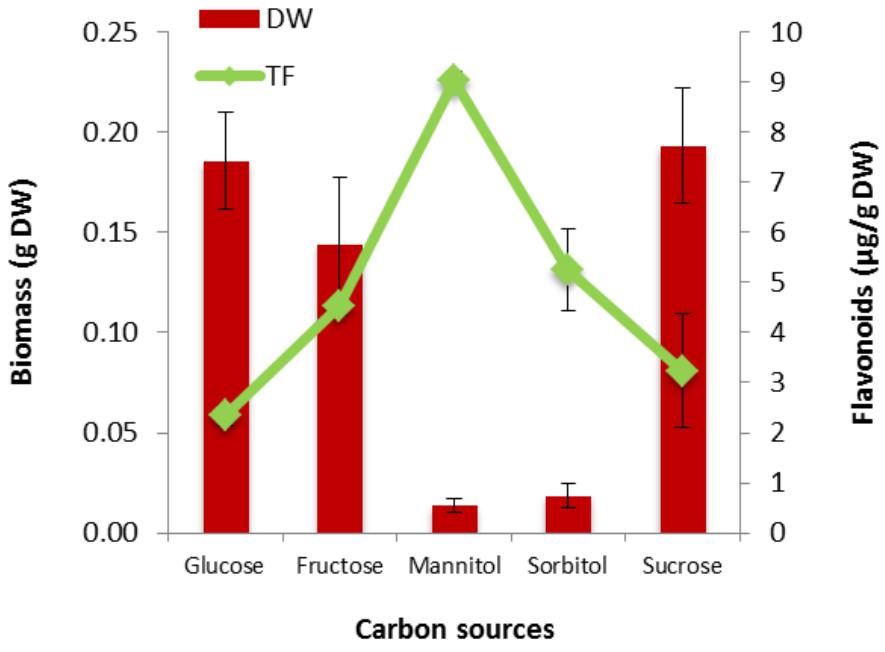


Figure 5.19: Effects of carbon sources on biomass and flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

For individual flavonoids productions, mannitol produced the highest alpinetin and pinocembrin accumulation at 0.15 ± 0.11 and 8.85 ± 0.14 µg/g DW respectively. The highest cardamonin production was achieved in medium supplemented with glucose (0.93 ± 0.04 µg/g DW), while sorbitol supplemented medium produced the highest

pinostrobin and panduratin A (0.44 ± 0.00 and 1.43 ± 0.00 µg/g DW respectively) (Figure 5.20).

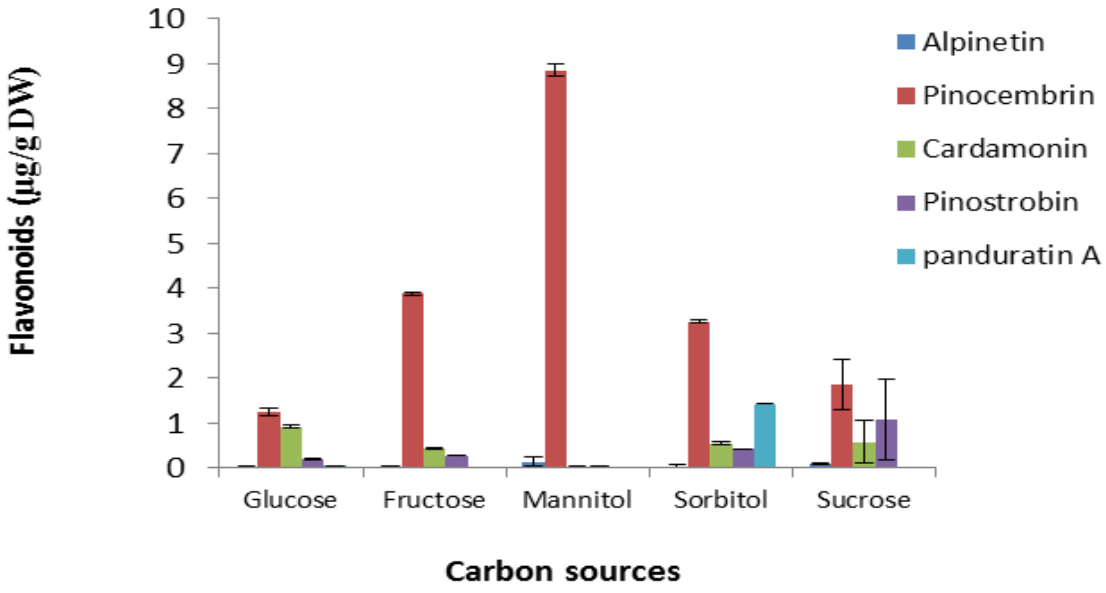


Figure 5.20: Effects of carbon sources on 5 individual flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

Studies were carried out by Wang and Weathers, (2007) and they verified the effect of sugars on artemisinin production in the hairy roots as well as seedling cultures of *Artemisia annua*. Maximum artemisinin were produced when hairy roots grown in the glucose augmented medium, however production of artemisinin were double than sucrose in the fructose supplemented medium. Based on their results, they concluded that the roles of sugars are not only as carbon sources but also as signals that affect the downstream production of artemisinin.

In this study, sucrose was found to be the best for growth of cell suspension cultures of *B. rotunda*, but not for the flavonoids accumulations. It is not practical to use fructose,

mannitol and sorbitol even though these carbon sources enhanced the accumulation of flavonoids but inhibited the cell growth. After 2 to 3 subcultures in media containing fructose the growth of cell suspension cultures were gradually reduced while in mannitol and sorbitol, the cell suspension cultures turned necrotic. Sucrose is the common carbon source for plant cell culture, serving as the principal energy source and a parent component for biosynthesis. The rate of biomass growth is usually directly correlated with sugar consumption (Wu and Zhong, 1999).

5.3.7.1 Effect of sucrose concentration

The effect of sucrose concentration was then further studied at 1% to 5% (w/v) and 0% as the control experiment. Figures 5.21 and 5.22 showed the effects of sucrose concentrations on *B. rotunda* cells growth and flavonoids production respectively. Our results showed that sucrose concentrations affected significantly the growth of cell suspension cultures ($P < 0.05$). Sucrose at 2% (w/v) produced the highest biomass at 0.21 ± 0.02 g DW/culture. However, sucrose concentrations higher than 2% gradually reduced growth of the cells in the culture media.

Decreased cell growth at high sucrose concentrations could be a result of high osmotic strength that probably affected the water content of the cells. A similar result was also shown in *Eriobotrya japonica* (Ho *et al*., 2010). Cell growth was inhibited by high initial sucrose concentrations which led to high osmotic pressure in cell cultures of *Panax notoginseng* (Zhang *et al*., 1996) and *Holarrhena antidysenterica* (Panda *et al*., 1992). Low sucrose concentrations also resulted in slow growth of cell cultures and this is most likely due to insufficient energy sources.

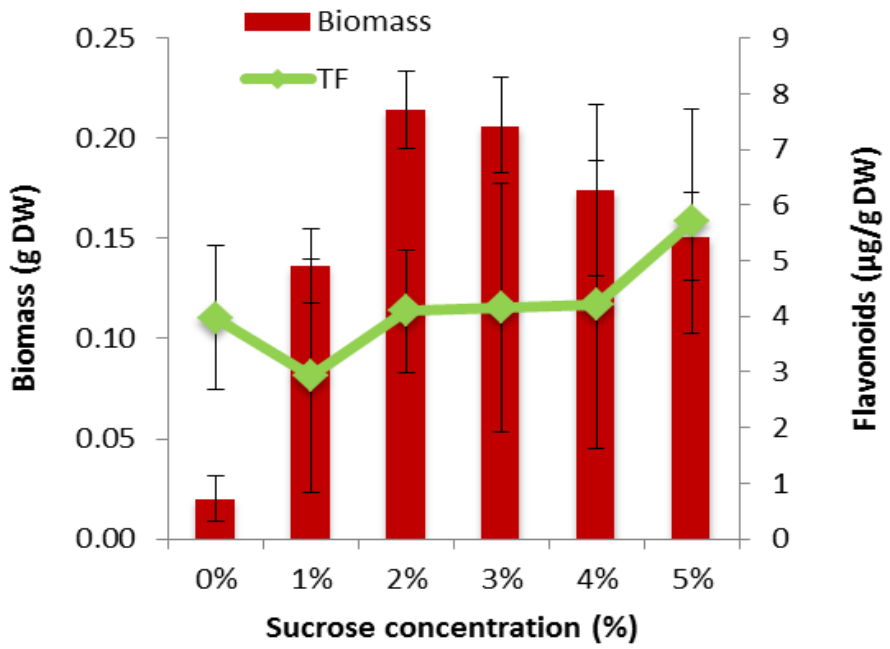


Figure 5.21: Effects of sucrose concentration on biomass and flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

Meanwhile, in determining the best sucrose concentration for flavonoids production in cell suspension cultures, it was found that sucrose concentrations did not significantly affect total flavonoids accumulation ($P < 0.05$). Five percent of sucrose concentration generated 5.70 ± 2.24 µg/g DW, 4% of sucrose concentration produced 4.21 ± 1.10 µg/g DW, 3% of sucrose concentration yielded 4.15 ± µg/g DW and 2% of sucrose concentration produced 4.10 ± 0.02 µg/g DW. Values of biomass of cell suspension cultures treated with 2 to 5% concentration of sucrose were significantly different from control (3.97 ± 1.29 µg/g DW) and 1% sucrose (2.92 ± 2.1 µg/g DW) ($P < 0.05$) (Figure 5.21).

Individual flavonoids showed highest production of alpinetin, pinocembrin, cardamonin, pinostrobin and panduratin A with 0.21 ± 0.02, 3.95 ± 1.71, 0.79 ± 0.65, 0.65 ± 0.65 and 0.90 ± 0.03 µg/g DW respectively. These values were observed within

2-5% of sucrose concentrations (Figure 5.22). The results also showed that the highest production of panduratin A at 5% of sucrose concentration was significantly different compared to other concentrations ($P < 0.05$) (Figure 5.22).

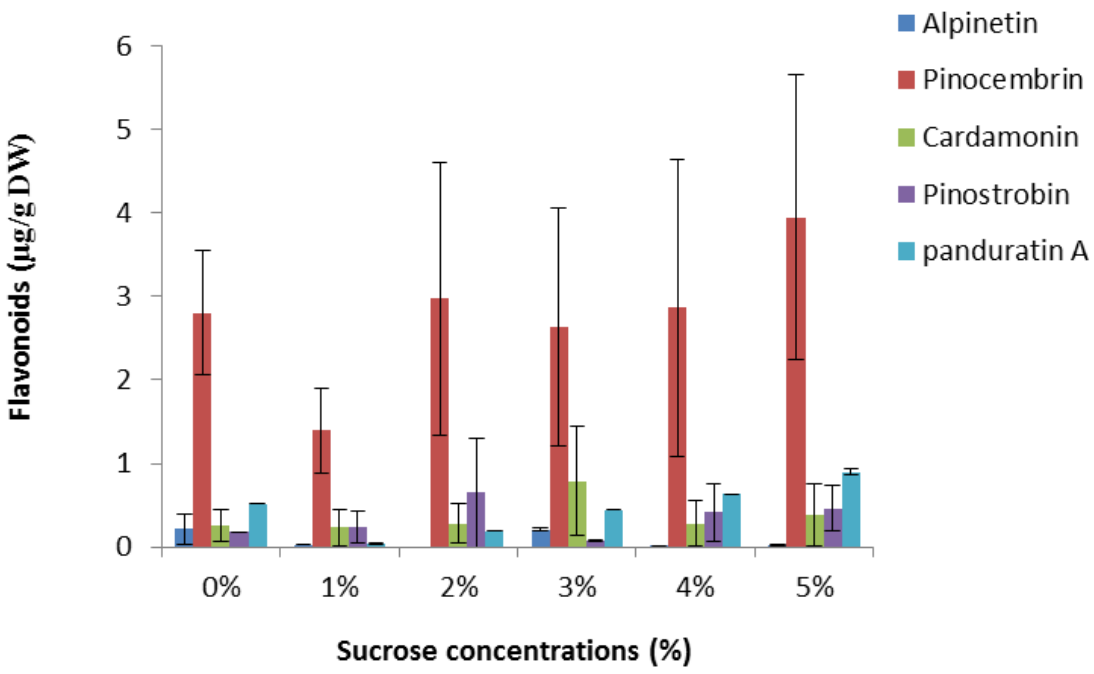


Figure 5.22: Effects of sucrose concentrations on individual flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

The present results showed that increase in concentration of sucrose resulted in increased production of individual flavonoids. Similar to our results, other researches have indicated that high sucrose level was favorable to the synthesis of secondary metabolites (Akalezi *et al*., 1999). Likewise, Weselake *et al*., (1998) have reported that the triacylglycerol content of oilseed rape cells increased about 8 times when the sucrose concentration in the growth medium was raised from 2 to 22% (w/v). Choi *et al*., (1994) also found that the optimum concentration was 3% to 5% but 7% of sucrose inhibited cell growth. On the other hand, Knobloch and Berlin (1980) showed that 8% sucrose was ideal for the indole alkaloid accumulation in cell cultures of *Catharanthus*

roseus. Ho *et al*., (2010) found that the optimum concentration was 3% to triterpine production by *Eriobotrya japonica.* An increase in initial sugar concentrations was also favorable to rosmarinic acid formation by *Coleus blumei* (Gertlowski and Petersen, 1993), *Agastache rugosa* (Kim *et al*., 2001) and *Lavandula vera* MM (Ilieva and Pavlov, 1997) cells, anthocyanin production by suspension cultures of *Perilla frutescens* (Zhong *et al*., 1994), saponin production by *Panax ginseng* (Wu and Zhong, 1999) as well as pinoresinol by *Forsythia x intermedia* (Schmitt and Petersen, 2002).

In contrast, *Aralia reptans* tend to show reduced secondary metabolites productions at higher sucrose concentration (Callebaut *et al*., 1990). In *Bacopa monnieri* shoot cultures, 2% sucrose favored biomass accumulation, whereas sucrose free medium accumulated maximum amount of bacoside A content (Naik *et al*., 2010).

Misawa, (1994) revealed that the most suitable carbon source and its optimal concentration for growth and the secondary metabolites accumulation are plant species and product dependent. Therefore, the conclusion from these contrasting results shows that the production of secondary metabolites was directly proportionately to the concentration of sucrose. Thus, this study reinforced the view that the response would depend on plant species.

5.3.8 Effects of combined optimized culture conditions on biomass and flavonoids production

Optimized parameters for biomass production were combined to see the final effects on cell growth (Table 5.1). As shown in figure 5.23, combined optimized culture conditions increased the biomass growth of cell suspension cultures of *B. rotunda*. In this study, combined optimized culture conditions and control experiment resulted in 0.25 ± 0.04 and in 0.20 ± 0.04 g DW/flask respectively. Whereas yield of flavonoids

was found significantly ($P < 0.05$) 3 folds higher under combined optimized culture conditions (26.01 ± 9.07 µg/g DW) compared to control experiment (7.77 ± 4.42 µg/g DW).

Table 5.1. Combination of optimized parameters.

Parameters	**Optimized**	**Control**
Inoculum size	2% cell suspension	1% cell suspension
Agitation	100 rpm	80 rpm
pH	5.8	5.8
Temperature	25	25
MS strength	Full	Full
PGRs	1 mg/l NAA and 1 mg/l BAP	Without PGRs
Sucrose concentration	20 g/l	30 g/l

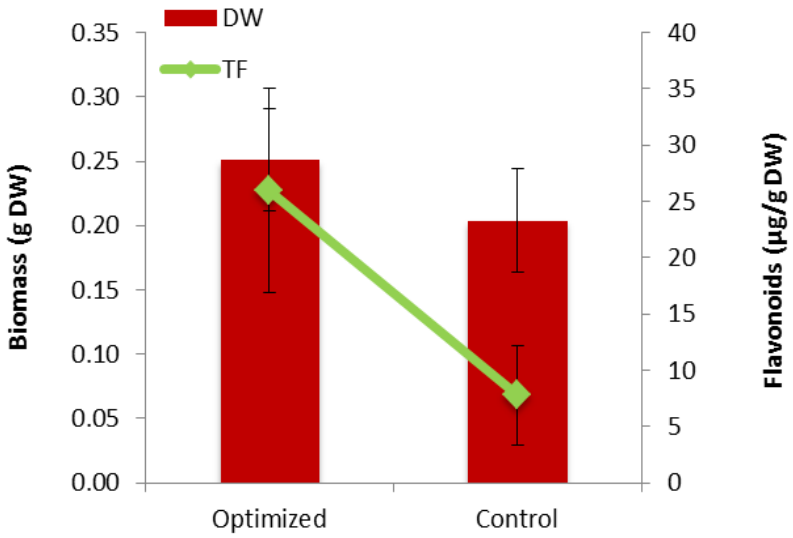


Figure 5.23: Effects of optimized culture condition on biomass and flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

The present results showed that combined optimized culture conditions produced significant ($P < 0.05$) increased in pinocembrin, pinostrobin and panduratin A production at 7.67 ± 2.26, 14.51 ± 2.41 and 1.56 ± 0.42 µg/g DW respectively (Figure 5.24). This optimized condition might be important to trigger the enzyme activities involved in the pathway.

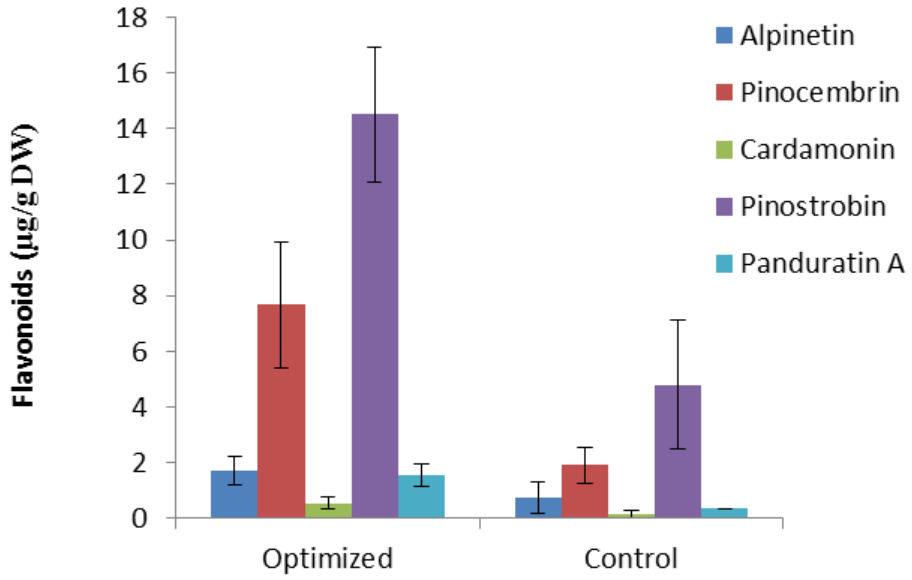


Figure 5.24: Effects of optimized culture conditions on individual flavonoids accumulation in cell suspension cultures of *B. rotunda* after 14 days of cultivation. Error bars indicates the standard deviation of mean values.

In conclusion, it is crucial to optimize various parameters of culture conditions in order to maximize biomass and flavonoids accumulation in cell suspension cultures of *B. rotunda*.

CHAPTER 6

SUSPENSION CULTURE ESTABLISHMENT AND PRODUCTION OF SELECTED FLAVONOIDS IN DIFFERENT REACTOR GEOMETRIES

6.1 INTRODUCTION

Boesenbergia rotunda suspension culture system displayed rapid growth and high metabolite synthesis, which provides possibility for exploiting resources by large-scale culture of cells. By using controlled bioreactor system, environment independent cultivation with several advantages is possible such as better control of the plant tissue and medium contact as well as aeration and medium circulation, filtration of the medium and the scaling up of the process. Five selected flavonoids with reported biological activity (Kirana *et al*., 2007, Tewtrakul *et al*., 2009) were detected in cell suspension cultures of *B. rotunda* in shake flasks. Following the establishment of these cell suspension cultures system, cell cultures with desired metabolites production were scaled up in controlled bioreactor in order to obtain high biomass and selected flavonoids accumulation in cell suspension cultures. This chapter describes the growth of cell suspension cultures and production of selected flavonoids *via* shake flasks, balloon, column and stir tank reactors. The objective of this chapter is to study the *B. rotunda* cell suspension cultures growth and their metabolites production in four types of reactors with different geometries.

6.2 MATERIALS AND METHODS

6.2.1 Cell suspension cultures

All cell suspension cultures were derived from the same source of established cell suspension cultures in Chapter 3 section 3.3.3.

6.2.2 Initial volume

Effects of initial inoculation volumes were observed at two levels of inoculums (15 ml of SCV as normal initial inoculation volume and 30 ml of SCV as double initial inoculation volume). The cells were left to settle down in Falcon tubes for 20 minutes prior to inoculation. This study was carried out in one liter total volume column reactor at 0.5 L/min air flow rate at room temperature and the SCV were observed at three days interval up to day 24. Maximum growth rate and final dry weight (DW) of cell cultures were recorded. Based on the growth development, initial inoculation volume were determined for all reactor studies.

6.2.3 Reactor cultivations

Cell suspensions were cultured in 250 ml shake flask (SF), 1 L column reactor (CR), 5 L balloon reactor (BR) and 2 L stir tank bioreactor (STR). The variables were listed in Table 6.1. To prevent the formation of large aggregates during cultivation, the cell suspension cultures were sieved prior to inoculation. After 14 days cultivation, cells were harvested for the biomass growth and determination of flavonoids content.

Table 6.1: Variables of bioreactor.

Reactor/Parameter	CR	BR	STR
Total working volume (ml)	700	700	1500
Air flow rate	0.5 (L/min)	0.5 (L/min)	10%
Cultivation temperature	Room temperature	Room temperature	25 ℃
Type of impeller	-	-	Two blade paddle impeller
Type of sparger	-	-	Bubble free aeration basket

6.2.3.2 pH and agitation

Effects of pH and agitation were determined in STR at four levels (pH 5 at 70 rpm, pH 5 at 90 rpm, pH 6 at 70 rpm and pH 6 at 90 rpm). The pH was regulated by automatic addition of 0.1 M NaOH or 0.1 M HCL throughout the culture period and the aeration was set at 10% O_2 pressure. The gas was fed *via* diffusion using aeration basket installed within the STR. This method of aeration allows for bubble-free aeration of the liquid medium while simultaneously reducing shear force due to bubble hydrodynamics.

6.2.4 Determination of cell biomass

The cells were harvested on day 14^{th} of cultivation. Determinations of cell biomass were carried out according to section 5.2.3, Chapter 5. The growth data were presented as g/l dry weight (DW). Three growth parameters were used for analyzing the biomass growth in suspension cultures *via* shake flasks and controlled reactors.

$$\text{Fold of biomass produced} = \frac{\text{Final DW - Initial DW}}{\text{Initial DW}}$$

$$\text{Growth yield } (Y_{x/s}) = \frac{\text{Final DW - Initial DW concentrations}}{\text{Initial sucrose – final sucrose concentrations}}$$

$$\text{Maximum growth rate } (\mu_{max}) \text{ (day)} = \frac{(\ln X_2 - \ln X_1)}{t_2 - t_1}$$

Where: X_1 was SCV at the end of log phase and X_2 represents SCV at early log phase.

6.2.5 Extraction and HPLC analysis

Extraction and flavonoids content were done as described in sections 4.2.4 and 4.2.5, Chapter 4. The production of flavonoids data were presented as µg compound(s)/g DW and yield of compound was calculated as below.

$$\text{Compound yield } (Y_{p/s}) = \frac{\text{Final compound - initial compound}}{\text{Initial sucrose – final sucrose concentrations}}$$

6.2.6 Statistical analysis

Statistical analyses were carried out as described in sections 5.2.5, Chapter 5, with 3 replicates.

6.3 RESULTS AND DISCUSSION

6.3.1 Effects of initial volumes on cells growth

Effects of initial inoculation volume were observed at two volumes of inoculums. The biomass data and growth profile are shown in Table 6.2 and Figure 6.1, respectively.

Table 6.2: Biomass production at different initial inoculation volumes.

Initial volumes (SCV)	g/l	Folds increase	μ_{max} (day)
Normal	5.96	8.68	0.11
Double	7.04	7.33	0.12

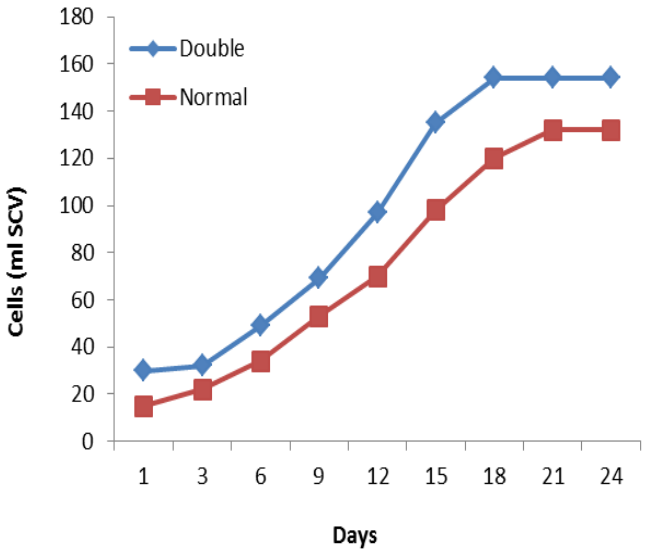


Figure 6.1: Effects of initial inoculation volume on biomass growth of cell suspension cultures after 14 days cultivation.

The biomass growth for two initial inoculation volumes exhibited similar growth profile, with insignificant lag phase (Day 1 to 3) during which the volume changed a

little, followed by a log phase (Day 4 to 18) when there was a rapid increase in cell volumes and finally in a stationary phase, the cell volume did not change further (Day 18-24).

Both volumes of inoculums used resulted in similar cell morphology and were light yellow in appearance until day 18. Cultures with normal initial inoculum volume (15 ml of SCV) turned light brown after 21 days of cultures. Inoculum volume of *B. rotunda* suspension cultures greatly affected cell vigour in column reactor. When the volumes of inoculums were doubled, *B. rotunda* suspension cells proliferated rapidly but the cells became brown relatively early (day 18). The cultures were then harvested at day 21 as the stationary phase is forced on the culture by depletion of the nutrients and possibly due to an accumulation of cellular wastes. If the culture is kept in stationary phase for a prolonged period the cells may die.

Both cultured cells showed a similar maximum growth rate i.e. 0.12/day and 0.11/day for doubled and normal SCV inoculums, respectively. Volume of inoculum was also found to affect the final yield of biomass. The final biomass for normal initial inoculation volume was 5.96 g/l DW with about 9 folds increase of initial volume whereas double initial inoculation volume produced final dry weight of 7.04 g/l DW with about 7 folds increase of initial volume. The results also suggested that log and stationary phases for single initial inoculation volume were extended in this experiment due to the small inoculation volume. By using single inoculation volume, stationary phase was delayed, log phase was extended, and delayed browning as compared to double inoculation volume of cell suspension. Based on these results, subsequent studies in reactor using cell suspension cultures of *B. rotunda* were inoculated at single initial volume.

6.3.2 Reactor cultivations

6.3.2.1 Biomass growth and flavonoids production

Studies on growth of the cultures revealed that the cells continuously divide in the entire reactor systems tested. Establishment of cell suspension cultures in SF, CR, BR and STR are shown in Figure 6.2a to b, Figure 6.3a to b, Figure 6.4a to b and Figure 6.5, respectively.

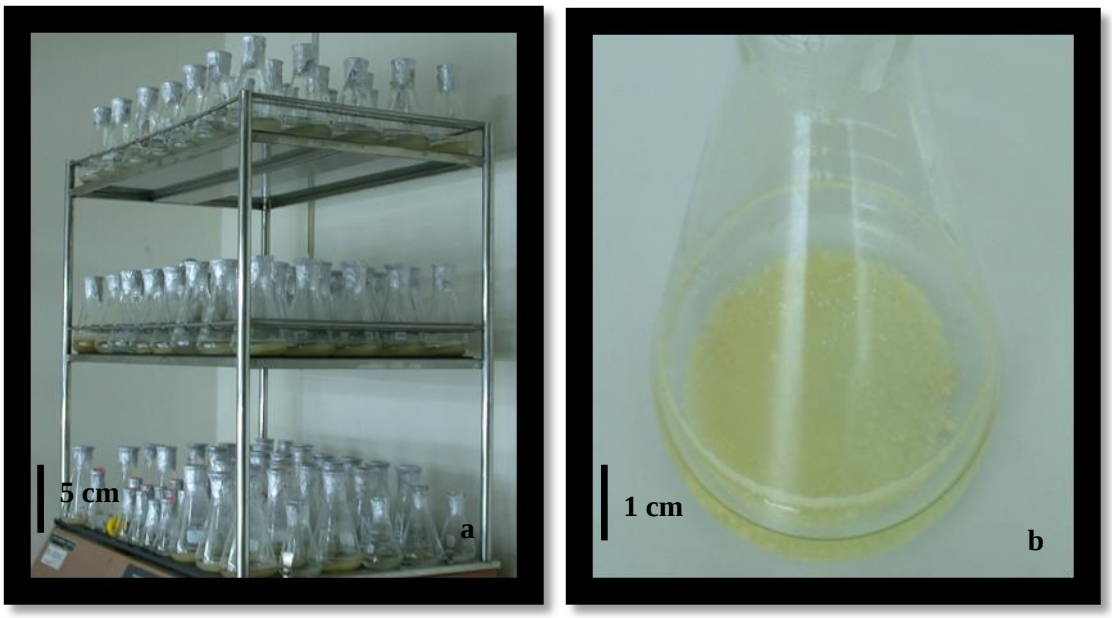


Figure 6.2: Establishment of cell suspension in 250 ml shake flasks. a) Cells were placed on shaker at 80 rpm. b) Cells in flask before transfer into reactors.

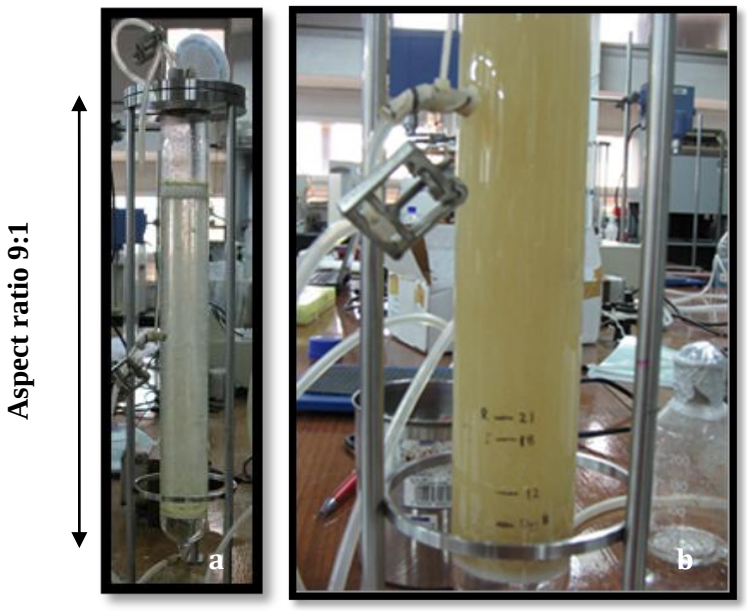


Figure 6.3: Establishment of cell suspension in column reactor. a) Cells at day 3 of inoculation. b) Cells after 21 days of cultivation. Aspect ratio (H:D) = X:X, where H; height and D; diameter.

Figure 6.4: Establishment of cell suspension in balloon reactor. a) Cells at day 3 of inoculation. b) Cells after 9 days of inoculation. Aspect ratio (H:D) = X:X, where H; height and D; diameter.

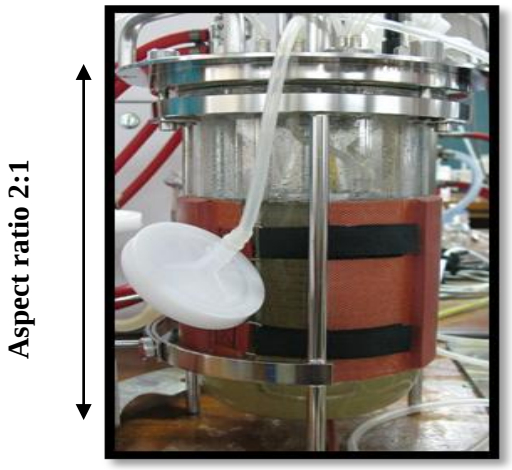


Figure 6.5: Establishment of cell suspension in stirred tank reactor at day 14 of cultivation. Aspect ratio (H:D) = X:X, where H; height and D; diameter.

The growth profiles of cell suspension cultures in SF, CR and BR are shown in Figure 6.6. In the BR, the cells grew exponentially from the start of batch cultivation and reached the stationary phase after 18 days. In the CR, the cells grew slightly slower compared to in BR. The cell growth in BR also reached the stationary phase after 18 days of cultivation. A dried deposit of *B. rotunda* cells on the circular wall above the liquid medium level in the reactor was observed, especially in the CR. The cells were cultured in MS medium for 14 days in the reactors before harvesting.

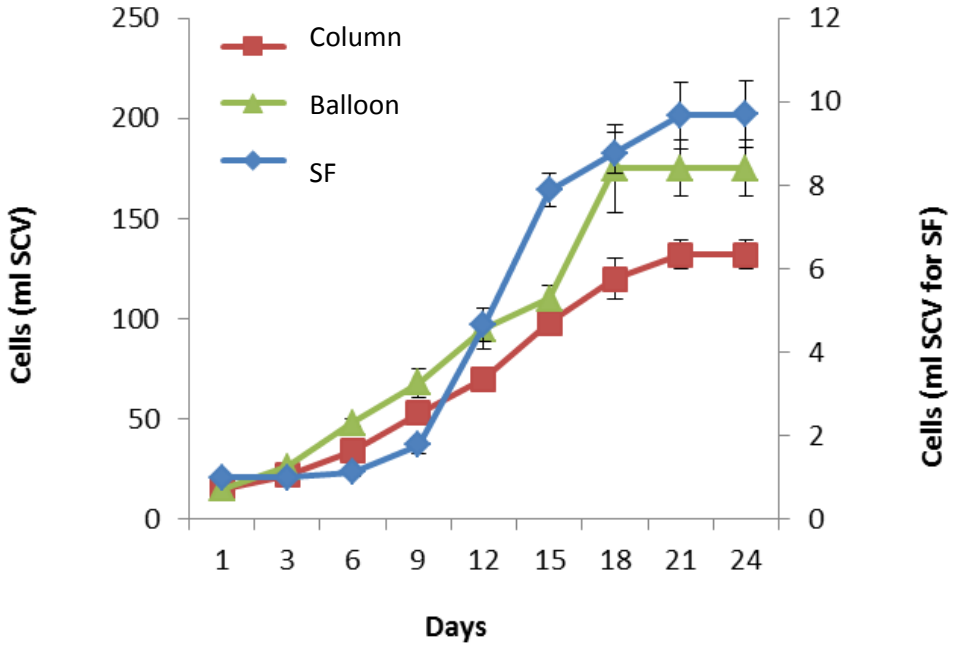


Figure 6.6: Growth profiles of cultured cells within 24 days of cultivation. Values are mean ± SD for 3 replicates.

Based on the profiles of growth, BR was a better bioreactor for the establishment of *B. rotunda* cell suspension cultures as compared to other reactor geometries tested. With normal initial inoculation volume, harvested cells in BR reached 6.36 ± 0.32 g/l DW whereas in CR 5.96 ± 0.19 g/l DW was obtained after 14 days of cultivation. Lowest final dry weight was found from STR, 2.26 ± 0.10 g/l DW. Eventhough total increment of cell growth in SF seem to be more than other reactors (CR, BR and STR, Table 6.3), there was no significant difference between the final dry weight of the cell suspension cultures in SF, CR and BR systems ($P < 0.05$). The final dry weight concentration of the SF cultivation was similar to that of the CR and the BR system (Table 6.3).

In terms of biomass folds, BR showed the highest increment followed by SF, CR and finally STR. Although it is not clear yet the exact reason for these observed differences,

it is suggested that enlarged cross-section of the top part of BR facilitated to dramatically decrease the generated foam layer from being accumulated and thickened, which could result in operational problems for air-driven reactors.

Highest maximum growth rate was observed in SF whereas the CR and BR showed similar maximum growth yield followed by the STR system. Although the growth yield was quite similar in SF, CR and BR, the rate was lowest in the STR at 0.08. The growth parameters are listed in Table 6.3.

Table 6.3: Comparative values of growth parameters in *B. rotunda* suspension cultures in shake flasks, column, balloon and stirred tank reactors.

Biomass	**SF**	**CR**	**BR**	**STR**
Final dry weight (g/l)	6.72 ± 0.28	5.96 ± 0.19	6.36 ± 0.32	2.26 ± 0.10
Folds	10.50	8.68	13.25	2.50
μ_{max}	0.25	0.11	0.12	0.07
$Y_{x/s\ (g/g)}$	0.22	0.20	0.21	0.08

Error bars indicates the standard deviation of mean values (*n*=3).

Currently, only a small number of plant cell cultures are commercially exploited for bioactive compounds production. The main problems are delaying the development of large scale cultivation of plant cell suspension cultures include poor throughput, cell line variability, and obstacles in the scale up process (Pan *et al*., 2000). In agreement with our result, a reduced productivity has often been reported during the scale up of plant cell cultures (Schlatmann *et al*., 1993). A decrease in biomass observed could be due to numerous factors for example shear stress, oxygen supply and gas composition.

Our studies showed that the growth differences between the air-driven reactors (CR and BR) and STR were significant ($P < 0.05$). This could be due to the application of mechanical agitation in STR which produces a high shear stress environment for plant cells (Pan *et al*., 2000). In air-driven reactors such as CR and BR, gas was introduced from the bottom to mix with the liquid medium and cells. In these air-driven reactors geometry, cells usually experience limited exposure to shear as compared to STR geometry.

Biomass and flavonoids production from different types of reactor systems are presented in Figure 6.7. Results showed that reactor types significantly affected the production of final biomass and total flavonoids ($P < 0.05$). The highest flavonoids concentration was observed in the CR followed by BR, SF and STR systems. The total flavonoids concentration of cells cultured in CR, BR, SF and STR was 9.95 ± 0.86, 8.97 ± 1.43, 4.66 ± 0.51 and 2.47 ± 0.41 µg/g DW, respectively.

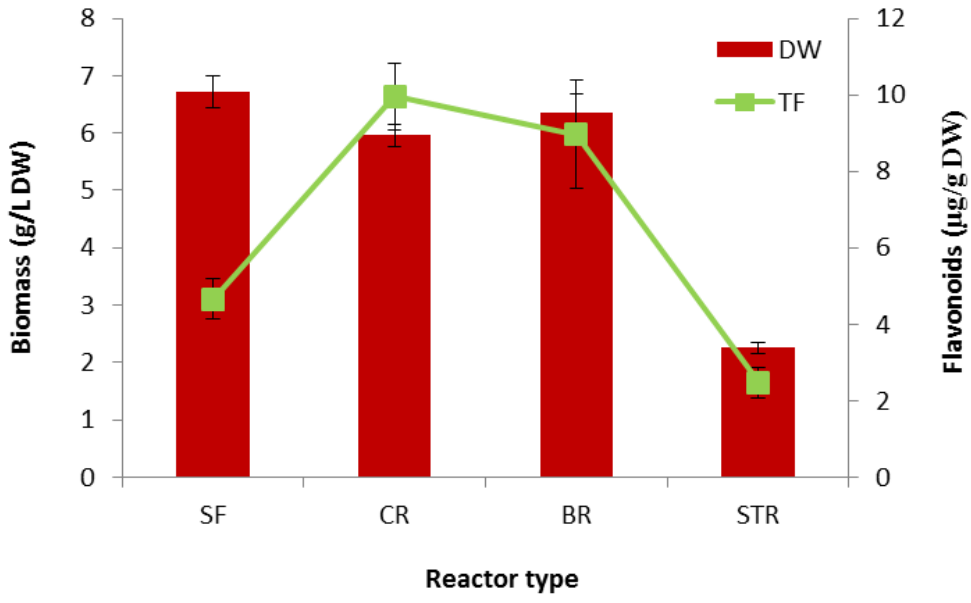


Figure 6.7: Biomass and total five flavonoids production from different types of reactors.

Comparison between the air-driven reactors (CR and BR) and STR, and also between air-driven reactors and SF were significant on flavonoids production ($P < 0.05$). However flavonoids content was similar between the CR and the BR systems. This could be due to the oxygen supply applied in these reactor systems which was significant in affecting secondary metabolite formation in plant cell cultures (Zhong *et al*., 1993).

One of the significant findings of this study was that the secondary metabolites production varied with the *in vitro* culture systems. In general, the secondary metabolite concentrations were inversely related with the biomass production; the highest biomass production system (SF>BR>CR) showed the lowest secondary metabolite concentration (SF<BR<CR). This observation did not apply to STR because it was observed that the culture turned brown and the metabolites production was inhibited upon transfer from shake flasks to a stirred tank reactor with mechanical agitation and diffusion-mode aeration. This result showed that good biomass growth was necessary for secondary metabolite production.

A similar trend was observed in the individual five selected flavonoids concentration where significantly high ($P < 0.05$) pinocembrin and cardamonin concentration was observed in air-driven reactors. Figure 6.8 and Table 6.4 showed five flavonoids formation in all types of reactors tested.

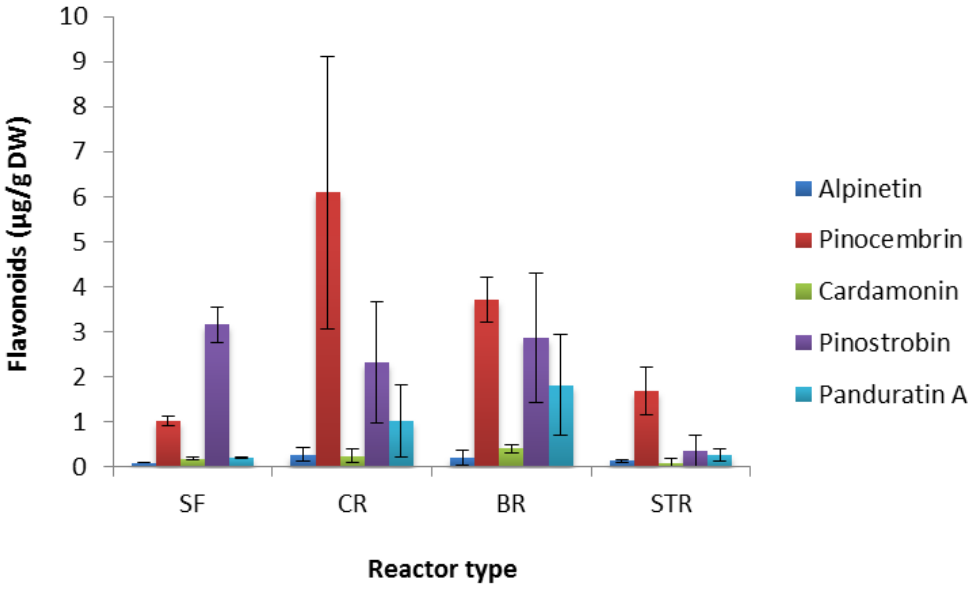


Figure 6.8: Five flavonoids produced from different types of reactor after 14 days of cultivation.

Table 6.4: Effects of reactor type and geometries on five flavonoids production in cell suspension cultures of *B. rotunda.*

	Alpinetin	**Pinocembrin**	**Cardamonin**	**Pinostrobin**	**Panduratin A**	**TF**
SF	0.09 ± 0.01	1.02 ± 0.11	0.18 ± 0.02	3.15 ± 0.39	0.20 ± 0.03	4.66 ± 0.51
CR	0.27 ± 0.14	6.09 ± 3.03	0.24 ± 0.14	2.33 ± 1.35	1.02 ± 0.79	9.95 ± 0.86
BR	0.19 ± 0.16	3.71 ± 0.51	0.40 ± 0.09	2.85 ± 1.43	1.81 ± 1.12	8.97 ± 1.43
STR	0.13 ± 0.02	1.67 ± 0.53	0.07 ± 0.13	0.34 ± 0.27	0.27 ± 0.14	2.47 ± 0.41

*Values are expressed in ug/g

In terms of the flavonoids production over sucrose ($Y_{p/s}$), air-driven reactors showed the highest production followed by SF and finally STR. These similar production trends of flavonoids were observed for almost all five selected compounds (Table 6.5).

Table 6.5: Flavonoids yields on sucrose in cell suspension cultures of *B. rotunda.*

	Alpinetin	**Pinocembrin**	**Cardamonin**	**Pinostrobin**	**Panduratin A**	**TF**
$Y_{p/s}$ (SF)	0.00	0.03	0.01	0.11	0.01	0.16
$Y_{p/s}$ (CR)	0.01	0.20	0.01	0.08	0.03	0.33
$Y_{p/s}$ (BR)	0.01	0.12	0.01	0.10	0.06	0.30
$Y_{p/s}$ (STR)	0.00	0.06	0.00	0.01	0.01	0.08

*Values are expressed in ug/g

The current studies examined different types of reactor geometries for their effects on the production of high quality *B. rotunda* cell suspension cultures with active metabolites. The optimization of these two main factors through biomass and bioactive compounds is required for an efficient, large-scale production of *B. rotunda* species. Reactor cultivation of *B. rotunda* cell suspension cultures is a relatively new area of research. Cell cultures of various species are used for mass production of specific metabolites in bioreactors but the production of Zingiberaceae species were scarce. To our knowledge, this is the first reported study of *B. rotunda* cells and bioactive compounds production in different reactor geometries. The first production of ginseng roots in a balloon-type bioreactor has been reported by (Choi *et al*., 2000).

This study demonstrated the possibility of growing cell suspension cultures in the BR and CR for large scale and rapid production of biomass which can be later exploited in order to optimize the flavonoids production. Further research is needed to assess the full potential of *in vitro* production of biomass growth for optimized production of flavonoid contents.

6.3.2.2 Combined pH and agitation

Effects of pH and agitation in STR on biomass growth and total flavonoids production are shown in Figure 6.9 and Table 6.4. Five selected flavonoids produced from different treatment combinations are shown in Figure 6.10.

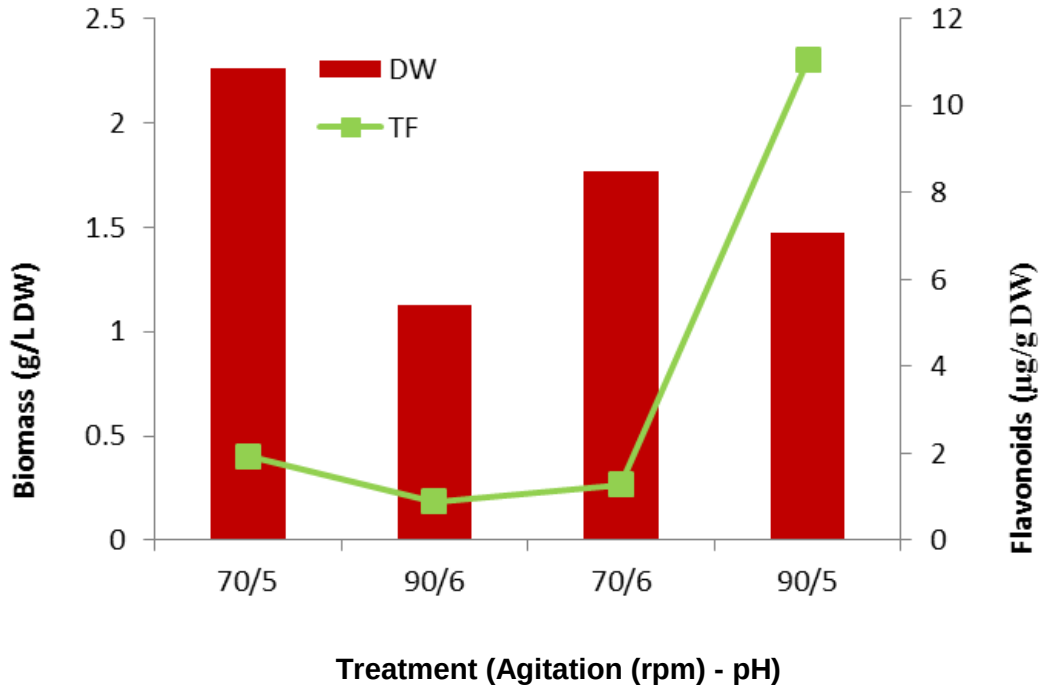


Figure 6.9: Biomass and total selected flavonoids production from treatment through STR.

To study the effect of combined agitation rate and pH value on the yield of biomass and flavonoids, the cells were cultivated using STR at 25 °C and 1 vvm aeration rate. The gas was fed *via* aeration bask*e*t allowing bubble-free aeration of the liquid medium. The final biomass growth reached a maximum value of 2.27 g/l under 70 rpm agitation and pH 5. The lowest biomass growth was at 1.13 g/l under 90 rpm agitation and pH 6. It was observed that the culture turned brown upon transfer from shake flasks to STR at this particular combination. The result suggested that low agitation rate i.e. 70 rpm (pH

5) was sufficient for mass transfer within the liquid cultures of *B. rotunda*. However, at the agitation rate up to 90 rpm (pH 5), elevated shear stress which may negatively influenced the biomass growth. pH 6 was not favorable towards the biomass growth as this pH both 70 and 90 rpm agitation rates produce low final yield of biomass (Table 6.6).

Table 6.6: Biomass and total flavonoids production in STR at different combinations of agitation and pH.

Agitation (RPM)/pH	DW (g/l)	TF (µg/g DW)
70/5	2.26	1.9
90/6	1.13	0.9
70/6	1.77	1.3
90/5	1.47	11.1

The flavonoids production at an agitation rate of 90 rpm, pH 5 reached maximum value at 11.05 µg/g DW. The lowest total flavonoids content was 0.89 µg/g DW extracted from cells agitated under 90 rpm at pH 6. From this study, the results suggested that increased agitation rate favors improved flavonoids production but at the same time greatly reduced the growth (Table 6.6). The increase in flavonoids production could be attributed to the elevated injury response of the cells when exposed to high shear hydrodynamics at 90 rpm. In this treatment pinocembrin reached maximum value followed by cardamonin, alpinetin, pinostrobin and finally panduratin A at 10.20, 0.51, 0.23, 0.11 and 0.0001 µg/g DW respectively (Figure 6.10).

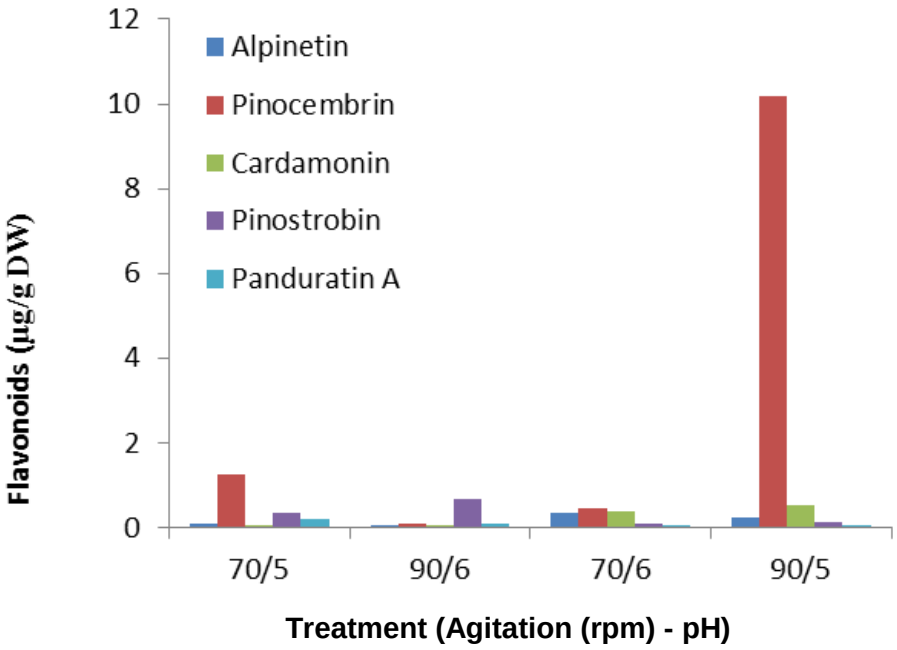


Figure 6.10: Selected five flavonoids from different combinations of agitation and pH in STR.

In the previous experiments, when the culture was grown without pH control, the pH in the medium varied in a range between 4.5 and 6.5 (data not shown). In the experiment with pH controlled, the biomass growth and flavonoids profiles were recorded. Similar with our study, Chandrashekar *et al*., (1999) showed that the controlled pH during cultivation process gave a better result than otherwise (Chandrashekar *et al*., 1999).

In the design of a reactor process, parameters such as agitation and aeration, pH, dissolved oxygen and inoculum levels were significantly important (Jüsten *et al*., 1996). Among them, the effects of agitation and pH carried out in this study were among the most critical parameters in the scale-up process and played significant roles in determining the productivity of the process.

The major roles of agitation is to improve mixing, mass and heat transfers in reactors. Although an increase in agitation offer better mixing and mass/heat transfer effects,

further increase could also result in high shear stress environment and may lead to many negative effects, such as rupture of cells, vacuolation and autolysis, and decrease in productivity (Cui *et al*., 1997).

In this study it can be concluded that some degree of compromise were required in order to balance between good biomass growth and flavonoids production. Based on the data, an obvious approach would be to cultivate the cell suspension at 70 rpm, pH 5. Once sufficiently high biomass was obtained, the agitation could be increased to 90 rpm (pH 5) to elicit more flavonoids production by the cells.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

7.1 GENERAL DISCUSSION

Plant secondary metabolites are the most significant source for drugs and medicines. They are of significant value to human because they form the basis of aroma, flavouring and colouring of food spices, drink and beverages when isolated and purified from their host plants (Collin, 2001). *In vitro* propagation of medicinal plants with enriched bioactive principles and cell culture methodologies for selective metabolite production is believed to be highly useful for commercial production of pharmacologically important compounds. In view of this, some of the approaches to enhance the yield of bioactive compounds have been examined and discussed in this study for *Boesenbergia rotunda* (L.) Mansf. Kulturpfl.

A successful protocol was developed for mass propagation of *B. rotunda* with 90% of the explants producing multiple shoots (5 shoots per explant) within 10 to 14 days on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l as a source of meristem explants for callus induction. Subsequently, callus were induced from the meristem explants on MS medium supplemented with 1.0 mg/l D-biotin, 1.0 mg/l IAA, 2.0 mg/l 2,4-D, 1.0 mg/l NAA, 30.0 g/l sucrose and 2.0 g/l gelrite which revealed that a meristem derived callus were capable of producing 100% embryogenic callus within three months. This protocol provides a complete efficient system for the establishment of cell suspension cultures for the production of targeted compounds.

Selected compounds (pinostrobin, pinocembrin, cardamonin and alpinetin) were profiled and compared to profiles obtained from field grown *B. rotunda* rhizomes. All the five targeted flavonoids were successfully detected in both callus and cell suspension cultures. However, flavonoids content was significantly lower in *in vitro* cultures compared to the intact rhizomes ($P < 0.05$). The presence of these compounds is significant for further exploitation in enhancing the accumulation of the flavonoids in *in vitro* cultures.

In an attempt to enhance biomass growth and flavonoids accumulation, different initial inoculation volumes, agitation, temperature, media strength, carbon sources and their concentrations, pH values and plant growth regulators (PGRs) were studied. Accumulation can be enhanced or reduced by changing the composition of nutrient medium, more specifically the concentration and nature of the inorganic nitrogen and phosphate, carbon supply and growth regulators.

In cell suspension cultures, the highest biomass growth production was observed at initial pH 5.8. Initial inoculation volume at 2% (v/v) produced the highest biomass yield and flavonoids. Studies were also performed to see the effects of cultivation temperature on biomass growth and flavonoids production, which showed the highest biomass was produced at 25 °C whereas cultivation temperature at 20 °C produced highest total flavonoids. Highest biomass growth and TF were achieved in shake-flasks cultured at 100 rpm. Plant growth regulator that helped to increase the cells biomass yield was the combination of NAA and BAP at a concentration of 1.0 mg/l. Flavonoids enhancement on the other hand required addition of 2,4-D and BAP at the concentration of 5 mg/l and 1 mg/l. Sucrose is the most important carbon source for the development of plant cell cultures and biosynthesis of secondary metabolites. In this study, sucrose was found to enhance growth of cell suspension cultures of *B. rotunda*, but not for the flavonoids

accumulations. Sucrose at 2% (w/v) produced the highest biomass. However, sucrose concentrations higher than 2% gradually reduced growth. Meanwhile, in determining the best sucrose concentration for flavonoids production in cell suspension cultures, it was found that different levels of sucrose concentrations tested did not significantly affect the flavonoids accumulation ($P < 0.05$).

Effects of the combined optimized culture conditions on the biomass and flavonoids production were also investigated. In this study, the biomass of cell suspension cultures was increased in the optimized treatments examined compared to control experiment. In terms of flavonoids production in cell culture, the five targeted flavonoid constituents were significantly ($P < 0.05$) enhanced in the optimized treatment with the highest enhancement shown in pinostrobin, followed by pinocembrin, alpinetin, panduratin A and finally cardamonin production. Therefore, it can be concluded that a combination of these optimized factors is a very useful strategy in enhancing the flavonoids production particularly the pinostrobin production in cell suspension cultures of *B. rotunda.*

This study have been able to demonstrate that cell suspension cultures of *B. rotunda* propagate successfully in a balloon reactor with an increase of 13 folds in biomass relative to the initial biomass. HPLC analysis revealed that the total flavonoids production was significantly higher ($P < 0.05$) in air-driven reactors (balloon and column geometries) as compared to shake flasks. The result indicated that an efficient protocol for the mass production of *B. rotunda* biomass could be achieved *via* reactor culture of cell suspension cultures and could be used as a source of medicinal raw materials. Effects of different volumes of inoculum in column reactor were also studied. Increased inoculation size, vessel size and geometries were correlated with biomass. Nevertheless, reactor feasibility studies need to be carried out in which the optimization

of the parameters such as oxygen and aeration affects, shear rate, number of impellers as well as antifoaming agent.

The results from this study provide more research avenues in the area of metabolomics, proteomics and transcriptomics. Cell suspension developed can be used as a system to provide reproducible and sustainable material for the respective studies. From proteomics and transcriptomics data, targeted enzymes in the pathway will be identified. The genes involved will be either down regulated or over expressed to enhance the targeted compounds. Technologies that could be adopted are genetic engineering and RNAi technology for knocking off gene expression to enhance the flavonoids production in *B. rotunda.*

7.2 CONCLUSIONS

In conclusion, this study has successfully established cell suspension cultures of *B. rotunda* for the production of biomass and five selected flavonoids. The productivity of biomass growth and selected flavonoids was found to be dependent on culture condition, growth regulators and carbohydrate sources. These findings suggested that *B. rotunda* cell cultures required full strength MS medium, combination of 1 mg/l NAA and BAP and moderate level of sucrose, cultivated at 25 °C and agitated at 100 rpm. Other parameter which was optimal for biomass growth and selected flavonoids production was inoculum density. Our study successfully demonstrated the production of selected flavonoids in large scale using reactor cultures. Viable procedure for the mass production of *B. rotunda* cell biomass is possible *via* reactor culture and can also be used as a source of secondary metabolites.

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