EVALUATION OF FACTORS AFFECTING THE CELL SUSPENSION CULTURES AND THE PRODUCTION OF BIOACTIVE COMPOUNDS OF *Spilanthes acmella* L.

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
2, 4-D	2, 4-dichlorophenoxyacetic acid
Picloram	4-amino-3, 5, 6-trichloropicolinic acid
Zeatin	4-hydroxy-3-methyl-trans-2-butenylaminopurine
2-iP	N^6-(2-isopentyl) adenine
BA	6-benzylaminopurine
IAA	3-indole-acetic acid
PBA	6-(benzylamino)-9-(2-tetrahydropyrany)-9H-purine
ANOVA	Analysis of Variance
w/v	Weight per volume

PENILAIAN FAKTOR-FAKTOR YANG MEMPENGARUHI KULTUR AMPAIAN SEL DAN PENGHASILAN BAHAN-BAHAN BIOAKTIF DARIPADA S*pilanthes acmella*L.

ABSTRAK

Dalam kajian ini, lima turunan kultur kalus telah diperbangunkan daripada petiol *Spilanthes acmella* L. Kultur ini diinduksikan pada medium pengaruhan kalus, medium Murashige dan Skoog (MS) yang ditambahkan dengan 0.5 mg/L 2, 4-D. Eksplan-eksplan petiol juga dikulturkan pada medium MS yang ditambahkan dengan 0.5 mg/L 2, 4-D serta kandungan pikloram yang berbeza (0.0 – 2.0 mg/L). Pengolahan kalus pada medium dengan kehadiran pikloram adalah kurang berbanding dengan kalus yang dihasilkan dengan kehadiran 2, 4-D sahaja. Kalus padat serta berbutiran dihasilkan secara berterusan pada setiap kitaran subkultur. Kalus berasal daripada sel turunan L4 yang bersifat lembut and rapuh digunakan sebagai bahan permulaan untuk penyediaan pengkulturan ampaian sel, dengan medium cecair yang mempunyai komposisi yang serupa. Pemerhatian terhadap kinetik pertumbuhan sel menunjukkan lengkungan 'sigmoid' dan kedua-dua berat basah dan kering telah menjangkau jisim sel yang tertinggi selepas 15 hari pengkulturan. Sel berwarna kuning yang dihasilkan pada kitar subkultur yang kelapan menghasilkan lebih kurang sepuluh kali ganda jisim permulaan 0.5 g. Penambahan sukrosa (30 g/L) dalam medium kultur didapati menyokong biojisim sel yang tertinggi berbanding dengan kepekatan sukrosa yang lain. Olahan sel dengan kasein hidrolisat (3.0 g/L) telah membuktikan peningkatan jisim basah dan kering apabila elisitor tersebut ditambahkan pada hari pengkulturan pertama dan kelapan. Pertumbuhan sel ditambahbaik apabila ekstrak yis digunakan dan

ditambahkan pada hari pengkulturan yang kelapan. Akan tetapi, penambahan chitosan ke dalam kultur sel menunjukkan kesan negatif kerana ia tidak menggalakkan sebarang pertumbuhan sel. Ekstrakan etanol daripada anak benih *in vitro*, sel kawalan serta sel olahan elisitor telah dianalisis dengan GC/MS. Kompaun seperti *N*-isobutyl-(2*E*, 4*Z*, 8*Z*, 10*E*)-dodecatetraenamide, stigmasterol, butylated hidroksitoluene dan asid 9, 12-oktadekadienoik telah ditemui pada semua sampel yang diuji.

EVALUATION OF FACTORS AFFECTING THE CELL SUSPENSION CULTURES AND THE PRODUCTION OF BIOACTIVE COMPOUNDS OF *Spilanthes acmella* L.

ABSTRACT

In this study, five lines of the callus culture were established from the petioles of *Spilanthes acmella* L. The culture was initiated on callus induction medium, Murashige and Skoog (MS) medium supplemented with 0.5 mg/L 2, 4-D. The petiole explants were also cultured on MS medium with 0.5 mg/L 2, 4-D and different concentrations of picloram (0.0 – 2.0 mg/L). Callus initiated on the medium with the presence of picloram induced lesser callus when compared with the callus initiated with only 2, 4-D. Compact and granular calluses were continuously produced with each subculture cycle. The soft and friable callus line L4 was used as the initial material for the establishment of cell suspension culture in the liquid medium with the same composition. Observation on growth kinetic of the cells showed a sigmoid curve and both fresh weight and dried weight reached the highest cell mass after 15 days of culture. The yellow-coloured cells produced nearly ten fold cell mass from the initial weight of 0.5 g at the 8^{th} subculture cycle. The addition of sucrose (30 g/L) in the medium was found to support the highest cell biomass compared to other concentrations of sucrose. Cells treated with casein hydrolysate (3.0 g/L) proved to enhance fresh and dried cell mass, when the elicitor was added on the first and the 8^{th} day of culture. The growth of cells was improved when the yeast extract treatment was applied on the 8^{th} day of culture. But, the addition of chitosan into the cell culture showed negative effect as it did not encourage any cell growth. Ethanol extracts from the *in vitro* plantlets, control cells

and elicited cells were analyzed by GC/MS. Compounds such as *N*-isobutyl-(2*E*, 4*Z*, 8*Z*, 10*E*)-dodecatetraenamide, stigmasterol, butylated hydroxytoluene and 9, 12-octadecadienoic acid were found in all the tested samples.

1.0 INTRODUCTION

Ever since the evolution from 300 million years ago, plants have been one of the most precious possessions on earth. Numerous plants have been playing a key position in human. Plants are natural sources of food additives, attires, flavours, shelters, colourings and medicines for the basis of human civilization and most importantly providing living creatures with unlimited fresh air. With the advanced technologies, novel and exciting findings, scientists all over the world have discovered and unfolded the uses of plants that are able to keep the survival of humans.

Our ancestors knew that plants have the powerful remedial properties through serendipitous observations and experiences with many trials and blunders. Gurib-Fakim (2006) noted a few of the traditional medicines systems, for example, the Ayurvedic, Unani and the Chinese and others since antiquity. Devanesen and Maher (2003) believed that the traditional health systems practiced by the Aborigines in Australia are related to their cultures and beliefs. Until today, some of these traditional medicines are still being practiced and becoming a trend as a need for daily health care or therapy. This indicates that in the near future more and more people would prefer plant-derived medicines instead of using synthetic drugs.

Countless plants from various families have been recognized and used as medicinal herbs in traditional practice of healing. *Spilanthes acmella* is one of them. According to Hind and Biggs (2003), plants from the genus of *Spilanthes* are related to the Sunflower. This is because *Spilanthes* spp. are members of the tribe Heliantheae, sub-tribe Ecliptinae (Hind and Biggs, 2003; Chandra *et al*., 2007). The major constituent from these species is an isobutylamide. Despite containing this

compound (also known as spilanthol), chewing the *Spilanthes acmella* will cause temporary numbness together with a tingling sensation. These plants are utilized for treating toothache, gum and throat infections. They are also widely used as insecticidal agent and are served as dishes (Chung *et al*., 2008).

Despite the demand for herbal medicines is increasing from time to time, Kumar and Gupta (2008) reported that the yearly demand for herbal medicines in the global market is 5 – 15 %. The primary and secondary products of medicinal plants with curing properties are greatly sought after, especially in the pharmaceutical industry. However, the bioactive compounds obtained from plants are limited and can be fluctuating (Wilken *et al*., 2005). Therefore, in the process of obtaining these high-value plant substances, there are a few obstacles that need to be beaten. For instance, these useful plants resources from the wild are diminishing, the deficiency of professional knowledge with incomplete information about each and every plant (especially on the biosynthesis pathway) as well as high in labour cost.

For that reason, plant tissue culture is playing an important role in order to overcome those barriers. Since the discovering of plant tissue culture, this technique has been used in plant propagation and conservation. Meanwhile, the cell suspension culture system is an alternative for producing bioactive compounds from the medicinal plants. Tissue culture technique requires less space, the conditions on growth are controllable, the clones and the mother plant are identical as well as the elimination of other factors such as climate, season and eradication of viruses, bacteria and fungi from the mother plant.

Studies have been done on determination of chemical compounds, from the field and cloned plants of *Spilanthes acmella*. However, not much published

research papers used plant cell culture technique to produce the active compounds from *S. acmella*. Therefore, the objectives of this research are:

1. To determine the subculture effect on stability of *S. acmella* callus cell line.
2. To initiate the cell suspension culture of *S. acmella*.
3. To study the effect of elicitors on cell suspension culture of *S. acmella*.
4. To detect the desirable compounds from the mother plant, *in vitro* plantlets, callus and cell suspension cultures of *S. acmella*.

2.0 LITERATURE REVIEW

2.1 *Spilanthes acmella* L.

2.1.1 Classification and Plant Morphology

Based on the plant taxonomy by United States Department of Agricultural (USDA), *S. acmella* is classified as follows:

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Asteridae
Order	:	Asterales
Family	:	Asteraceae (daisy family)
Genus	:	*Spilanthes* Jacq.
Species	:	*S. acmella* (L.)
Binomial name:		*Acmella oleracea* (L.) R. K. Jansen

S. acmella (Figure 2.1) is a member of the tribe Heliantheae. This species is a flowering annual (in temperate provinces) or could be a short-lived perennial herb. It can grow and reaches 60 cm tall. This plant has green to red, erect and usually decumbent stems. Leaves are simple, opposite in pairs, with long but flattened petioles (2 – 6.5 cm). The blade is broadly ovate to deltate (Chung *et al*., 2008). Leaf margins are dentate and hair appendages are found on the upper and lower leaf surfaces. Numerous disc florets are yellow in colour. Each flower with five petals

form tight cones. Inflorescences of *S. acmella* are solitary, located at the terminal and axillary pedunculate capitula (Hind and Biggs, 2003). Tan (2004) noted that roots are often seen at lower nodes. This plant can be propagated either by seeds or stem cuttings (Chandra *et al*., 2007).

Figure 2.1 *Spilanthes acmella* L.

2.1.2 Habitat and Distribution

S. acmella L. (syn. *Acmella oleracea* L.), belongs to the Asteraceae family (formerly being recognized as Compositae). To date, about sixty species of *Spilanthes* have been reported from various parts of the world including India (Chandra *et al*., 2007). Chung *et al*. (2008) documented the occurrence of five species of *Acmella* (*A. brachyglossa, A. ciliate, A. oleracea, A. paniculata, and A. uliginosa*) in Taiwan. *S. acmella* is native to the tropics of both Africa and South America. It is believed that the species is extremely ancient, dating perhaps to the time before the drifting of the continents.

S. acmella is also known as paracress (after the Brazilian province, Pará) or toothache plant. The other common names include Eyeball Plant, Spot Plant, Brazil Cress, Alphabet Plant and Australian Cress. In Hindi, it is known as Akarkara (Chandra *et al*., 2007). The French named this plant as 'brède mafane' and 'cresson de Para' while in Portuguese as agrião do Pará and jambú (Bosch, 2004). *S. acmella* are called 'subang nenek' by the local Malays and 'phak khraat huawaen' in Thai (Pitasawat *et al*., 1998).

This plant crops up in the wild and can be cultivated as well. This plant has been growing in the northern and southern hills and plateaus in India (Pandey and Agrawal, 2009). It is widely scattered throughout all the provinces. It requires moist soil and can be found in damp pastures, at swamp bounds, on rocks near the sea and also cultivated in herbal gardens (Chan *et al*., 2005).

2.2 Plant Derived Active Compounds

2.2.1 Bioactive Compounds From *Spilanthes acmella*

Alkamides can be obtained from plants in the Aristolochiaceae, Asteraceae, Brassicaceae, Convolvulaceae, Euphorbiaceae, Menispermaceae, Piperaceae, Rutaceae and Solaraceae families (Rios-Chavez *et al*., 2003). The pungent principle that caused the taste of *S. acmella* plant to be 'hot' is believed from the compound known as spilanthol (Figure 2.2). Yasuda *et al*. (1980) elucidated spilanthol from *S. oleracea* as *N*-isobutyl-2, 6, 8-decatrienamide. The same component was isolated from *S. acmella* (Baruah and Leclercq, 1993).

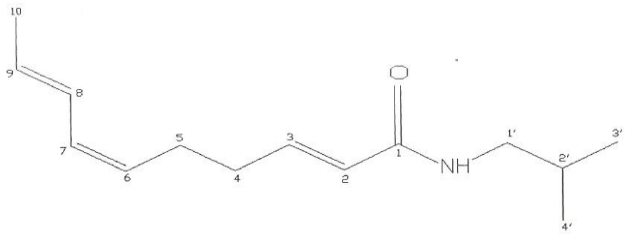

Figure 2.2 Spilanthol (Moulik, 2006)

Besides spilanthol, new amides, 2-methyl-butylamide, (*Z*)-non-2-en-6, 8-diynoic acid isobutylamide and (*Z*)-dec-2-en-6, 8-diynoic acid isobutylamide were isolated from *S. oleracea* (Greger *et al*., 1985). Nakatani and Hagashima (1992) determined three alkamides, (2*E*)-*N*-(2-methylbuthyl)-2-undecene-8, 10-diynamide, (2*E*, 7*Z*)-*N*-isobuthyl-2, 7-tridecadiene-10, 12-diynamide and (7*Z*)-*N*-isobuthyl-7-tridecene-10, 12-diynamide from *S. acmella* L. var. *olearacea* Clarke. Both studies also reported that spilanthol was identical with affinin (Figure 2.3). Molina-Torres *et al*. (1999) noted that the roots of *Heliopsis longipes* contained the affinin.

Figure 2.3 Affinin (Molina-Torres *et al*., 1999)

The isolation of spilanthol, undeca-2*E*, 7*Z*, 9*E*-trienoic acid isobutylamide and undeca-2*E*-en-8, 10-diynoic acid isobutylamide from *S. acmella* were carried out by Ramsewak *et al*. (1999). These compounds were isolated from the hexane extract of the flower buds of the plant.

A total of eight alkylamides were identified through HPLC (High Performance Liquid Chromatography) separation and later confirmed with NMR

(Nuclear Magnetic Resonance) spectroscopy on the ethanolic *S. acmella* extracts (Bae, 2007). Those alkylamides were: (2*Z*)-*N*-isobutyl-2-nonene-6, 8-diynamide, (*2E*)-*N*-isobutyl-2-undecene-8, 10-diynamide, (2*E*)-*N*-(2-methybutyl)-2-undecene-8, 10-diynamide, (2*E*, 7*Z*)-*N*-isobutyl-2, 7-tridecadiene-10, 12-diynamide, (2*E*, 6*Z*, 8*E*)-*N*-(2-methylbutyl)-2, 6 8-decatrienamide, (7*Z*)-*N*-isobutyl-7-tridecene-10, 12-diynamide, (2*E*, 4*Z*)-*N*-isobutyl-2, 4-undecadiene-8, 10-diynamide and (2*E*, 4*E*, 8*Z*, 10*E*)-*N*-isobutyl-dodeca-2, 4, 8, 10-tetranamide. Other than that, Moulik (2006) suggested that the bioactive compounds from *S. acmella*, the isobutylamides, also present in *Echinacea purpurea*. There was no bolt from the blue with the finding as both plants are from the same family (Asteraceae).

2.2.2 Medicinal Usage of *Spilanthes acmella*

S. acmella has been used as a masticator for toothache and gum diseases. Revathi and Parimelazhagan (2010) reported the use of *S. acmella* (L.) Murr. by the Irula tribal in Hasanur Hills (Southern Western Ghats), Tamil Nadu, India to cure oral ailments. Through chewing onto the plants, mainly the flower heads and the leaves will cause a tingling buzz of the tongue, lips and gum, followed by numbness to the mouth with profuse salivation. Older generations had discovered the utility of this ancient plant for treating toothaches (Hebbar *et al*., 2004).

Singh *et al*. (2009b) reported that *S. acmella* possess anti-inflammatory, antibacterial, antifungal and antimicrobial properties. Chakraborty *et al*. (2009) also found that *S. acmella* showed anti-inflammatory property and with analgesic potential as well. Traditionally, this plant is also used in treatment of flu, cough and tuberculosis (Burkill, 1966; Singh *et al*., 2009a). Stammering children were treated with this plant (Saraf and Dixit, 2002). In addition, the same plant was used for the healing of dysentery and rheumatism (Baruah and Leclercq, 1993).

2.2.3 Larvicidal and Anti-Malarial Potential

In Thailand, there are at least 24 species belonging to four genera of mosquitoes that are responsible in transmission of malaria, filariasis, Japanese encephalitis and dengue (Insun *et al*., 1999). *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* are among the vector mosquitoes which are responsible for the transmission and caused outbreak of dengue fever and filariasis in tropical and subtropical regions. The latest trend in producing larvicidal, pesticidal or insecticidal agents is focused on their effectiveness and safety to human being and their toxicity level that may introduce harmful consequences to the environment and the future generations. However, the relying on many synthetic compounds has caused the resistance from the common vectors. Therefore, plant-derived compounds that possessed the properties against mosquito larvae, pupae and adults are remarkable alternatives. Further research with improved formulations unquestionably would be eco-friendly and more sustainable where it should be utilized in any vector control curriculum.

Methanol extract of *S. mauritiana* (Jondiko, 1986), petroleum ether extracts of *Vitex negundo* L. (Karunamoorthi *et al*., 2008), acetate extract from leaves of *Achyranthes aspera* (Bagavan *et al*., 2008) and chloroform-methanol extracts of mature leaves from *Solanum villosum* Mill (Chowdhury *et al*., 2008) showed effectiveness against mosquito larvae. Pavela (2008) reported that the methyl alcohol extracts from *Otanthus maritimus* and *Ammi visnaga* showed promising larvicidal effect on *C. quinquefasciatus*. β-sitosterol found in *Abutilon indicum* (Linn) Sweet was found to possess mosquito larvicidal activities (Rahuman *et al*., 2008a). *Ficus racemosa* Linn tree bark proved to have larvicidal effects against *C. quinquefasciatus* (Rahuman *et al*., 2008b). Rios-Chavez *et al*. (2002) reported that

species from Asteraceae family possessed insecticidal, fungicidal and antibacterial properties. Pitasawat *et al.* (1998) investigated ten species of carminative plants on killing *C. quinquefasciatus*. Amongst the ten plants, *S. acmella* Murr. showed potential of larvicidal properties (Insun *et al*., 1999).

Saraf and Dixit (2002) used the isolation of spilanthol from *S. acmella* Murr to test the larvicidal activity on *A. aegypti* Linn, *Anopheles culicifacies* Giles and *C. quinquefaciatus* Say. The result showed complete mortality of the eggs, larvae and pupae from the three species of mosquitoes tested.

The isolation of artemisinin from *Artemisia annua* L. (a Chinese medicinal herb) is another well-known antimalarial plant that showed amazing results against the mosquitoes (Liu *et al*., 2006). Other than that, Goel *et al*. (2007) proved that *A. annua* is the only source which is not resisted by *Plasmodium falciparum*, a malarial parasite. From that study, they also concluded that monoterpenes is rich in the essential oils obtained from the petals and leaves of this plant. According to Aivazi and Vijayan (2009), the acetate extract from *Quercus infectoria* was found to be the most effective against the fourth instar larvae of *Anopheles stephensi* Liston.

2.2.4 Other Usages of *Spilanthes acmella*

Recently, plant extract from *S. acmella* was found to contain one of the constituents required in anti-wrinkle products that claimed to have 'botox-like effect' (Demarne and Passaro, 2008). *S. acmella* extracts have also been used in several products under the label Jurlique®, such as Herbal Recovery Night Mist, Calendula Lotion, and Arnica Cream and the rest (www.jurlique.com). Other than that, *S. acmella* extracts were also marketed for daily oral consumption by a few

herbal extract companies, which were reported to be able to improve our immune system (Bae, 2007).

2.3 *In Vitro* Culture Techniques

2.3.1 Plant Tissue Culture

From time to time, the practical application of new technologies in agricultural trounce over problems faced, especially the conventional way in propagating plants. The first scientific paper in plant tissue culture published by Gottlieb Haberlandt in 1902 has been an eye-opener to the world (Collin and Edwards, 1998). Since then, lots of plants have been studied and from there, plant tissue culture technique is considered as a very useful tool in pushing the advance of propagation practices to the extreme.

The principle of totipotency refers the capability of plant cells and tissues to divide and to regenerate. That explained the capability of small group of aseptic cells, tissues or even organs to transform into a new plantlet. However, it is not easy to obtain disinfected tissue from non-sterile surroundings and the proliferation will only continue when transfer the tissues in antiseptic condition to new fresh medium regularly.

The success of a culture system is highly depended on the nutrient media. The composition of a culture medium consists of major inorganic nutrients, micro elements, iron, vitamins, carbon source, organic nitrogen and plant growth regulators. The macro nutrients comprise of nitrogen, phosphorous, calcium, potassium, magnesium and sulphur while the micronutrient salts are ferum, manganese, zinc, boron, cuprum, cobalt and sodium. The vitamins stock is made up of thiamine, glycine, nicotinic acid and pyridoxine acid. Generally, small amount are

needed from both the micronutrients and vitamins. It is essential to provide the *in vitro* plants, calluses or cells with carbon source as energy. According to George and Sherrington (1984), sucrose is the best carbon source along with other options such as glucose, fructose, galactose, maltose, raffinose or mannose. The most commonly used basic medium in plant micropropagation is Murashige and Skoog (MS) (1962). Later on, many designated media were formulated from this medium by manipulating the concentrations of elements involved.

Plant growth regulators are applied to the medium, in order to improve the development of the plants and the process of morphogenesis. These plant growth regulators are only needed in a very low quantity. A number of plant growth regulators, such as auxins, cytokinins, gibberellins, ethylene and abscisins, are available for plant growth. However, auxins and cytokinins are among the most commonly used in plant tissue culture.

In general, auxins are used to enhance the growth of callus or cells, promote the formation of roots and embryogenesis. 3-indole-acetic acid (IAA) is the only natural auxin whereas synthetic auxins include 2, 4-dichlorophenoxyacetic acid (2, 4-D), 3-indolebutyric acid (IBA), 1-naphthylacetic acid (NAA), and 4-amino-3, 5, 6-trichloropicolinic acid (picloram). Cytokinin, another important plant growth regulator, encourages the division of cells as well as adventitious shoot formation. There are two natural cytokinins, 4-hydroxy-3-methyl-trans-2-butenylaminopurine (zeatin) and N^6-(2-isopentyl) adenine (2-iP). Kinetin (KN), 6-benzylaminopurine (BA), 6-(benzylamino)-9-(2-tetrahydropyranyl)-9H-purine (PBA), tidiazuron and adenine are some of the synthetic cytokinins found in the market.

Over the past few years, several studies were done on *S. acmella*. Saritha *et al*. (2002) were able to get multiple shoots formation of *S. acmella* Murr. in MS

medium supplemented with 0.5 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA. With the similar use of plant growth regulators, Saritha and Naidu (2008) obtained direct shoot regeneration using leaf as explant. Ang and Chan (2003) micropropagated *S. acmella* L. using the aseptic axillary buds as explants and multiple shoots were formed on MS medium containing only 2.0 mg/L BA.

Singh *et al*. (2009b) regenerated the *S. acmella* L. plants using nodal segments with transverse thin cell layer (tTCL) culture system in MS supplemented with 5.0 mg dm^{-3} BA. Other than that, shoot tips of *S. acmella* L. were rejuvenated into new plants by alginate-encapsulated technique (Singh *et al*., 2009a). Leaf explants from *in vitro S. acmella* L. seedlings were used and formed calluses on MS + 1 µM NAA + 10 µM BA. Later on, differentiation occurred where shoot buds transformed to shoots without the application of NAA (Pandey and Agrawal, 2009).

2.3.2 Callus Culture

Callus is recognized as morphologically unstructured tissue. Callus can be obtained through wounding on most plant parts. Normally, this kind of injury is used as defense mechanism and happens due to the existence of birds, animals or insects from the wild. However, callus can also be induced via culture under germ-free environment. Unorganized development of cells could be seen when explants were cut and placed on the culture medium. Types of tissue may be varied and explant such as leaves, stems, roots, seeds and others can be used for initiation of callus. Lim (2008) reported that the presence of either endogenous or exogenous of plant growth regulators can also trigger the induction of callus by controlling the process of differentiation.

Different plant parts may produce dissimilar characteristics of callus, which may diverse from soft and friable to hard and compact or coexistence of both in the same culture system. The colours of calluses may differ from each other as well, depending on the plant parts, concentrations of plant growth regulators used and most importantly the species of the studied plant. As stated by Salman (2002), green callus derived from the leaf explants of *Gypsophila paniculata* was initiated by inoculating onto solid MS medium supplemented with 1.0 mg/L 2, 4-D and 0.2 mg/L BA. The fresh weight of the hard callus increased twice the amount after four weeks of culture. The increment of biomass only stopped when reached the fifth week. With a few passages of subculture, the callus became soft and attained the highest growth on the same initiation medium. Llamoca-Zárate (1999) reported that more calluses were induced from the cotyledons of *Opuntia ficus-indica* compared to the hypocotyls. The optimum growth of the soft, white-yellow calluses was obtained when subcultured on solid MS medium with 0.9 µM kinetin, 2.3 µM 2, 4-D, 1.0 µM picloram, 400 mg/L casein hydrolysate and 3 % sucrose. The calluses were subcultured on this selected friable callus medium (FCM) every three to four weeks before the establishment of its cell suspension culture system. Callus from *Fagopyrum esculentum* was red in colour due to the presence of anthocyanin. The callus was initiated from the hypocotyl segments of the plant (Moumou *et al*., 1992). The callus was maintained on Gamborg's B5 medium supplemented with 100 mg/L myo-inositol, 100 mg/L casein hydrolysate, 0.5 mg/L pyridoxal, 0.5 mg/L nicotinic acid, 0.1 mg/L thiamine, 0.2 mg/L glycine, 0.5 mg/L kinetin and 2.0 mg/L 2, 4-D. The callus was transferred to the new medium at every three week intervals and two procyanidins were synthesized, procyanidin B2 and procyanidin B2-3'-*O*-gallate. Zhao *et al*. (2001) stated that three types of calluses were produced from the stems

and leaf explants of *Catharanthus roseus*, comprising the compact callus clusters, friable half-closed and hollow callus clusters. The induction medium was MS medium supplemented with either of the combinations: NAA and kinetin or 2, 4-D and kinetin. Surprisingly, the content of indole alkaloids was higher in the compact callus clusters. Therefore, it showed that compact calluses obtained from studies should not be ignored. Young leaves taken from the *in vitro* seedlings of *Eucommia ulmoides* were used to induce callus production (Miyanaga *et al*., 2004). Three different callus aggregates, daughter aggregate (DA), meristemoid-like aggregate (MA) and old aggregate (OA), were formed on Gamborg' s B5 medium supplemented with 0.1 mg /L 2, 4-D and 40 g sucrose.

2.3.3 Cell Suspension Culture

Although unorganized cells could be inoculated on solid medium as callus, it can be dispersed into liquid medium with the culture vessel agitated on rotary shaker operating from 80 – 120 rpm. The motion of agitating is to break up small clumps of cells into more single cells, provide better air circulation for the cells and improve the absorption of nutrients from the medium. The cell suspension culture system has better growth rate compared to the solid medium system. Normally, the composition of nutrients in solid medium is similar to the liquid medium except the addition of agar for gelling purpose.

The growth of cells normally shows a typical sigmoid curve and divided into the initial lag phase, exponential phase and stationary phase. Cells divide quite slowly at the beginning of the culture, when the cells are first transferred to a new and fresh medium. The vigorously divided cells at the exponential phase will reach the optimum or highest yield and followed by very slow or non-dividing of cells at

the stationary phase. From the growth curve, it is possible to determine or identify the optimum time of harvesting the cells as well as the time for subculturing. The measurement for cell growth can be determined by determining the fresh and dry weight of the cells.

The initiation of cell suspension culture is usually from callus. Moreover, the composition of liquid medium normally similar to the callus culture medium. From the study conducted by Cusidó *et al*. (1999), callus induced from stems of *Taxus baccata* was inoculated on Gamborg's B5 medium supplemented with double concentrations of vitamins, 0.02 mM 2, 4-D, 0.004 mM kinetin and 0.001 mM GA_3. The medium for maintaining the *T. baccata* cells was unchanged; the only alteration was the use of triple concentrations of B5 vitamins and obliteration of the phytagel. In a similar study, Tan (2004) found that the best medium for cell suspension culture of *S. acmella* was the same as the induction medium for its callus.

2.4 Production of Secondary Metabolites via *In Vitro* Culture Technique

2.4.1 Cell Suspension Culture For Production of Secondary Metabolites

Plant products like alkaloids, anthocyanins, flavonoids, quinines, lignans, steroids and terpenoids have been considered as marketable products from the plants. These secondary metabolites are usually used in the pharmaceutical industries as well as in manufacturing flavors, perfumes, colourants and others (Verpoorte *et al.*, 2002). However, desirable product yields from plants are not always satisfactory. Therefore, optimization of the growth media (nutrient composition) and elicitation could be applied for consistent and higher production of secondary metabolites via *in vitro* culture systems.

Many studies on *in vitro* production of secondary metabolites have been carried out. Sánchez-Sampedro *et al*. (2005) reported the accumulation of silymarin from cell culture of *Silybum marianum* L. Through the study done by Wong *et al*. (2004) the production of ajmalicine from plant cell culture of *C. roseus* was accumulated. Songsak and Lockwood (2004) noted that both *Nasturtium montanum* and *Cleome chelodonii* produced higher level of glucosinolates via the cell suspension culture technique. Paclitaxel, an anticancer drug, could be extracted from the cell suspension culture of *Taxus chinensis* (Zhang and Xu, 2001; Luo and He, 2003; Wang and Wu, 2004). In a recent study conducted by Kang *et al*. (2009), production of bilobalide, ginkgolides A and ginkgolides B were accumulated in the cell culture of *Ginkgo biloba*.

Other than obtaining plant secondary metabolites through the technique of cell suspension culture, organ culture can be another option. Palazón *et al*. (2003) reported that the secondary product obtained from hairy root lines of *Panax ginseng* was the best way for ginsenosides (triterpenic saponins) accumulation after a few attempts on different culture techniques were used. Betalaines, as described by Savitha *et al*. (2005) as a red water-soluble food dye were found in hairy root culture of *Beta vulgaris*.

A consistent and higher production of secondary metabolites from plant cell cultures can be achieved with elicitation. There are two classes of elicitors, the biotic and the abiotic. Biotic elicitors are recognized as compounds from microbial origin while physical factors such as temperature, UV light and metal ions are considered as abiotic elicitors. They are believed to be able to enhance plant secondary products. As proposed by Savitha *et al*. (2005), the utilization of elicitors commercially is reflected as a strategically important approach. Park *et al*. (2006) also commented on

the usage of elicitors to increase the yield of secondary metabolite effectively. There are plenty of successful studies which proved that elicitation feeding efficiently increased the production of secondary metabolites. Dignum and Verpoorte (2002) reported the production of glucovanillin from *Vanilla planifolia*. The study was conducted by using three different plant families, *V. planifolia, Ilex dumosa* and *Catharanthus roseus*. Vanillin was fed into the three lines of cells on the tenth day, 12 hours before the harvesting process. With that, 49.7 % of glucovanillin was obtained. Chong *et al*. (2005) applied various types of elicitors into the cell suspension culture of *Morinda elliptica*. For that study, the elicitation treatments affected the cell growth as well as the content of anthraquinone. The enhancement of tocopherol accumulation was successfully presented in both suspension culture of *Helianthus annuus* L. and *Arabidopsis thaliana* (Gala *et al*., 2005).

Sugar, in the form of sucrose, fructose or glucose, is frequently used in boosting the production of plant secondary metabolites. Larronde *et al*. (1998) concluded that with the existence of sucrose in cell suspension culture of *Vitis vinifera* had increased the accumulation of anthocyanins. Sucrose added into freshly sub-cultured cells (five to seven days), which was at the end of the exponential period, produced higher cellular response. In the study conducted by Davoren *et al*. (2002), *Brassica napus* L. cells were cultured in three different concentrations of sucrose, consisting 2 %, 6 % and 14 % (w/v) in the growth media. From the results, they concluded that sucrose did enhance the yield of tricylglycerol and acyl-CoA: diacylglycerol acyltransferase. As reported by Park *et al*. (2006), the amount of sanguinarine from cell culture of *Eschscholtzia californica* also increased as high as 5.7 folds due to the addition of sucrose.

Chen and Chen (2000) used yeast extract on *Salvia militiorrhiza* cells with the increased production of cryptotanshinone but the accumulation of rosmarinic acid was reduced. For the induction of phyenylethanoid glycosides (PeGs) in cell suspension culture of *Cistanche deserticola*, Cheng *et al*. (2005a) also utilized yeast extract as the elicitor. The cells were repeatedly treated with addition of yeast extract on the 15, 17 and 19 days of culture. From there, there was a significant improvement on the production of PeGs. In the cell suspension culture of *Glehnia liltoralis*, yeast-extract treated S-type plant cells (from southern past of Japan) accumulated six-fold higher of bergapten production than the yeast-extract treated N-type cells (from northern part of Japan) (Kitamura *et al*., 1998). Yan *et al*. (2005) also conducted a study on the hairy root cultures of *S. miltiorrhiza* using yeast extract. The results showed that the total content of three tanshinones (cryptotanshinone, tanshinone I and tanshinone II A) was increased with 100 mg/L of yeast extract.

Chitosan may be a distinguished weight loss supplement for humans. It also can be used for improving the growth and secondary metabolites accumulation in cell suspension cultures. In general, chitosan is derived from the shells of crustaceans (crabs, shrimps and others). Cheng *et al*. (2005b) reported that by adding chitosan as elicitor into the cell suspension culture of *Cistanche deserticola* resulted in the increased production of PeGs with 3.4 times higher than the control cells. Zhang *et al*. (2007) compared the elicitor-treated and non-elicitor-treated cell suspension culture of *Taxus chinensis* with chitosan and found that the growth and the productivity of paclitaxel were increased with the addition of chitosan. The addition of 50 g/L chitosan into *Azadirachta indica* cells produced the highest accumulation of azadirachtin content (Prakash and Srivastava, 2008).

Chen *et al*. (2006) pointed out that casein hydrolysate is not necessarily required in the cell suspension culture of *Vitis labrusca*. The result from this study was varied from other similar studies besides the exception of using cells from *V. vinifera*. Other than that, jasmonic acid is also extensively used in many cell suspension culture studies. For instance, treatment of jasmonic acid (JA) in cell suspension culture of *Helichrysum kraussii* produced α-amyrin and β-amyrin (terpenoids) but were never found in cells which were not treated (Prinsloo and Meyer, 2006). Miguel-Chavez *et al*. (2007) also compared the elicited cells (by adding JA) with the control. The results from their study showed the productivity of alkaloids in the cell culture of *Erythrina americana* Miller increased.

2.4.2 Detection of Secondary Metabolites via Chromatographic Techniques

Chromatographic methods such as paper chromatography (PC), thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC) can be used in general separation and purification of plant compounds. Normally, either single or combined operations from the different techniques may be needed in order to separate certain classes of compound (Harborne, 1998).

There are two types of GC, gas-liquid chromatography (GLC/GC) and gas-solid chromatography (GSC). The use of GC can be used on both qualitative and quantitative analysis.

Compounds from tested samples will be separated in between two phases, the mobile gaseous phase and the liquid or solid stationary phase which is in the column. In the mobile gaseous phase, helium is the most commonly used but other gases like argon, nitrogen and hydrogen can be a substitute as carrier gas. Generally,

a packed column or capillary column can be utilized in GC. Each type of column is classified by the length, inside diameter, efficiency, sample size, relative pressure, relative speed, flexibility and the inertness from the chemical.

Mass Spectrometry (MS) is one of the many detectors that can be incorporated into the GC. In the research done by Safaei-Ghomi *et al*. (2009), the antioxidant activity of essential oil and methanol extracts of *Thymus caramanicus* Jalas was detected by both GC and GC/MS analysis. From that study, 15 components were identified and quantified. The major component contained in the oil is carvacrol. In another study done by Hu and Zhong (2007), GC/MS was used on the quantification of JA in the cell culture of *Panax notoginseng*. Li *et al*. (2009) studied the essential oil from *Smallanthus sonchifolia*. The chemical analysis was performed using GC/MS. They claimed their study was the first investigation on the leaves of the plant, where a total of twenty one chemical constituents were identified.

GC/MS is proposed for the detection of bioactive compounds (spilanthol) in plants, callus and cell culture of *S. acmella*.

3.0 MATERIALS AND METHODS

3.1 Callus Culture of *Spilanthes acmella*

3.1.1 Plant Material

The *in vitro* plantlets of *S. acmella* were obtained from Plant Tissue and Cell Culture Laboratory of School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The plantlets were maintained on MS + 3 % sucrose + 0.8 % (Agar Type 100, ALGAS, Chile) but without any plant growth regulator. The pH of the medium was adjusted prior to 5.75 with 0.1 N NaOH (Natrium hydroxide) solution or 0.1 N HCl (Hydrochloride acid) before sterilized using autoclave machine (Tomy SS-325 Autoclave) at 121 ℃ and 1.05kg/cm^3 pressure for 11 minutes. Single nodes excised from the *in vitro* plantlets were used for subculturing. The cultures were placed in the culture room under continuous lighting with cool, white fluorescent tubes at an intensity of 2000 - 2500 Lux (measured with Lux meter Litron model LX-101) regulated at 25 ± 2 °C. Regular subculturing were performed every five weeks on the same fresh medium. Abnormal plantlets (with short internodes or twisted stems) were eliminated.

3.1.2 Selection and Induction of Callus Culture

Petioles of 1 cm in length were excised from four weeks old *in vitro* plantlets of *S. acmella*. The petiole explants were placed on solid MS medium supplemented with 0.5 mg/L 2, 4-D, a callus induction medium formulated by Tan (2004). Approximately 30 mL medium of the culture medium added with 0.8 % (w/v) agar was used in each 150 mL glass vessel. Two petiole explants from each different plantlet were weighed and inoculated into each glass vessel and six experimental units were used for each individual plantlet. After six weeks of culture, the calluses

were weighed, separated from the petiole explants and transferred onto free callus culture medium. The calluses induced from each plantlet were considered as a callus line. A total of five lines was created and the amount of callus produced after six weeks of culture was determined. The data was analyzed using One-Way Analysis of Variance (ANOVA) and followed by mean comparison using Tukey (HSD) Test at $p \leq 0.05$ to determine where the callus induction ability from the different callus line was similar.

3.1.3 Establishment of An Optimum Callus Induction Medium

Petioles (1 cm in length) were excised from four weeks old *in vitro* plantlets of *S. acmella*. They were cultured into 150 mL glass vessel containing MS medium supplemented with 0.5 mg/L 2, 4-D and picloram (0, 0.5, 1.0, 2.0 and 2.5 mg/L). Six replicates were used for each combination of plant growth regulators and two explants were cultured into each culture vessel. The initial weight of the petiole explants was recorded. The cultures were placed in the culture room maintained in the same condition as mentioned as in section **3.1.1**. The biomass of the induced calluses was weighed after six weeks of culture. Data collected were statistically analyzed using One-Way Analysis of Variance (ANOVA) followed by a comparison of mean using Tukey (HSD) at $p \leq 0.05$.

3.2 Proliferation of Callus

3.2.1 Effect of Subculture on Proliferation of Callus

The petiole-derived callus (0.5 g) of the five selected lines (L1-L5) was subcultured onto fresh solid MS medium supplemented with 0.5 mg/L 2, 4-D every four weeks. The callus cultures were maintained under the same condition described

as in **3.1.1**. The increased in callus mass was measured at every subculture cycle until the 5th subculture cycles. Six experimental units were used for each callus line and the growth for each line was recorded at every four week subculture interval. The texture and the colour of the calluses were also observed.

3.2.2 Effect of Subculture Frequency on *Spilanthes acmella* Callus on MS Medium Supplemented with 0.5 mg/L 2, 4-D and Picloram

Friable calluses (0.5 g) of line M1 – M5 were inoculated onto MS medium + 0.5 mg/L 2, 4-D and different concentrations of picloram (0 - 2.5 mg/L). The calluses were subcultured every four weeks. The fresh biomass of the calluses was determined from the 1st until the 5th subculture cycles. Six experimental units were used on each combination medium. The morphology and the colour of the calluses were also observed at each subculture cycle.

3.3 Establishment of Cell Suspension Culture of *Spilanthes acmella*

3.3.1 Preparation of Cell Suspension Culture

Cell suspension culture was established by transferring 1.0 g of soft, friable callus (line L4) into 100 mL Erlenmeyer flasks containing 25 mL of liquid MS medium supplemented with 30 g/L sucrose and 0.5 mg/L 2, 4-D, the optimum callus proliferation medium. The cultures were continuously agitated on a rotary shaker (G10 Gyrotary Shaker®, New Brunswick Scientific Co. Inc., N. J., U.S.A) at 120 rpm (revolution per minute). They were maintained at 25 ± 2 °C with 24 hours photoperiod with light intensity of 2000 - 2500 Lux. After three weeks of culture, the cells were filtered through a metal sieve (diameter: 850 µm). Filtered cells were then separated from the liquid medium through vacuum filtration on a Buchner

funnel layered with Whatman® No. 1 filter paper (90 mm diameter) and joined with a water pump (Eyela Aspirator 3A-S, Tokyo Rikakikai Co. Ltd.). Subcultures were done on a 14-day cycle by using 0.5 g of cells and inoculated into 25 mL of fresh medium.

3.3.2 Establishment of Growth Kinetics of *Spilanthes acmella* Cells

Cell biomass of 0.5 g of loose and fine cells were transferred to each 100 mL Erlenmeyer flask cointaining 25 mL of liquid MS medium + 0.5 mg/L 2, 4-D. The cultures were placed on a rotary shaker in the culture room with the same conditions as in **3.3.1**. Three flasks of cells were harvested and weighed every three days for a period of 30 days. The harvesting process was exactly the same as described in **3.3.1**, except the cells were not to be filtered through the metal sieve again. The fresh and dried cell biomasses were determined at each harvesting time. The dried cell biomass was recorded after air-dried at 25 ± 2 °C until constant weight was attained.

3.3.3 Effect of Subculture Frequency on Cell Growth

A fresh weight of 0.5 g of cells was cultured into MS + 0.5 mg/L 2, 4-D medium. The cells were subcultured every 14 days until the 7 subculture cycles. On each subculture cycle, the cells of six experimental units were weighed. The growth index (GI) was determined at each subculture cycle based on the following formula:

GI = [final weight – initial weight] / initial weight

3.4 Elicitation Effect on Cell Suspension Culture of *Spilanthes acmella*

3.4.1 Effect of Sucrose

A fresh cell biomass of 0.5 g of 14 days old homogenous cells were cultured into 100 mL Erlenmeyer flasks containing 25 mL of liquid MS medium + 0.5 mg/L