MICROPROPAGATION, ESTABLISHMENT OF CELL SUSPENSION CULTURES AND EVALUATION OF ANTIOXIDANT ACTIVITIES OF *Gynura procumbens*

by

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ABSTRAK

Eksplan tunas *Gynura procumbens* yang aseptik berasal daripada tunas aksilari pokok induk yang tumbuh di ladang, boleh dihasilkan melalui pensterilan-permukaan selama 20 minit dengan 20 % Clorox®. Pucuk berbilang dapat dihasilkan dalam medium MS yang dibekalkan dengan 2.0 mg/L BA dengan min pucuk sebanyak 14.7 ± 0.7 daripada setiap tunas eksplan selepas empat minggu pengkulturan. Apabila kepekatan BA yang dibekalkan ke dalam medium MS dikurangkan kepada 1.5 mg/L BA, min pucuk berbilang yang agak tinggi masih dihasilkan (13.9 ± 1.3 pucuk per eksplan) dan penghasilan pucuk abnormal yang kurang. Kitar pengsubkulturan yang optimum ialah empat minggu. Semua pucuk mikro berumur empat minggu menghasilkan akar selepas dikulturkan dalam medium MS pepejal selama enam hari. Semua anak pokok *in vitro* hidup selepas aklimatisasi di dalam rumah hijau. Medium MS yang dibekalkan dengan 2 mg/L Picloram merupakan medium yang terbaik untuk penghasilan kalus daripada eksplan daun. Kalus membiak dengan baik dalam medium MS yang dibekalkan dengan 0.5 mg/L Picloram dengan menghasilkan min biomassa sebanyak 7.874 ± 0.413 g selepas empat minggu pengkulturan. Dalam 14 kitar pengsubkulturan, penghasilan kalus menjadi mantap dari segi biomassa selepas pengsubkulturan yang ke-empat. Kalus yang terhasil adalah kuning muda dan berciri rapuh. Kultur ampaian sel boleh didapati dengan menginokulasikan 0.5 g kalus rapuh ke dalam 20 mL medium MS yang dibekalkan dengan 0.5 mg/L Picloram dan 60 g/L sukrosa. Sel membiak

dengan baik di bawah pembekalan cahaya yang berterusan dengan intensiti cahaya 2000 - 2500 lux. Ekstrak methanol kultur sel menunjukkan jumlah kandungan flavonoid, jumlah kandungan fenolik dan aktiviti pengyingkiran radikal bebas DPPH yang lebih tinggi berbanding dengan pokok induk, anak pokok kultur tisu dan kalus.

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ABSTRACT

Aseptic bud explants of *Gynura procumbens*, obtained from the axillary buds of field grown mature plants, could be established via surface-sterilization with 20 % Clorox® for 20 minutes. The formation of multiple shoots could be induced on MS medium supplemented with 2.0 mg/L BA with an average of 14.7 ± 0.7 shoots produced from each bud explant after four weeks of culture. When the concentration of BA supplemented into the MS medium was reduced to 1.5 mg/L BA, reasonably high number of shoots (13.9 ± 1.3 shoots per explant) can still be produced with less production of abnormal shoots. The optimum subculture cycle was four weeks. All the four weeks old micro shoots produced roots when cultured on gelled basic MS medium after six days of culture. All the *in vitro* plantlets survived after acclimatization in the plant house. MS medium supplemented with 2 mg/L Picloram was the best medium for induction of callus from the leaf explants. The induced calli proliferated well on MS medium supplemented with 0.5 mg/L Picloram with the average biomass of 7.874 ± 0.413 g after four weeks of culture. Throughout the fourteen subculture cycles, the production of callus in term of biomass was found to become constant after the 4th subculture cycle. The calli produced were light yellow and friable in nature. Cell suspension culture could be established by inoculating 0.5 g of this friable callus in 20 mL of MS medium supplemented with 0.5 mg/L Picloram and 60 g/L sucrose. The cells proliferated well under continuous light with light intensity of 2000 - 2500 lux. The methanolic

extracts of the cultured cells showed higher total flavonoid content, total phenolic content and free radical scavenging activity compared to the mother plants, tissue-cultured plantlets and calli.

1.0 INTRODUCTION

Plants playing an important role in human history. We have relied on plants for basic needs such as food, clothing and shelter over centuries. Plants have also been used for other purposes like poisons for hunting, production of stimulant beverages and as the basis of sophisticated traditional medicine (Salim *et al.*, 2008).

Plants produce biochemicals which are important to healthcare, food, flavour and cosmetics industries. Many pharmaceutical compounds are produced from the secondary metabolites of plants. Medicinal plants contain the secondary metabolites that give its value for herbal remedies and healthcare preparations. Examples are digitalis, L-DOPA, morphine, codeine, reserpine, and the anticancer drugs vincristine, vinblastine and taxol used in treatment of ovarian and breast cancers (Dicosmo and Misawa, 1995). The World Health Organization (WHO) had reported that around 80% of world's population especially those from developing countries are relying on plant-derived medicines for their healthcare (Gurib-Fakim, 2006). In the industrialized countries, plant-based traditional medicines are becoming the alternative medicine and their uses increased steadily over the last ten years (Salim *et al.*, 2008).

In the year of 1999, Ong and Norzalina had studied the usage of herbal medicine by the villagers in Gemencheh, Negeri Sembilan, Malaysia. They used the various plant species found in the forests, gardens, farms and abandoned lands as herbal medicine to treat various ailments. The plant species used by the villagers are *Annona muricata*, *A. squamosa*, *Carica papaya*, *Psidium guajava*, *Curcuma domestica*, *Zingiber officinale*, *Centella asiatica* and *Sauropus androgynus*. Various weeds like *Eupatorium odoratum*, *Hyptis suaveolens*, *Mimosa pudica*, *Oxalis*

corniculata, *Phyllanthus niruri* have also been used. These medicinal plants play an important role in the lives of Malaysian local communities.

Gynura procumbens has also been proven as a medicinal plant with antihyperglycaemic, antihypertensive, anti-inflammatory, antiviral and anticancer properties (Zhang and Tan, 2000; Akowuah *et al.*, 2001; Akowuah *et al.*, 2002; Rasadah *et al.*, 2006; Hoe and Lam, 2003; Kim *et al.*, 2006; Iskander *et al.*, 2002; Jiratchariyakul *et al.*, 2001; Agustina *et al.*, 2006; Jenie *et al.*, 2006 and Meiyanto *et al.*, 2007). The methanolic extract of its leaves was found to have antioxidant property (Rosidah *et al.*, 2008). The antioxidant property was reported to reduce the oxidative stress in diabetics (Lollinger, 1981; Collier *et al.*, 1990). *G. procumbens* (Lour.) Merr. is a plant species of family Compositae. It grows in Southeast Asia especially Indonesia, Malaysia and Thailand. In Malaysia, the local name for *Gynura procumbens* is ‘sambung nyawa’ (Burkill, 1966; Wiart, 2000).

To monitor the importance of our local medicinal plants, Malaysia Natural Products Society was formed in 1994 to oversee the co-ordination of activities that involve medicinal plants and the release of *Malaysian Pharmacopoeia*. The activities of the society are to screen the marine and terrestrial biochemical diversity for medicinal principles, phytomedicinals and nutraceuticals (Hoareau and DaSilva, 1999).

Due to the increasing demands of the medicinal plants, there is a risk that many of these medicinal plants may face extinction as a result of exploitation, environment-unfriendly harvesting techniques, loss of growth habitats and unmonitored trade of medicinal plants. So there is a need of using modern biotechnological techniques to nurture and conserve these medicinal plants.

Plant tissue culture method has been used to mass propagate many plant species. *In vitro* culture techniques, have the advantage of reducing the inconsistency of biotic and non-biotic factors such as soil, geographical, weather and humidity that can affect the contents of plant secondary metabolite and uniformity of plantlets (Bhojwani and Razdan, 1996). The production of plant biochemicals via cell suspension culture offers more advantages since the cells are physiologically more homogenous and therefore potentially more controllable than that of the whole plant (Bourgaud *et al.*, 2001). In the *in vitro* culture condition, the expression of secondary metabolite pathways is easily altered by external factors such as nutrient levels, stress factors, light and growth regulators (Rao and Ravishankar, 2002).

Application of tissue culture for medicinal plants has been proven to be useful in the production of therapeutic compounds. Nowadays, plant cell culture is essential to maintain the adequate and continuous supplies of plant-derived bioactive compounds to meet the market demand especially for the compounds that are only available in small quantities from plants. There are a few plant-derived drugs in the market that has been successfully produced by cell culture techniques such as antitumor drug paclitaxel and diosgenin as important starting materials for production of various steroid hormones and *Panax ginseng* ginsenosides (Salim *et al.*, 2008).

Hence to ensure sustainable production of plant materials, this study was carried out with the following objectives:-

1. To establish a micropropagation protocol for the production of *G. procumbens* plantlets.
2. To develop callus and cell suspension culture of *G. procumbens* for the production of bioactive compounds with antioxidant properties.

3. To compare the antioxidant activities of the mother plant, *in vitro* plantlets, callus and cell cultures of *Gynura procumbens*.

2.0 LITERATURE REVIEW

2.1 *Gynura procumbens*

2.1.1 Distribution and habitat of *Gynura procumbens*

Gynura procumbens (Lour.) Merr. is a vigorous climbing herb belonging to the family Compositae (Asteraceae) (Burkill, 1966). It is also named as *Gynura sarmentosa* DC. and *Calacia procumbens* Lour (Wiart, 2000). It is locally known as sambung nyawa, akar sebiak, daun dewa, kelemei merah and kacham akar in Bahasa Malaysia, bai bing cao in Mandarin (Burkill, 1966; Wiart, 2000) and Pra kum dee kwai in Thailand (Gale *et al.*, 2007).

G. procumbens is a scandent or erect and glabrous herb growing in various parts of Southeast Asia especially Borneo, Java, Philippines and Malaysia (Perry and Metzger, 1980; Wiart, 2000). It can be found in West Peninsular Malaysia but very rare in East Peninsular Malaysia (Burkill, 1966; Henderson, 1959).

*G. procumben*s produces simple and exstipulate leaves with medium size leaf blade of 2.5-7.5 cm in length and 0.8-2.5 cm in width. The succulent leaves are elliptic to lanceolate in shape with cuneate base and dentate margin. Petioles are short, range from 0.2 cm to 0.8 cm. The flowers are bisexual and orange in colour with narrow bracts and purple involucres. The style is slender and hairy. The mature achenes are narrow and puberulous while the pappus are white and silky (Wiart, 2000).

2.1.2 Uses of *Gynura* species

G. procumbens has long been used as traditional medicine for various types of ailments. It is used as a remedy for kidney problems in Indonesia and as a

febrifuge in Indo-China (Perry and Metzger, 1980; Wiart, 2000). The dried leaves rubbed with oil and mashed are used as poultice for rashes (Perry and Metzger, 1980). It is used for the treatment of dysentery in Malaysia. The leaves are edible and also used as food flavour. It is also known as a Cambodian febrifuge (Burkill, 1966). The plant is also widely used for the relief of migraine and constipation and treatment of hypertension, diabetes mellitus and cancer. The extracts of *G. procumbens* was reported to have antihyperglycaemic (Zhang and Tan, 2000; Akowuah *et al.*, 2001; Akowuah *et al.*, 2002; Rasadah *et al.*, 2006), antihypertensive (Hoe and Lam, 2003; Kim *et al.*, 2006), anti-inflammatory (Iskander *et al.*, 2002), antiviral (Jiratchariyakul *et al.*, 2001) and anticancer (Agustina *et al.*, 2006; Jenie *et al.*, 2006; Meiyanto *et al.*, 2007) properties.

Besides *G. procumbens*, other *Gynura* species are also well known for their therapeutic values. *G. pseudo-china* (L.) DC. a native plant of India, Thailand, and China is cultivated in Java for the treatment of breast tumors and erysipelas. The sap of the leaves is used to treat throat inflammation and the root is used as a remedy for uterine hemorrhages, dysentery and inflamed wounds. The powder of the plant is mixed with tea and given to parturient women. It also used to regulate menstruation or as a medicine to correct irregular menses. *G. aurantiaca* (Bl.) DC. is used to cure ringworm in Indonesia. *G. divaricata* (L.) DC. is used to combat craving for opium (Perry and Metzger, 1980). While the sap of the leaves of *G. japonica* (Thunb.) Juel is applied to treat poisonous insect bites and the combination of leaves and root or the root alone are applied to the poisonous snakes' bites. It is also used to cure muscular-skeletal system disorders, injuries and ulcers (Perry and Metzger, 1980; Weckerle *et al.*, 2009). It is also used to heal bone fracture and tonsillitis (Lee *et al.*, 2008). *G. scandens* O. Hoffm., consumed by chimpanzees in the Budongo Forest

Reserve in Uganda (Tweheyo *et al.*, 2004), is used to heal patients suffering from orchiocele, cystitis and urethristis (Chifundera, 2001).

G. formosana, a popular vegetable planted throughout Taiwan, and used as a folk medicine was found to contain active chromanone (Jong and Chou-Hwang, 1997). While, *G. elliptica*, a succulent herb endemic to Taiwan showed anti-platelet aggregation activity (Lin *et al.*, 2000). Chen *et al.* (2009) had isolated a new cerebroside,1-O-β-D-glucopyranosyl-(2S,3S,4R,10E)-2-[(2′R)-2′-hydroxyltricosano-ylamino] -10-octadecene-1,3,4-triol from the aerial parts of *G. divaricata* DC.

2.1.3 Medicinal properties of *Gynura procumbens*

The extract of *G. procumbens* was reported to show the antihyperglycaemic and antihyperlipidaemic activities in diabetic rats. The elevated serum glucose levels in the diabetic rats were suppressed by the leaves extract but did not affect the elevated serum glucose levels in normal rats. The extract also significantly reduced serum cholesterol and triglyceride levels in the rats when given the optimum dose (Zhang and Tan, 2000). The n-butanol fraction of *G. procumbens* extract reduced the blood glucose levels of the streptozotocin-induced type 2 diabetic rats. However, the fraction did not give significant effect to the normal rats (Akowuah *et al.*, 2002). Anti-diabetic properties of *G. procumbens* was again proven by the evaluation on the anti-hyperglycaemic effect using oral glocuse tolerance test (OGTT) in normal rat and insulin release activity using insulin-secreting cell line, BRIN-BD 11. There is no cytotoxicity effect observed at the effective dose (Rasadah *et al*., 2006).

Pauliena *et al.* (2006) also examined the effect of *G. procumbens* extract on glucose uptake in 3T3 adipocyte cell lines. They reported that the hexane fraction and etyl acetate fraction of the plant extract increased the glucose uptake activity. It

was found that the antidiabetic action of *G. procumbens* might be acting through the stimulation of glucose uptake and the insulin action.

Hakim *et al.* (2008) reported that the diabetic rats force-fed with *G. procumbens* extract (100mg/kg) had increased the sperm count and mortality compared to the controls. Sani *et al.* (2008) also reported that *G. procumbens* had the ability to decrease blood glucose level, restored the fertility and increased spermatogenesis in male diabetic rats.

The ethanolic extract of *G. procumbens* was found to have antiangiogenesis (inhibition of new blood vessels formation) effect which believed to inhibit cancer development by reduce the nutrition and oxygen supply to the cells. This effect has been investigated by using chick embryo chorioallantoic membrane (CAM) (Jenie *et al.*, 2006). The ethanol extract of *G. procumbens* leaves had also been proven to have anticarcinogenesis effect on 4 nitroquinoline 1-oxide (4NQO)-induced tongue carcinogenesis in rat (Agustina *et al.*, 2006). The ethanolic extract of *G. procumbens* was also found to suppress lung cancer, and breast cancer development (Meiyanto *et al.*, 2007). Lee *et al.* (2007) reported that *G. procumbens* has antiproloferative and antipyretic effect in fetal bovine serum-activated human mesangial cells (MCs).

The aqueous extracts of *G. procumbens* administered orally to spontaneously hypertensive rats for 4 weeks had significantly reduced the blood pressure of the hypertensive rats (Kim *et al.*, 2006). The preliminary studies of Hoe *et al.* (2001) have shown that an aqueous extract of *G. procumbens* consistently and significantly decreases the systolic (SBP) and diastolic (DBP) blood pressure of rats at various doses.

Studies had also been done for the crude ethanolic extract *of G. procumbens* to prove its anti-inflammatory action by using a croton oil-induced mouse ear

inflammation model. The administration of the extract inhibited the increase in ear thickness in response to croton oil significantly. Through the thin layer chromatography method, steroids had been found and it might be one of the anti-inflammatory compounds in *G. procumbens* (Iskander *et al.*, 2002).

The toxicology evaluation had been done on the standardized methanol extract of *G. procumbens* administrated orally to male and female Sprague Dawley rats for 13 weeks. The results did not cause death to the rats and there were no adverse effects on the general condition, growth, body and organ weights, haematology, or clinical biochemistry values of the rats. It also did not result in histopathological abnormalities (Rosidah *et al.*, 2009).

Sadikun *et al.* (1996) had isolated a mixture of sterols containing β-sitosterol and stigmasterol, and sterol glycosides consisted of 3-O-β-D-glucopyranosyl β-sitosterol and 3-O-β-D-glucopyranosyl stigmasterol from *G. procumbens* leaves. The butanol fraction of *G. procumbens* leaves with antihyperglycaemic properties was found to contain quercetin, kaempferol 3-O-glucoside, quercetin 3-O-rhamnosyl (1&6) glucoside, quercetin 3-O-rhamnosyl (1&6) galactoside, kaempferol 3-O-rhamnosyl (1&6) glucoside (Akowuah *et al.*, 2001; Akowuah *et al.*, 2002).

The different fractions of the methanol extract of *G. procumbens* were found to have different antioxidant potential. Among the fractions, the ethyl acetate fraction displayed higher antioxidant properties. The results indicated that phenolic compounds in the plants provide substantial antioxidant activity (Rosidah *et al.*, 2008). The total phenolic contents and free radical scavenging activity of *G. procumbens* leaf extract was found to be affected by temperature. The extraction obtained at lower temperature had higher radical-scavenging activity when compared to extraction at higher temperatures (Akowuah *et al.*, 2009).

2.2 *In vitro* culture technology

In vitro regeneration of plants is a powerful tool for plant growth and development and as a conservation tools. For the recent decades, plant tissue culture had drawn the attention worldwide due to the production of high-yield transgenic crops, with disease-resistant traits and production of plant biochemicals that are importance in the healthcare, food, flavour and cosmetics industries (Dicosmo and Misawa, 1995).

2.2.1 Culture medium

Formulation of a suitable medium is very important in plant tissue culture, so as to provide the satisfactory growth of the plant tissue. Basically, a basic culture medium contains inorganic nutrients, organic nutrients, plant growth regulators, and gelling agents. The inorganic nutrients are the mineral elements that are essential for plant growth. The organic nutrients are vitamins (thiamine, niacin and pyridoxine), amino acids (glycine and cysteine), undefined supplements (casein hydrolysate, malt extract and yeast extract) and carbon source (sucrose) (Bhojwani and Razdan, 1996).

It is necessary to add the plant growth regulators in order to support good growth of plant tissues and organs. Plant growth regulators are consisting of two major group, auxins and cytokinins. Auxins, generally involved in the elongation of stem and internodes, tropism, apical dominance, abscission, rooting and cell division. The examples of auxin are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA) and dichlorophenoxyacetic acid (2,4-D). While cytokinins are concerned with cell division, modification of apical dominance and shoot differentiation, branching and compactness, a desired characteristic for some ornamentals, and delayed leaf and plant senescence. Commonly used cytokinins are

benzylamino purine (BAP), furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin (Bhojwani and Razdan, 1996). The effects of plant growth regulators on plant tissue are depending on the amount used, the target tissue, and their inherent activities.

Generally, the gelling agents are used to avoid the plant tissue submerged in the liquid medium and die due to lack of oxygen. The gelling agents are agar, agarose and gelrite. Usually the pH of the medium is adjusted between the range of 5.0 and 6.0 before sterilization. pH that higher than 6.0 will gives a fairly hard medium and pH below 5.0 does not promise the solidify of gelling agent (Bhojwani and Razdan, 1996).

2.2.2 Micropropagation

Micropropagation is often used for large-scale propagation of fruits trees, ornamental plants and medicinal plants. Micropropagation has the significant advantages over the conventional propagation techniques because small amount of original germplasm is needed to achieve large-scale propagation and regeneration of pathogen-free propagules in a relatively short time (Altman, 1999).

Micropropagation can be used as a tool to save endanger species via production of large number of plantlets. *Anthemis xylopoda* O. Schwarz (Asteraceae), an endangered species had been propagated through *in vitro* techniques. The shoot multiplied an average of 7.8 shoots per explant on MS medium supplemented with 0.005 mg/L Thidiazuron (Erdağ and Emek, 2005). The immature seeds of *Dendrobium fimbriatum* Hook., an endangered epiphytic forest orchid which are valued for its long lived beautiful sweet scented flowers, were successfully germinated in Vacin and Went medium with 0.1 mg/L NAA and 15%

coconut water (Sharma *et al.*, 2005). Two threatened Tasmanian members of the Asteraceae, *Calocephalus citreus* Less. and *Calocephalus lacteus* Less, were micropropagated through disinfected cold-treated capitula. Shoot multiplication was achieved in both species on MS with various concentrations of IAA (0.01–0.5 mg/L) and BAP (0.1–1 mg/L) (Jane *et al.*, 2003).

Micropropagation techniques also frequently applied to the mass propagation of crops, fruit trees and ornamental plants. For examples, micropropagation was used to mass multiply the superior coffee genotypes (Kumar *et al.*, 2006). Chrysanthemum, one of the most important global cut flower and pot plants, has also been mass propagated with the *in vitro* culture techniques (Silva, 2003).

Regeneration of plantlets via micropropagation techniques requires four main steps. The first step involves selection and establishment of aseptic tissues. For initiating an *in vitro* plant, the ability to get the clean tissue or pathogen-free tissue is very important and crucial.

Many kinds of disinfectants have been used to obtain the aseptic tissue. However, the commonly used disinfectants are sodium hypochlorite, alcohol, saturated solution of calcium hypochlorite, and mercuric chloride solution. The adding of wetting agent such as Tween 20 helps to increase the effectiveness of surface-sterilization of tissues. The plant tissue need to be rinsed with sterile distilled water to remove the residue of disinfectants before culture into medium (Beyl, 2000). The aseptic axillary buds of *Spilanthus acmella* were successfully obtained via surface sterilization using mercury chloride and Clorox® (Ang and Chan, 2003). There were a few methods that could eliminate the virus such as heat treatment, meristem culture and *in vitro* shoot-tip grafting (Bhojwani and Razdan, 1996). For example, a micropropagation protocol was developed to obtain virus-free plant from

sweet potato mother plant which was infected with Tobacco Mosaic Virus. This has been achieved through *in vitro* grafting method on apical meristems (Katoh *et al.*, 2004).

Second stage involves the multiplication of shoots or axillary bud. Normally, plant growth regulators are required to induce the formation of multiple shoots. For some plant species, only single plant growth regulator is supplemented in the basal medium for this purpose. For example, *Schizandra chinensis* regenerate shoots from the cotyledonary nodes of *in vitro* seedlings with the use of 1.0 mg/L BA supplemented into the woody plant media (Hong *et al.*, 2004). *Spilanthes acmella* L., was successfully micropropagated using MS medium supplemented with only 2.0 mg/L N^6- Bezyl adenine (BA). The addition of auxin had no significant effect for shoot proliferation (Ang and Chan, 2003). The MS medium containing 0.5 µM thidiazuron was highly recommended for the induction of *in vitro* shoots of *Cannabis sativa* by using nodal segments (Lata *et al.*, 2009). *In vitro* shoot multiplication from shoot tip explant of *Trichosanthes cucumerina* L. var. *cucumerina* was achieved on the semisolid MS basal medium with BA (Devendra *et al.*, 2008).

On the other hand, combination of plant growth regulators were frequently used in shoots regeneration of some plant species. Adventitious shoots were successfully regenerated from hypocotyl explants of *Euonymus japonicus* Cu zhi on MS basal medium supplemented with 1.5 mg/L 6-BA and 0.05 mg/L NAA (Shang *et al*., 2006). *Eclipta alba* (Linn.) hassk, a valuable medicinal herb, was mass propagated using the apical and axillary buds from selected plants. The best multiple shoot induction medium was MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA which produced 18 shoots per culture (Sayeed Hassan *et al.*, 2008).

Asparagus ofjicinalis L. had produced thick storage roots through i*n vitro* propagated stem explants by using high concentration of gellan gum for solidifying Murashige and Skoog (MS) medium containing 0.1 mg/L NAA, 0.1 mg/L kinetin and 30 g/L sucrose (Shigeta *et al*., 1996a).

The hypocotyl explants from 22 cultivars of *Catharanthus roseus* were cultured on MS medium supplemented with combination of auxin and cytokinin (zeatin, IBA, BA, NAA or TDZ). The optimum medium for shoot multiplication was MS plus 14 µM zeatin and 2.5 µM NAA (Choi *et al.*, 2003). Combination of plant growth regulators of TDZ and NAA has been used to establish an efficient and reproducible protocol for production of *in vitro* shoot of *Lilium davidii* var. unicolor (Lanzhou Lily). The highest frequency of regeneration (93.3 %) and the largest number of shoots per leaf explant was obtained on Nitsch & Nitsch (1969) medium supplemented with 0.5 mg/L TDZ and 1.0 mg/L NAA (Xu *et al.*, 2009).

After conversion from tissue into shoots, normally the shoots required an additional step by transferring the *in vitro* shoots to another medium, commonly supplemented with low concentration of auxin or different medium composition for elongation and rooting purpose (Bhojwani and Razdan, 1996). The *in vitro* shoots of *Lilium davidii* var. unicolor (Lanzhou Lily) were rooted successfully in the culture medium supplemented with IBA treatment and 92% of regenerated plantlets survived in the greenhouse (Xu *et al.*, 2009). The rooting medium for *Schizandra chinensis* was supplemented with low auxin, 0.5 mg/L NAA (Hong *et al.*, 2004).

The regenerated shoots of *Euonymus japonicus* Cu zhi were rooted on half-strength MS basal medium supplemented with 1.0 mg/L IBA and 100 mg/L activated carbon. The successful survival rate of acclimatized plantlets was 96% after transferred to greenhouse (Shang *et al.*, 2006). Half-strength MS medium

supplemented with 500 mg/L activated charcoal and 2.5 µM IBA was used for rooting of *Cannabis sativa* (Lata *et al.*, 2009). Rooting of *in vitro* plantlet of *Vriesea reitzii* was obtained on the MS medium free of plant growth regulators (Alves *et al.*, 2006).

The final step of micropropagation is the hardening-off process and acclimatization of *in vitro* plantlets for growing in soil mixtures under greenhouse conditions for later transplanting to the field. This step is very important to commercial micropropagation as the ultimate success is depending on the ability to transfer the *in vitro* plantlet out to the field on a large scale, low cost and with high survival rate. Normally, the low survival rate of *in vitro* plantlets after transfer to the field is due to the plantlets are unable to survive under ex vitro conditions. This is because plantlets are exposed to unique *in vitro* growth conditions normally with high levels of inorganic and organic nutrients, plant growth regulators, carbon source, high humidity and poor gaseous exchange. Therefore, gradual acclimatization process is necessary to prevent water loss and heterotrophic nutrition (Bhojwani and Razdan, 1996). For example, after the rooting process of *Crassula arborescens* plantlets, the covers of Erlenmeyer flasks gradually removed during the one week acclimatization. The plantlets were then transferred to the soil and sand mixture and acclimatized in greenhouse. The survival rate was almost 100 % after one month. The plants were transplanted to the field and grew to 27cm in 8 months averagely (Liu *et al.*, 2007). *In vitro* plantlets of *Vriesea reitzii* that longer than 3.0 cm after rooting process were transferred to trays containing carbonized rice coat and Turfa Fertil® mineral supplement (1:1) in a nebulization tunnel. The survival rate was 90% after 60 days (Alves *et al.*, 2006). The survival rate of 90% had been obtained for *in vitro* plantlets of *Citrus limon* (L.) Burm, f. cv. Interdonato after

acclimatization under a mist system (Kotsias and Roussos, 2001). The rooted *in vitro* plantlets of *Arbutus unedo* L. were planted in containers with the sterilized mixture of sand and *Siro* 30 (a commercial substrate of composted pinus bark and peat with ratio of 7:3 supplemented with *Osmocote* slow release fertilizer). The containers were covered with plastic to maintain high degree of humidity and the levels of humidity were gradually decreased. The rooted *in vitro* plantlets of *Arbutus unedo* L. showed survival rates from 84% to 98% according to different clones after four months in the greenhouse (Gomes and Canhoto, 2009).

2.2.3 Callus culture

Callus is defined as unorganized tissue that arising from the division of plants cells. Callus is normally induced from the cut edges of explants on the culture medium with plant growth regulators at various levels (Endress, 1994). Different types of plant growth regulators such as abscisic acid, gibberellins, auxins and cytokinins have been applied into the basic culture medium for callus initiation.

However, auxins are more frequently used for callus induction from explants. For examples, callus culture of barley (*Hordeum vulgare* L. var. BL-2) was raised from immature embryos on MSB_5 medium, based on MS medium (Murashige and Skoog, 1962) and B_5 vitamins (Gamborg *et al.*, 1968) supplemented with picloram 20.70 µM (Chauhan and Kothari, 2004). Ketchum *et al.* (1995) also reported that picloram is suitable for maximize callus biomass of *Taxus brevifolia*. Wheat (*Triticum* spp) had achieved its optimum embryogenic callus induction on MS medium supplemented with 2,4-D (Rashid *et al.*, 2009).

Combinations of plant growth regulators also act effectively for callus formation. According to Park *et al.* (2002), the induction of embryogenic callus from

young stem explants on MS medium supplemented with 0.5 mg/L 2,4-D and 1.0 mg/L kinetin was very effective for *Vigna radiate* W. For groundnut (*Arachis hypogaea* L.), the highest rate of callus production was recorded with the addition of 3.0 mg/L IAA and 1.0 mg/L BAP into the MS medium (Palanivel *et al.*, 2002). Yan *et al.* (2009) reported that BA higher than 2.0 mg/L inhibited callus proliferation of *Allium chinensis* G. Don. In the callus culture of *Euphorbia helioscopia*, the calli showed brown in colour when the concentration of 2,4-D rose to 4.0 mg/L (Yang *et al.*, 2009).

For some plant tissues, two different medium were required for callus initiation and callus proliferation. For instant, the maximum callus induction of *Allium chinensis* G. Don was obtained with culture medium supplemented with 0.1 mg/L BA and 1.0 mg/L 2,4-D, while the best callus proliferation was observed on the MS medium supplemented with 1.5 mg/L 2,4-D (Yan *et al.*, 2009). For *Cardiospermum halicacabum*, callus was induced on the MS medium supplemented with 5µM 2,4-D but maintained on the MS medium supplemented with lower concentration of 2,4-D (2 µM) (Thomas and Maseena, 2006).

Different type of plant growth regulators could affect the structure of calli. For example, the calli of *Euphorbia helioscopia* induced on the MS medium supplemented with 0 to 3.0 mg/L 2,4-D, the cells were small, high in cytoplasm ratio and abundant in cell contents. However, the calli induced on the MS medium supplemented with BAP were compact, green in colour. The cells were organogenic with relatively large, loose, low in cytoplasm and lacked of cell contents (Yang *et al.*, 2009).

Besides plant growth regulators, callus formation can also be influenced by type of explants. The older parts of plant are not selected to initiate a callus culture

as those explants have minimal content of merismatic cells and the higher accumulation of pathogenic substances. For *Allium chinensis* G. Don, after three weeks of culture, the basal plates of leaf showed the earliest signs of callus formation if compared with leaf and root explants which only started to initiate callus after five weeks of culture (Yan *et al.*, 2009).

Sometimes, callus culture was found to accumulate secondary metabolites more than the mother plant (Dicosmo and Misawa, 1995). High amount of isofavones was produced in the callus culture of *Genista. tinctoria* L., *G. radiata* L., *G. aethnensis* D.C., *G. sagittalis* L., *G. germanica* L. and *G. monospessulana* L. (Luczkiewicz and Glód, 2003).

2.2.4 Cell suspension culture

A cell suspension culture is composed of cells in liquid medium with continuous agitation to segregate the cells for optimum growth. Generally, a friable type of callus is used to initiate the cell suspension culture. The direct contact of the cells with the nutrients in the medium has resulted for higher biomass in the cell culture. Hence, cell suspension technique is always used to produce plant secondary metabolites.

Plant secondary metabolites play a major role in the adaptation of plants to environment and also represent as an important source of active pharmaceutical compounds. Plant secondary compounds are classified base on the biosynthetic pathways and they are phenolics, terpenes and steroids, and alkaloids (Bourgaud *et al.*, 2001).

The production of plant secondary metabolites has been achieved through field cultivation of medicinal plants with large variation in the contents. Plant cell

culture has been used as an alternative way to produce the secondary metabolites with standardized plant material and extracts in a confined environment (Su and Lee, 2007). By using this method, the secondary metabolite can be produced under controlled and consistent conditions, independent of geographical and climate factor and more cost-effective (Kieran *et al.*, 1997; Dicosmo and Misawa, 1995). Before initiating the cell culture, the selection of the hyper-productive plant that produces the most valuable secondary metabolites is very important. After that, establishment of *in vitro* cell culture will begin with the callus initiation.

Cell suspension cultures have been successfully implemented for the production of secondary metabolite. The cell cultures of cucurbitaceous plants had produced higher amount of triterpenoid if compared to the mother plants (Cho *et al.*, 1993). Rosmarinic acid which has antiviral, antibacterial, anti inflammatory and antioxidant effects was produced much higher in the cell cultures of *Coleus blumei* or *Salvia officinalis* (Petersen and Simmonds, 2003). Saponins (ginsenosides) of *Panax quinquefolium* were produced through cell suspension culture techniques and were found 68 % higher than naturally cultivated 4-6 year-old plant (Zhong *et al*., 1996). Anthocyanins also produced in the suspension cultures of *Vitis vinifera* (Qu *et al*., 2006).

The production of cell biomass and secondary metabolites depends a lot on the medium constituents. Hence, optimization of the mineral composition and organic constituent of the culture medium and the type of plant growth regulators are very important to obtain high yield products (Bourgaud *et al.*, 2001). Auxins and cytokinins are crucial for the proper stimulation of biosynthetic pathways. In cell culture of *Glehnia littoralis*s, NAA at 1 mg/l and the addition of 0.1 mg/l kinetin further improved cell growth and anthocyanin biosynthesis (Miura *et al.*, 1998). The

highest content of ginsenoside saponins were obtained in the cell culture of *Panax quinquefolium* with a combination of 2.5 mg/L IBA and 0.1 mg/L kinentin (Zhong *et al.*, 1996).

For the enhancement of biomass or bioactive compounds production, the chemical and physical stresses are applied for better yield. This is because secondary pathways are activated in response to stress. Elicitors usually used to trigger the formation of secondary metabolites (Rao and Ravishankar, 2002).

There are biotic elicitors (chitosan, autoclaved mycelium of pathogenic fungi, various protein extracts) or abiotic factors (temperature, UV light, heavy metal salts, and pH) that can stimulate secondary metabolite formation in plant cell culture. Manipulation of medium nutrients such as sugar, nitrate level, phosphate level and growth regulators could improve the yield (Bourgaud *et al.*, 2001; Rao and Ravishankar, 2002).

Lim (2008) reported the increased of free radical scavenging activity, total phenolic compound and rosmarinic acid in the cell culture of *Orthosiphon staminues* Benth by adding the elicitors (yeast extract, chitosan and casein hydrolisate) into the cell cultures. The use of metal-based elicitors like vanadium salts increased the accumulation of rosmarinic acid in lavender cell cultures (Georgiev *et al.*, 2006).

Sucrose was found to affect the cell culture of *Gymnema sylvestre*. The cell growth was increased by 9-fold when the culture medium was supplemented with 3% sucrose compared to the sucrose free medium (Lee *et al.*, 2006). In *Vitis vinifera* cell culture, the production of anthocyaninis increased 12-fold with the addition of 0.15 M sucrose (Larronde *et al.*, 1998).

The medium pH is usually adjusted between 5 and 6 before autoclaving. In the culture medium, the concentration of hydrogen ions changes during the

development of the culture (Rao and Ravishankar, 2002). The cell growth and secondary metabolite production could be affected by the changes of H^+ ions (Endress, 1994). For example, the cell culture medium with pH 5.75 had induced optimum cell biomass for *Eurycoma longifolia*, while the culture medium with pH 4.75 and 5.25 had induced high alkaloid content (Luthfi *et al.*, 2004).

Combination of conditioned medium and elicitation has also been used to enhance taxoid production in bioreactor cultures of *Taxus chinensis* cells. The addition of conditioned medium and methyl jasmonate elicitation had stimulated on the production of physiologically active taxuyunnanine C (TC) in cell cultures of *Taxus chinensis* (Wang and Zhong, 2002).

2.3 Antioxidant properties in plants

For these few recent years, research on relationships between antioxidants and prevention of various diseases like cardiovascular disease, cancer, diabetes, inflammation, neurodegenerative diseases and accelerated aging has been increasing. Free radicals have been claimed for its serious effect to human health by causing severe diseases by cell degeneration. These free radicals could be generated during normal body function or acquired from the environment (Stadtman, 1992; Dasgupta and De, 2007). Antioxidants were found to play an important role to protect the human body against damage by reactive oxidative stress (Lollinger, 1981).

Antioxidant capacity refers to the capability of a compound to protect a biological system against the reactive oxygen and nitrogen species (Percival, 1998). In the food industries, antioxidants have been widely used as food additives to against food oxidative degradation and extension of shelf-life (Djeridane *et al.*, 2006). Antioxidants are necessary to human as free radical scavengers, chelating

agents for transition metals, reducing agents, quenchers of singlet oxygen molecules and activators of anti-oxidative defense enzyme system in biological system (Yu *et al.*, 2002).

The synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are commonly used in food processing have been proven by exhibiting carcinogenic effects in living organism (Ito *et al.*, 1986). Hence, the findings of antioxidant properties in plants have become more important nowadays. Various phenolic compounds especially flavonoids are known to be involved in the healing process of diseases that caused by oxidative stress (Czinner *et al.*, 2000).

There are many methods to measure the antioxidant capacity. The methods differ in terms of reaction mechanisms, oxidant and target species, reaction conditions, and expression of results. Examples are Oxygen Radical Absorbance Capacity assay (ORAC), total phenol assay by using Folin-Ciocalteu reagent, Trolox Equivalent Antioxidant Capacity (TEAC) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay and β-Carotene or Crocin-Bleaching assay (Karadag *et al.*, 2009).

Many researchers have carried out research on plant species which owned its medicinal value with their antioxidant properties. The aromatic wild plants, *Pistacia lentiscus* L. (Anacardiaceae) and *Myrtus communis* L. (Myrtaceae), which traditionally used as anti-hypoglycaemic agent were reported for their antioxidant activity and high total phenolic content from the methanolic extracts (Chryssavgi *et al.*, 2008). The extracts of fresh and dried whole fruit of *Momordica charantia* L. (Bitter gourd) were able to reduce blood sugar in diabetic rats (Virdi *et al*., 2003). The leaves and stem bark fractions of *Scutia buxifolia* Reissek, popularly used as a

cardiotonic, antihypertensive and diuretic, showed to have a great potential to prevent diseases caused by overproduction of free radicals (Boligon *et al*., 2009). The polyphenols in *Orthosiphon stamineus* are the dominant constituents in the leaves and were found to be effective in reducing oxidative stress by inhibiting the formation of lipid peroxidation products in biological systems (Hollman and Katan, 1999).

The free radical scavenging activity and total phenolic compound had been determined in the *in vitro* cell culture of *Orthosiphon stamineus* Benth by Lim (2008). The antioxidant properties were found in the ethyl acetate and methanolic extracts from cell culture of *Lavandula vera* MM which mainly contains rosmarinic and caffeic acids. The antioxidative potency was discovered higher than the synthetic antioxidant (BHT) (Kovatcheva *et al.*, 2001). Thus, plant cell culture technique is an alternative way for natural antioxidative additives production. Hence, in this study, cell culture of *Gynura procumbens* was proposed and the antioxidant activities was compared between the clonal propagated and the mother plants.

3.0 MATERIALS AND METHODS

3.1 Establishment of aseptic plant materials

Gynura procumbens plants were planted at the plant house of School of Biological Sciences, Universiti Sains Malaysia, Penang. Nodal segments with axillary buds were cut into about 1 cm pieces and washed with detergent then rinsed under running tap water for 30 minutes. They were immersed in 70 % ethanol for 30 seconds. They were then surface-sterilized with 10 %, 20 %, and 30 % Clorox® solution for 20 minutes respectively. After rinsing three times with sterile distilled water, they were inoculated in test tubes containing solid Murashige and Skoog (MS) medium (1962) for 12 days. Each test tube contained one axillary bud and 30 test tubes were used for each treatment. The cultures were placed in the tissue culture room maintained at 25 ± 2 °C with continuous cool white fluorescent tubes with light intensity of 2000-2500 lux. The percentages of aseptic bud explants established and their survival were determined after 12 days of culture.

4.2 Induction of multiple shoot formation

4.2.3 Optimization of IBA and BA combination treatment on shoot proliferation

The nodal segments containing the axillary buds of three months old *in vitro* *G. procumbens* plantlets were used as explants. They were cut approximately 1 cm pieces and inoculated onto solid MS medium supplemented with combination of 0, 2, 4, 6, 8 and 10 mg/L of indole-3-butyric acid (IBA) and 6-benzylaminopurine (BA) respectively in 350 mL glass jar covered with plastic cap. The experiment was carried out in a 6 x 6 factorial block design with three experimental units for each combination treatment. The experiment was repeated three times. The number of

shoots produced from each explant was determined and recorded after four weeks of culture. The data was analyzed using Two-Way ANOVA followed by comparison of means using Tukey Test (HSD) at $p \leq 0.05$ to determine the best IBA and BA combination for multiple shoot formation.

3.2.2 Effect of reduced BA concentration on shoot proliferation

In vitro nodal segments axillary buds of three months old *G. procumbens* plants were cut into 1 cm pieces and inoculated onto solid MS medium supplemented with BA (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) in 350 mL glass jar. Nine experimental units with two explants per unit were used for each treatment. The experiment was carried out using complete randomized design. The number of shoot produced from each bud explant was determined after four weeks of culture. The data was analyzed using One-Way ANOVA followed by comparison of means using Tukey Test (HSD) at $p \leq 0.05$ to determine the best BA concentration for induction of multiple shoot formation.

3.2.3 Determination of optimum subculture interval for shoot proliferation

Three months old *in vitro* axillary buds were inoculated on solid MS medium supplemented with 1.5 mg/L of BA, the selected shoot proliferation medium. The number of shoots formed from each explant was recorded after four weeks, six weeks and eight weeks subculture cycle. Ten replicates were used for each subculture cycle. The best duration cycle for optimum production of shoots was then determined.