UNIVERSITI PUTRA MALAYSIA

ESTABLISHMENT OF TISSUE CULTURE AND EVALUATION OF BIOLOGICAL ACTIVITIES OF JARUM TUJUH BILAH (PERESKIA BLEO KUNTH)

NORRIZAH JAAFAR SIDIK

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Chairman : Associate Professor Norihan Mohd. Saleh, PhD

Faculty : Biotechnology and Biomolecular Sciences

Pereskia bleo is a leafy cactus which belongs to Cactaceae family and it is commonly used among the traditional medicine practitioners to prevent or treat cancer by consuming the leaves. As there is lack of information available on this plant, this research was carried out to determine the biological activities in natural plants and tissue culture materials. In order to produce standardize plant material for secondary metabolite production, tissue culture systems for *Pereskia bleo* was initiated and established. *In vitro* plantlets and tissues of *Pereskia bleo* were micropropagated in order to provide enough uniform explants for further tissue culture work and for extraction. The highest number of sterile explants (65.6%) was observed using fungicide for 20 minutes followed by 20% Chlorox® for 10 minutes. The growth characteristics on different types of basal media containing plant growth regulators were analyzed for maximum production of biomass: plantlets, calli and cell suspension. The highest number of multiple shoots

(5.3 ± 0.5) was formed in MS basal medium supplemented with 2.22 µM BAP using shoot tips explants. Using node explants cultured onto MS supplemented with 8.88 µM BAP gave the highest number of multiple shoots (6.7 ± 0.58). Cell suspension growth was highest in MS basal media supplemented with 2.26 µM 2,4-D. Hairy root experiments revealed that both leaf and stem explants of *Pereskia bleo* were susceptible to all Ag*robacterium rhizogenes* (TR 105, LBA 9402, 8196, ATCC 15834) tested. Addition of acetosringone in the *Agrobacterium rhizogenes* culture increased the transformation frequencies of hairy root in *Pereskia bleo.* Results from brine shrimp lethality test showed no toxicity of the *Pereskia bleo* extracts occurred. The petroleum ether, chloroform and methanol leaf extracts showed strong *in vitro* antiproliferative activities (IC_{50} : 6.5 - 25.4 µg/ml) for MCF-7 (human hormone-dependent breast cancer cell line) and (IC_{50} : 19.3 - 26.8 µg/ml) for MDA-MB-231 (human hormone non-dependent breast cancer cell line**)**. The IC_{50} value against HL-60 (human leukemia cell line) between 6.91 and 9.98 µg/ml for chloroform, petroleum ether and methanol extracts. Non-cytotoxic activity towards the non-tumour 3T3 mouse fibroblast indicated that the extracts exhibited selective mode of inhibition between tumor and non-tumor cells. The findings of antioxidative and antiproliferative activities of the plant extracts *in vitro* suggested that this species does contain antioxidant and cytotoxic compounds. The results obtained support the use of this species in traditional medicine for the prevention and treatment of cancer.

Abstrak disertasi yang dikemukakan kepada Senat Universiti Putra Malaysia bagi memenuhi keperluan ijazah Doktor Falsafah

PEMBANGUNAN KULTUR TISU DAN KAJIAN AKTIVITI BIOLOGI JARUM TUJUH BILAH (*PERESKIA BLEO* KUNTH)

Oleh

NORRIZAH JAAFAR SIDIK

Mac 2008

Pengerusi : Professor Madya Norihan Mohd. Saleh, PhD

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Pereskia bleo adalah kaktus berdaun daripada famili Cactaceae dan kebiasaannya digunakan oleh pengamal perubatan tradisional bagi mencegah dan mengubati penyakit kanser dengan cara memakan daunnya. Memandangkan tiada kajian dibuat ke atas spesies *Pereskia bleo*, penyelidikan ini telah dijalankan untuk mengenalpasti aktiviti biologi dan kultur tisu tumbuhan tersebut. Sistem kultur tisu *Pereskia bleo* dijalankan bagi memperolehi bahan tumbuhan yang piawai untuk penghasilan metabolit sekunder. Mikropropagasi plantlet *in vitro* dan tisu *Pereskia bleo* dilakukan bagi memperolehi eksplan yang uniform bagi kerja kultur tisu tumbuhan dan seterusnya untuk ekstraksi. Bilangan tertinggi eksplan yang steril (65.6%) diperolehi dengan penggunaan fungisid selama 20 minit diikuti penggunaan 20% Chlorox® selama 10 minit. Ciri-ciri pertumbuhan di dalam medium basal yang mengandungi hormon tumbuhan berlainan dianalisis untuk penghasilan biomassa plantlet, kalus dan sel suspensi yang maksimum.

Bilangan pucuk berganda yang paling tinggi (5.3 ± 0.5) diperolehi daripada medium asas MS yang mengandungi 2.22 µM BAP menggunakan eksplan hujung pucuk. Manakala dari eksplan nodal, bilangan pucuk berganda yang tertinggi (6.7 ± 0.58) diperolehi daripada medium asas MS yang mengandungi 8.88 µM BAP. Pertumbuhan sel ampaian adalah tertinggi dalam medium asas MS yang mengandungi 2.26 µM 2,4-D. Eksperimen menunjukkan kedua-dua eksplan daun dan batang Pe*reskia bleo* mengeluarkan akar rerambut menggunakan kesemua strain Ag*robacterium rhizogenes* (TR 105, LBA 9402, 8196, ATCC 15834). Penambahan acetosringone di dalam kultur *Agrobacterium rhizogenes* telah meningkatkan frekuensi transformasi akar rerambut. Keputusan menggunakan bioasai ujian kematian anak udang menunjukkan tiada toksiksiti terhadap ekstrak *Pereskia bleo*. Ekstrak daun petroleum eter, kloroform dan metanol menunjukkan aktiviti antiproliferatif yang tinggi terhadap sel kanser payu dara iaitu IC_{50} bernilai 6.5 - 25.4 µg/ml untuk sel MCF-7 dan IC_{50} bernilai 19.3 - 26.8 µg/ml untuk sel MDA-MB-231. Tiada aktiviti antiproliferatif terhadap sel normal 3T3. Ini menunjukkan ekstrak *Pereskia bleo* adalah selektif terhadap perencatan di antara sel kanser dan sel normal. Keputusan pemerhatian secara *in vitro* menunjukkan ekstrak *Pereskia bleo* ini mengandungi kompaun antioksidan dan sitotoksik. Keputusan kajian menyokong penggunaan spesis ini dalam perubatan tradisional untuk mencegah dan mengubati penyakit kanser.

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I certify that an Examination Committee has met on date of to conduct the final examination of Norrizah binti Jaafar Sidik on her Doctor of Philosophy thesis entitled "Establishment of Tissue Culture and Evaluation of Biological Activities of *Jarum Tujuh Bilah* (*Pereskia bleo* Kunth) in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Date: 10 July 2008

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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NORRIZAH BINTI JAAFAR SIDIK

Date: 5 May 2008

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LIST OF ABBREVIATIONS

IAA	Indolacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
A.rhizogenes	*Agrobacterium rhizogenes*
ANOVA	Analysis of variance
B5	Gamborg medium
BAP	6-Benzylaminopurine
^{o}C	Degree celsius
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleotic acid
EDTA	Ethylenediaminetetraacetic acid
FTC	Ferric thiocyanate
H^{+}	Hydrogen ion
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloride acid
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butric Acid
K^{+}	Potassium ion
KI	Potassium iodide
Kinetin	6-Furfurylaminopurine
L•	lipid free radical
LOO•	lipid peroxyl radical

L	Liter
LS	Linsmaier and Skoog
MS	Murashige and Skoog
MW	Molecular weight
NAA	Napththalene acetic acid
•OH	Hydroxyl radical
O_2•	Superoxide radical
OD	Optical density
Ri	Root inducing
rpm	Revolution per minute
T-DNA	Transferable –DNA
T_L	Left border of T-DNA
T_R	Right border of T-DNA
tms	transmembrane segments
w/v	Weight per volume
Vir	Virulence
v/v	Volume per volume

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The plant kingdom represents an enormous reservoir of chemical compounds. Malaysia posses an extremely rich biodiversity and these provide numerous plants with medicinal value. In developing country like Malaysia, medicinal plants continue to be the main source of medication. In recent years, traditional system of medicine has become a topic of global importance. Malaysia is a unique country because of the presence of three active streams of traditional system of medicine which are indigenous Malay, Ayurvedic (Indian) and the Oriental (Chinese). Many of the medicinal plants have been scientifically evaluated for their possible medical applications. The researchers can take a random approach to plant selection or can limit their search to plant of a certain species or genus (the taxonomic approach), plant that contain specific chemicals (the chemotaxonomic approach), or plant that are already known as traditional medical cures (the ethnobotanical approach). Current developments in phytotechnology, phytochemistry and biotechnology have facilitated rapid progress in natural product research. Bioactive compounds extracted from plant can be used as stimulants, hallucinogens, insecticides, poisons, therapeutic agents, food additives, pesticides, dyes, flavors, cosmetic and pharmaceuticals.

The biological activities of the secondary metabolites can be tested using different kinds of bioassays. Bioassay is a biological testing procedure for estimating the concentration of active substance in the extract. A large number of plant secondary metabolites have been shown to possess antioxidative and anticancer properties. As an example, a new anticancer drug, taxol, is derived from the bark of *Taxus brevifolia.* Taxol is a complex diterpene alkaloid known due to its unique mode of action on the microtubular cell system (Jordan and Wilson, 1995). There are many bioassay systems to evaluate the plant chemicals such as *in vitro* antimicrobial, antiproliferative, antioxidant and radical scavenging test (Attaur-Rahman and Choudhary, 2001). Over the last few decades, biological assays (bioassays) have become more important to an effective quality control program especially in biopharmaceutical development. *In vitro* bioassay systems are becoming more popular on the grounds of speed, reproducibility, ethics and convenience as opposed to the *in vivo* animal models, which are expensive, time consuming and ethically sensitive.

Traditionally, plants have been collected for medicinal use from wild areas. In natural product research, the presence of large amount of plant biomass is necessary to provide enough bioactive compounds from the plant tissue. This presents an unfeasible solution due to the lack of reliable and abundant supply of the plant material. Natural habitats for medicinal plant are disappearing fast and together with environmental instabilities, it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to

consider the possibilities of using cell cultures as an alternative supply for the production of plant natural products (Dicosmo and Misawa, 1995). Plant cell cultures have the potential of providing a low cost route to numerous plant derived natural products that are very expensive to synthesize chemically or that occur naturally at very low concentration.

During the past few decades, tissue culture techniques have been manipulated for many purposes such as for the improvement of plants growth, secondary metabolites production and biological activities and transformation. A significant advance in plant tissue culture techniques have led to the use of callus and cell suspension culture (undifferentiated cells) of some plant species for the study of biological activities and production of valuable secondary metabolites (Mulabagal and Tsay, 2004; Bestoso *et al*., 2006). Cell suspension cultures have been sought to deal with problems of low concentrations secondary metabolites in whole plants, like artemisinin (Basile *et al*.,1993) and paclitaxel (Christen *et al*., 1991; Luo *et al*., 2001).

Several widely commercial medicinal plants in Malaysia such as *Eurycoma longifolia, Centella asiatica and Morinda citrifolia* had been successful cultured and their secondary metabolites and biological activities were analyzed (Ali *et al*., 2000; Jasril *et al*., 2003; Maziah, 2005). Technology for large-scale plant cell cultures has been demonstrated in Japan with the production of shikonin by

Mitsui Chemicals Industry Ltd., and production of ginseng saponins by Nitto Denko Co. (Ushiyama, 1991).

Tissue culture is the technique of maintaining plant tissue in an artificial medium *in vitro* under control condition. Propagation of plants through tissue culture has become an important and popular technique. The continuous supply of sterile plantlets will overcome the contamination problem and reduce the time for sterilization process. Tissues from various organs such as stem and leaf of the axenic plantlets can be induced to form callus. Callus tissue can serve as an experimental system to investigate the biological activities using specific bioassays. However, many factors contribute to the ability of a specific tissue to form callus such as medium and plant growth regulators. At present, researchers aim to produce substances with antitumor, antiviral, hypoglycaemic, anti-inflammatory and antimicrobes through tissue culture technology. However, establishment of tissue culture system is required prior to further exploration of the biosynthetic capabilities of various cell cultures.

In vitro propagation of medicinal plants with bioactive principles and cell culture methodologies for selective metabolite production is found to be highly useful for commercial production of medicinally important compounds. The increased use of plant cell culture systems in recent years is perhaps due to an improved understanding of the secondary metabolite pathway in economically important plants. A continuation and intensified efforts in this field will lead to controllable