



UNIVERSITI PUTRA MALAYSIA

***COLLECTION, MORPHOLOGICAL CHARACTERIZATION, BIOACTIVITY
EVALUATION AND MICROPROPAGATION OF SELECTED TACCA
SPECIES (DIOSCOREACEAE)***

MOHD ZULHILMI MISROL

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By

MOHD ZULHILMI MISROL

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of the Requirements for the Degree of Master of Science**

April 2016

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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EVLUATION AND MICROPROPAGATION OF SELECTED *TACCA* SPECIES
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April 2016

Chairman : Associate Professor Thohirah Lee Abdullah, PhD
Faculty : Agriculture

Studies were carried out with the objectives of establishing a germplasm collection of selected *Tacca* species from various accessions in Malaysia, to describe the morphological characters of selected *Tacca* species, to evaluate anti-cancer, anti-histamine and anti-inflammatory activity using various parts of plant crude extract and to improve and develop tissue culture protocol for *T.integrifolia*. *Tacca* (Dioscoreaceae) is a native understorey medicinal plant with a great potential to be developed as an ornamental plant and is considered rare in Malaysia. A germplasm collection consisting of 60 samples representing four species from three states of Peninsular Malaysia and one of Sarawak was established in Field 2 of the University Farm, UPM. *Tacca* species was found growing under various environment conditions where *T. integrifolia*, *T. chantrieri* and *T.nivea* thrived on the moist soils of the forest floor in well-drained areas, high humidity and under more than 70% shade. In contrast, *T. leontopetaloides* was found in dappled shade under coconut trees and under full sun (0% shade) along coastal areas. The conserved *Tacca* species has been characterized for important vegetative and morphological characters for utilization as an ornamental and medicinal plant.

The most variation among *Tacca* species differed in their seed shape, apices of innermost bracts, bract and bracteoles color. The bract and bracteoles color were purple color in *Tacca integrifolia*, dark purple color in *Tacca chantrieri*, white purplish in *Tacca nivea* and green color in *Tacca leontopetaloides*. Three different groups were determined from group cluster and dendrogram based on 23 qualitative and 12 quantitative characteristics among *Tacca* species using MVSP programme.

Various plant parts of selected *Tacca* species [*Tacca integrifolia* (TI), *Tacca chantrieri* (TC) and *Tacca nivea* (TN)] were extracted and tested for their *in vitro* cytotoxicity in cancer cell lines using MTT assay. Results obtained showed that the rhizome extracts were the most potent among the various parts of the plants. Among the rhizome extracts, TI showed the most promising anti-tumour activity, followed by TN and TC. Further investigation on TI revealed that HCT116 and PC-3 cells were the most

sensitive towards the rhizome extracts, with GI_{50} values of $3.3 \pm 1.3 \mu\text{g/mL}$ and $4.0 \pm 0.8 \mu\text{g/mL}$ respectively. In conclusion, the rhizome extract of TI emerged as the most potent and further study on the plant is warranted.

Antihistamine and nitric oxide (NO) inhibitory activity were carried out using methanolic extract from various plant parts (PPCE) and selected *Tacca* species (TSCE). The antihistamine activity was tested in rat mast cell (RBL-2H3) line. Results obtained shows mild antihistamine activity among PPCE and TSCE without showing cytotoxicity activity on RBL-2H3 cells after 4 h using MTT assay. The NO inhibitory activity was determined in a murin macrophage cell line (RAW 264.7). The results demonstrated weak NO inhibitory activity by *T. integrifolia* rhizome extract (TIR) compared with others TSCE and PPCE. Cell exposed to the extracts showed viability with a range of 60 to 90%.

The sterile seeds for the propagation of sterile seedlings were prepared by surface sterilization of loose seed (LS) in 10% commercial bleach, CLOROX and aseptic removal of sterile seeds from fruits pre-sterilized (FS) by burning using 95% ethanol. The sterile seeds from both sources were cultured on the sterile $\frac{1}{2}$ MS and full MS media containing 30 g/L sucrose, pH 5.6 for 4 months. The highest percentage of germination of *T.integrifolia* seeds were recorded from LS in $\frac{1}{2}$ MS media (56%), follow by FS with $\frac{1}{2}$ MS media (16%), LS with full MS (10%) and FS with full MS media (8%) after four months of cultured.

The effect of *in vitro* vertical cutting and decapitation on efficient shoot multiplication of *T. integrifolia* was investigated via shoot tip in MS medium and MS medium fortified with 1 mg/L of 6-benzylaminopurine (BAP). *In vitro* seedlings (2 to 4 cm in height) were cut directly by vertical and decapitated 0.5-0.7 cm above from cotyledon node. After 12 weeks of culture, plantlets regenerated from decapitation in MS medium fortified with 1 mg/L BAP were able to produce new healthy shoots higher than intact and vertical cutting plantlet in the same medium.

The protocol for *in vitro* propagation of *T. integrifolia* through shoot was carried out. The shoot derived from sterile seedling was used as explants for shoot induction and multiplication. The explants were harvested from 16 weeks old seedling and cultured on solidified MS medium supplemented with various concentration of BAP (0, 1, 2, 3, 4, 5 mg/L) for shoot induction and multiplication. The highest number of new shoots and leaves were obtained in MS medium supplemented with 3 mg/l BAP. MS medium with 0.3 mg/L IBA was recommended for root induction after eight weeks of culture with highest number of healthier root.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

**KOLEKSI, PENCIRIAN MORFOLOGI, PENILAIAN BIOAKTIVITI DAN
MIKROPROPAGASI *TACCA* SPESIS TERPILIH (DIOSCOREACEAE)**

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April 2016

Pengerusi : Profesor Madya Thohirah Lee Abdullah, PhD
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Kajian telah dijalankan dengan objektif untuk mewujudkan koleksi germplasma spesies *Tacca* terpilih dari pelbagai tempat penemuan baharu di Malaysia, untuk menghuraikan ciri-ciri morfologi bagi spesies *Tacca* terpilih, untuk menilai aktiviti anti-kanser, anti-histamine dan anti radang dengan menggunakan pelbagai ekstrak mentah dari bahagian tumbuhan dan untuk penambahbaikan dan mewujudkan protokol kultur tisu untuk *Tacca integrifolia*. *Tacca* (Dioscoreaceae) adalah tumbuhan ubatan di kaki hutan yang berpotensi besar untuk dibangunkan sebagai tumbuhan hiasan dan sukar ditemui di Malaysia. Satu koleksi germplasma yang terdiri daripada 60 sampel yang mewakili empat spesies daripada tiga negeri di Semenanjung Malaysia dan satu di Sarawak, telah dibangunkan di Lapangan 2, Ladang Universiti Putra Malaysia. Spesies *Tacca* telah ditemui tumbuh di bawah pelbagai keadaan persekitaran di mana *T. integrifolia*, *T. chantrieri* dan *T. nivea* hidup subur di kawasan lantai hutan bertanah lembap dan bersaliran baik, berkelembapan tinggi dan di bawah lebih daripada 70% teduhan. Sebaliknya, *T. leontopetaloides* didapati di bawah naungan belang-belang pokok kelapa dan di bawah sinaran matahari penuh (0% naungan) sepanjang kawasan pantai. Spesies *Tacca* yang dipulihara dikenalpasti ciri-ciri morfologi dan vegetatif yang penting bagi penggunaan sebagai tumbuhan hiasan dan ubatan.

Kebanyakan variasi bagi spesies *Tacca* telah dikenalpasti dengan ketara pada bentuk biji benih, bentuk hujung pelepah bunga yang terdalam, warna pelepah bunga dan misai pelepah bunga. Warna pelepah bunga dan misai pelepah bunga saling berinteraksi antara satu sama lain di mana warna ungu pada *T. integrifolia*, warna ungu gelap pada *T. chantrieri*, ungu keputihan pada *T. nivea* dan warna hijau pada *T. leontopetaloides*. Tiga kumpulan yang berbeza telah ditentukan dari kelompok kluster dan dendrogram berdasarkan 23 ciri kualitatif dan 21 ciri kuantitatif antara spesies *Tacca* terpilih menggunakan program MVSP.

Pelbagai bahagian tumbuhan daripada species *Tacca* terpilih [*T. integrifolia* (TI), *T. chantrieri* (TC) dan *T. nivea* (TN)] telah diekstrakkan dan kesitotoksikan *in vitro* diuji dalam tiga bahagian sel kanser [HCT116 (usus), PC-3 (prostat) dan MCF-7 (payudara)] dengan menggunakan ujian MTT. Saringan awal mendapati ekstrak rizom adalah yang

paling berkesan di kalangan pelbagai bahagian tumbuhan untuk perencatan bahagian sel kanser. Di antara ekstrak rizom bagi spesies *Tacca* terpilih, TI menunjukkan aktiviti anti-tumor yang paling menjanjikan, diikuti oleh TN dan TC. Siasatan lanjut mengenai tindak balas dos TI menunjukkan bahawa sel HCT116 dan PC-3 adalah yang paling sensitif terhadap ekstrak rizom, dengan nilai GI_{50} masing-masing sebanyak $3.3 \pm 1.2 \mu\text{g/mL}$ dan $4.0 \pm 0.8 \mu\text{g/mL}$. Kesimpulannya, ekstrak rizom daripada TI muncul sebagai yang paling mujarab dan kajian lanjut mengenai tumbuhan ini adalah wajar dilakukan.

Aktiviti antihistamin dan penghalang nitric oksida (NO) telah dijalankan menggunakan ekstrak methanol dari pelbagai bahagian tumbuhan (PPCE) dan spesies *Tacca* terpilih (TSCE). Aktiviti antihistamin telah diuji dalam jalur sel leukemia basofilik tikus (RBL-2H3). Keputusan yang diperolehi menunjukkan aktiviti antihistamin yang lemah di antara PPCE dan TSCE tanpa menunjukkan aktiviti sitotoksik terhadap sel RBL-2H3 selepas 4 jam menggunakan ujian MTT. Aktiviti penghalang NO diukur dalam jalur sel makrofaj murin (RAW 264.7). Keputusan menunjukkan aktiviti penghalang NO yang lemah oleh ekstrak rizom *T. integrifolia* (TIR) berbanding dengan TSCE dan PPCE. Sel yang dirawat dengan ekstrak menunjukkan daya maju sel dari 60 hingga 90%.

Biji benih yang steril untuk pembiakan anak benih yang steril telah disediakan dengan pensterilan permukaan biji benih longgar (LS) di dalam 10% peluntur komersial, CLOROX, dan penyingkiran aseptik biji benih yang steril daripada buah (FS) yang terlebih dahulu disterilkan dengan membakar menggunakan 95% etanol. Biji benih steril daripada kedua-dua sumber dikulturkan di atas media $\frac{1}{2}$ MS dan MS penuh yang steril, mengandungi 30 g/L sukrosa, pH 5.6 selama empat bulan. Peratusan tertinggi bagi percambahan biji benih *T. integrifolia* telah direkodkan dari biji benih longgar dalam media $\frac{1}{2}$ MS (56%), diikuti dengan biji benih steril dari buah dengan media $\frac{1}{2}$ MS (16%), biji benih longgar dengan media MS penuh (10%) dan biji benih steril dari buah dengan media MS penuh (8%) selepas empat bulan dikulturkan.

Kesan pematangan *in vitro* secara menegak dan pemenggalan dalam penggandaan pucuk *T. integrifolia* yang efisien dikaji menggunakan hujung pucuk dalam media MS dan media MS diperkaya dengan 1 mg/L 6-benzylaminopurine (BAP). Anak benih *in vitro* (2 hingga 4 cm tinggi) dipotong terus secara menegak dan dipenggal 0.5-0.7 cm dari atas ruas kotiledon. Selepas 12 minggu pengkulturan, anak pokok dijana dari pemenggalan di dalam media MS diperkaya dengan 1 mg/L BAP dapat menghasilkan pucuk baru yang sihat, lebih banyak daripada anak pokok yang dipenggal dan utuh di dalam medium yang sama. Protokol bagi pembiakan *in vitro* *T. integrifolia* melalui hujung pucuk telah dijalankan. Hujung pucuk yang berasal dari anak benih steril digunakan sebagai eksplan untuk penjanaan dan penggandaan pucuk. Eksplan dituai dari anak benih yang berusia 16 minggu dan dikulturkan dalam media MS pejal yang dibekalkan dengan pelbagai kepekatan BAP (0, 1, 2, 3, 4, 5 mg/L) untuk induksi dan penggandaan pucuk. Bilangan tertinggi pucuk dan daun baru telah diperolehi dalam media MS diperkaya dengan 3 mg/L BAP. Media MS diperkaya dengan 0.3 mg/L IBA telah disyorkan untuk induksi akar selepas lapan minggu dikulturkan dengan jumlah akar sihat tertinggi.

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

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
CRD	Completely randomized design
°C	Degree celcius
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Foetal bovine serum
FS	Seed from fresh fruit
GI ₅₀	Concentration of 50% growth inhibition
HCT116	Colon cancer line
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IC ₅₀	Half maximal inhibitory concentration
iNOS	Inducible nitric oxide synthase
LC ₅₀	Lethal concentration
LPS	Lipopolysacharide
LS	Loose seed
MCF-7	Breast cancer cell line
µg	microgram
µM	micromolar
MS	Murashige and Skoog's medium
MSH	Murashige and Skoog's medium+1 BAP
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVSP	Multivariate System Package
N	north
NAA	1-naphthalene acetic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffer saline
PC-3	Prostate cancer cell line

PPCE	Plant part crude extract
S	Second
SAS	Statistical analysis system
S.D	Standard deviation
S.E	Standard error
TC	<i>Tacca chantrieri</i>
TCR	<i>Tacca integrifolia</i> rhizome crude extract
TGI	Concentration that produces total growth inhibition
TI	<i>Tacca integrifolia</i>
TL	<i>Tacca leontopetaloides</i>
TN	<i>Tacca nivea</i>
TSCE	<i>Tacca</i> species crude extract
UPGMA	Unweighted pair group method using arithmetic averages
UV	Ultra violet

CHAPTER I

INTRODUCTION

As one of the 12 mega-diversity countries in the world, Malaysia has not less than 15,000 species of vascular plants (Jamadon *et al.*, 2007). *Tacca* comes from the family Dioscoreaceae and is a native understorey ornamental and medicinal plant that has a great potential to be developed as a commercial plant in Malaysia. *Tacca* is an evergreen herbaceous and perennial plant with thick rhizomes or tubers and possess stunning inflorescences. Typically, *Tacca* inhabits the moist and shaded understory environment in the tropical lowland forest and hilly areas, at an altitude of 1300m above sea level (Chooi, 2004). A great diversity of *Tacca* species can be found in Malaysia where five of the species are distributed in this region namely *Tacca integrifolia*, *Tacca chantrieri*, *Tacca nivea*, *Tacca leontopetaloides* and *Tacca palmata*.

The inflorescence has whisker-like filiform bracteoles and the colour of the two conspicuous inner involucral bracts range from white, green, purple, brown to near black colour. The true flowers of *Tacca* are dark purple, brown, or near black in colour and they are actinomorphic, hermaphroditic with six stamens (Zhang *et al.*, 2005). Unfortunately, *Tacca* species population in tropical forests are threatened by human development and its habitat preservation in many cases is not considered important. The natural habitat of many species have been totally destroyed, and those of many others have been reduced in size and highly fragmented that the species are in imminent danger of extinction. If the natural populations become extinct, *ex situ* conserved population can be used to maintain the evolutionary process of the endangered species, and will be released back to nature for its habitat restoration (Cohen *et al.*, 1991). In such cases, *ex situ* conservation is essential to preserve a species that has left to extinction in nature (Maxted *et al.*, 1997). Thus, *ex-situ* conservation is required to preserve a species that is in danger of extinction in nature (Li *et al.*, 2002).

The *ex situ* conservation of *Tacca* species is to keep records of the plant characteristics for identification purposes and historical data. It is also for identification of the economically important varieties on their multiplication in order to reintroduce the species into their natural population and for the aim of commercialization so that the industries are less dependent on harvesting this particular species directly from the wild nature.

The abundance of medicinal plants serves as an ideal resource that can unleash new discoveries in the medicine industry. They have proven beneficial because they contain various phytochemicals which are natural molecules produced by plants for protection. These plants serve as an alternative to modern medicine for most local and tribal communities. Traditional use of herbal medicine consist of herbs, herbal material, herbal preparations and finished herbal products that have active ingredients in various plant parts.

The most serious threats to human health in the world is a cancer and chemotherapy is still the standard treatment method. Most of the anticancer drugs presently applied in chemotherapy are cytotoxic to normal cells and cause immunotoxicity which have an effect not only tumor development, but also aggravates patient's recovery. The finding and identification of new antitumor drug with low side effects on immune system has become an important goal in many studies of immunopharmacology (Xu *et al.*, 2009). With this objective, many researches have been carried out to natural compounds in plants, marine organism and microorganisms. Relating to the low side effects of plants and other natural compounds, many researchers and scientists are concerned in working on them to find new medications. Discovery of anticancer agents from plant sources started in the earliest 1950s with the finding and development of vinblastine and vincristine, vinca alkaloid and the isolation of cytotoxic podophyllotoxins (Cragg and Newman, 2005). Rhizomes of *Tacca* have been applied in Chinese folk medicine for the treatment of gastric ulcer, hepatitis and enteritis (Yokosuka *et al.*, 2005). Previously, the structural characterization and isolation of diarylheptanoids, steroidal glycosides and diarylheptanoid glucosides, such as furostan, spirostan, pseudofurostan, withanolide glucoside and pregnane glycosides from the rhizomes of *T. chandleri* have been carried out, as well as their cytotoxic activities against cultured normal and tumor cells were reported by Yokosuka *et al.* (2002a,b,c,d).

Allergy is a serious health problem worldwide due to disfunction of immune system. This term was emerged initially from 20th century and describe about acute hypersensitive immune responses (anaphylaxis) to allergens by predisposed individuals. Allergen is a substance that causes organ disfunction and tissue inflammation (allergic reaction) such as food, moles spores, cosmetics, animal hairs, dust mites and pollen. Mast cell is an important mediator of allergy, which is constituent of virtually all tissue and organ. Activation of mast cell will results in the release of mediators such as histamine and serotonin within minutes (Matsuda *et al.*, 2004). The enzyme (β -hexosaminidase) was released a histamine and used as a marker of mast cell and basophil degranulation, which are stored in the secretory granules (Kraithep *et al.*, 2008). There are numerous pharmacological agents that available for allergic treatment. The mechanism of drug that effective against allergy was acted on a target, influenced by multiple mediators within the allergy cascade. The establishment of compound as powerful tools for allergy treatment was succeeded, if the activity of antigen (IgE) interfering compound against a diverse group of allergy mediators was achieved. Therefore, novel approaches are warranted by screening some useful candidates as anti-allergic drugs, so that a few novel therapeutic candidates can be identified.

Mast cells play an important role in the number of physiological process of homeostasis and disease. Nitric oxide (NO), produced by nitric oxide synthase (NOS), is diatomic radical with cytotoxic properties. Many mast cell functions can be influenced by NO including degranulation, leukotriene production, early mediator release and adhesion (Mc Cauley *et al.*, 2005). Nitric oxide might occurred as a source of free radicals, leading to infiltration and damage of tissue of lymphocytes and inflammatory reactions. Some medicinal plants significantly showed inhibition of nitric oxide level, are performed as potential candidates for new anti-inflammatory drug. Inflammation is a biological reaction to noxious stimuli such as pathogens, which cause cell and tissue

damage. It is acted as a protective measure by removing harmful stimuli and to start process of healing.

Tissue culture technique has been used for the *in vitro* mass propagation serves the future requirement instead of the conventional propagation method. *In vitro* conservation can be performed by normal *in vitro* culture preservation, slow growth conservation and cryopreservation. Normal preservation and cryo-preservation are not practical due to the frequent subculture requirement (Peschke and Phillips 1992). The micropropagation of *Tacca chantrieri* has been investigated using young leaves and leaf stalks of seedling via callus induction (He *et al.*, 2002). Axillary and adventitious shoots of seedling as explants for *in vitro* propagation of *T. Chantrieri* also have been reported (Choraensub *et al.*, 2008). In 2011, the micropropagation *Tacca leontopetaloides* was studied by Brokini *et al.* (2013) using seed embryos.

This study was designed to achieve the following general objectives:

- a) To investigate morphological characterization among selected *Tacca* species
- b) To evaluate cytotoxicity potential, anti-histamine and anti-inflammatory activities of selected *Tacca* species
- c) To establish a tissue culture protocol for *T. integrifolia*

From each experiment, the specific objectives of this study were as follows:

- a) To establish a germplasm collection of selected *Tacca* species from Peninsular Malaysia and Borneo.
- b) To describe the morphological characters among selected *Tacca* species for identification.
- c) To screen cytotoxicity activities of selected *Tacca* species in MCF-7 (Breast), HCT116 (colon) and PC-3 (prostate) cancer cell lines using MTT assay.
- d) To screen anti-allergy activity of selected *Tacca* species using antihistamine assay.
- e) To screen anti-inflammatory of selected *Tacca* species using Griess assay.
- f) To optimize sterilization protocol and concentration of MS medium *in vitro* seed germination of *T. integrifolia*.
- g) To improve shoot multiplication technique of *T. integrifolia*.
- h) To determine optimum concentration of 6-benzylaminopurine (BAP) for shoot regeneration.
- i) To determine optimum concentration of 1-naphthaleneacetic (NAA) and indole-3-butyric acid (IBA) for root induction.

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