PHARMACOLOGICAL STUDIES AND ESTABLISHMENT OF TISSUE CULTURE FOR *Hymenocallis littoralis*

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By

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LIST OF ABBREVIATIONS

$^{\bullet}OH$	Hydroxyl radical
µl	Micro litter
µM	Micro molar
$^{1}O_2$	Singlet oxygen
5-FC	5-fluorocytosine
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
$AlCl_3$	Aluminum chloride
AmB	Amphotericin B
ANOVA	Analysis of variance
BHA	Butylhydroxyanisole
BHT	Butylated hydroxytoluene
C. albicans	*Candida albicans*
CE mg/g	Catechin equivalent (mg) per gram
CFU	Colony forming unit
DNA	Deoxyribonucleic acid
DPPH	Diphenyl-1-picrylhydrazyl

ET	Single electron transfer
EtOH	Ethanol
Fe (II)-TPTZ	Ferrous tripyridyltriazine
Fe (III)-TPTZ	Ferric tripyridyltriazine
$Fe(SO_4)_2$	Ferric sulphate
$FeCl_2$	Iron (II) chloride
$FeCl_3$	Ferric chloride
FRAP	Ferric ion reducing antioxidant parameter
g	Gram
g/L	Gram per liter
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric acid
H. littoralis	*Hymenocallis littoralis*
HA	*Hymenocallis littoralis* anther
HB	*Hymenocallis littoralis* bulb
HF	*Hymenocallis littoralis* flower
HL	*Hymenocallis littoralis* leaves

HR	*Hymenocallis littoralis* root
HS	*Hymenocallis littoralis* stem
HAT	Hydrogen atom transfer
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
HSV-I	Herpes Simplex Virus type I
IC_{50}	Concentration providing 50% inhibition
L/min	Liter per minute
LC_{50}	Lethality Concentration at 50 %
LD_{50}	Median lethal dose
M	Molar
MFC	Minimum Fungicidal Concentration
MeOH	Methanol
mg GAE/g	Milligrams of Gallic acid equivalent per gram
mg/kg	Milligrams per kilogram
mg/mL	Milligram per milliliter
MH broth	Mueller Hinton Broth

MHA	Mueller Hinton agar
MIC	Minimum inhibitory Concentration
min	Minutes
mL	Milliliter
mL/kg	Milliliter per kilogram
ml/min	Milliliter per minutes
mM	MilliMolar
mm	Millimeter
mol/L	Mol per liter
mRNA	Messenger RNA
NA	No activity
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NaNO_2$	Sodium nitrate
NaOH	Sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
nm	Nanometer
O_2^-	Superoxide anion

OD	Optical density
OECD	Organization of Economic and Cooperative Development
OsO_4	Osmium tetroxide
PBS	Phosphate buffer solution
R_f	Relationship to the Front
RM	Ringgit Malaysia
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rt	Retention time
SARS-Cov	Severe Acute Respiratory Syndrome-Associated Coronavirus
S.E.M	Standard error of the mean
SD	Standard deviation
SEM	Scanning Electron Microscopy
spp.	Species
sp.	Species
TBHQ	Tert-butylhydroquinone

TCA	Trichloroacetic acid
TCM	Traditional Chinese Medicine
TEM	Transmission Electron Microscopy
TLC	Thin Layer Chromatography
TPTZ	Tripyridyltriazine
U.K	United Kingdom
US$	United States Dollar
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultra violet
v/v	Volume/volume
v/w	Volume/weight
WHO	World Health Organization

Kajian Farmakologi dan Pembangunan Kultur Tisu bagi Tumbuhan *Hymenocallis littoralis*

ABSTRAK

Hymenocallis littoralis merupakan sejenis pokok hiasan yang mempamerkan pelbagai aktiviti terapeutik seperti aktiviti anti-Candida, antioksidan, sitotoksiti dan penyembuhan luka. Sehubungan itu, bahagian-bahagian pokok ini seperti anter, batang, umbisi, bunga, daun dan akar telah diekstrak menggunakan methanol melalui kaedah sonikasi. Kajian aktiviti anti-Candida telah dijalankan dengan menggunakan ekstrak pokok ini ke atas yis *Candida albicans* (ATCC 10231). Ekstrak metanol bagi bunga dan akar pokok ini mempamerkan aktiviti anti-Candida yang baik pada 6.25 mg/mL sebagai kepekatan perencatan minimum (MIC). Analisis oleh mikroskop elektron pengimbasan dan transmisi berjaya mendedahkan bahawa sel Candida telah kehilangan integriti dinding sel dan kandungan nukleusnya selepas 36 jam dirawat dengan menggunakan ekstrak berkenaan. Ekstrak metanol bagi bunga dan anter menunjukkan kuasa antioksidan penurunan ferric (FRAP) pada 555.12 ± 1.67 dan 568.09 ± 0.42 µmol ($Fe2^{+}$)/g. Selain FRAP, ujian 2,2-diphenyl-1-picrylhydrazyl (DPPH) yang telah dijalankan menunjukkan bahawa kepekatan perencatan pada kadar 50 % (IC_{50}) bagi bunga: 1.29 mg/mL, daun: 5.07 mg/mL, batang: 1.33 mg/mL, umbisi: 5.42 mg/mL, akar: 3.68 mg/mL dan anter: 0.31 mg/mL. Akhirnya, kandungan fenolik dan flavonoid daripada semua ekstrak telah dianalisis. Kandungan fenolik secara meningkat terkandung dalam akar < daun < batang < umbisi < bunga < anter, manakala kandungan

flavonoid pula adalah pada umbisi < akar < daun < bunga < batang < anter. Penemuan ini menunjukkan bahawa ekstrak metanol bagi anter mempamerkan aktiviti antioksidan yang lebih baik berbanding semua ekstrak lain dan mempunyai kandungan fenolik dan flavonoid yang tinggi. Ujian kematian udang air garam telah dijalankan bagi mengetahui aktiviti sitotoksiti dan ekstrak metanol bagi daun menunjukkan 100 % kematian terhadap kesemua anak udang selepas 12 jam menjalani ujian. Kepekatan kematian (LC_{50}) median telah dikira dan ianya menunjukkan nilai sebanyak 8.50 µg/mL bagi umbisi, 11.73 µg/mL bagi akar, 15.95 µg/mL bagi anter, 16.92 µg/mL bagi batang dan sebanyak 64.58 µg/mL bagi bunga. Ekstrak metanol bagi daun dan akar mempunyai aktiviti sitotoksiti yang kuat. Aktiviti farmakologi kemudiannya difokuskan pula kepada kandungan bahan-bahan penyembuhan luka. Didapati, ekstrak daripada umbisi, akar, batang, dan anter mempunyai aktiviti penyembuhan luka yang aktif pada kepekatan 1 µg/mL selepas 36 jam menjalani ujian. Oleh kerana ekstrak pokok ini mempamerkan aktiviti farmakologi yang menonjol, teknik mikropropagasi *in vitro* telah digunakan untuk manghasilkan kalus dan pucuk tumbuhan *Hymenocallis littoralis* menggunakan kombinasi 2,4-Dichlorophenoxyacetic acid (2,4-D) dan Benzylaminopurine (BAP) pada kepekatan yang ditetapkan. Kepekatan 13.5 µM 2,4-D dan 4.5 µM BAP telah dikenal pasti untuk menginduksikan kalus pada masa yang tersingkat dalam peratusan yang tertinggi. Kombinasi dan kepekatan hormon ini berjaya untuk membentuk kalus dalam hanya 15 hari di samping menghasilkan 93.75% kalus. Hormon 2,4-D pada kepekatan 13. 5 µM mengambil masa selama 10 hari untuk meghasilkan pucuk tumbuhan dengan ketinggian 6.2 cm manakala hormone BAP pula pada kepekatan yang sama mampu menghasilkan 4.2 pucuk tumbuhan daripada satu tisu meristem umbisi yang digunakan.

Ekstrak ini seterusnya digunakan dalam analisis kromotografi cecair berprestasi tinggi (HPLC) menggunakan kaedah ultraungu (UV) untuk mengesahkannya. Lycorine telah digunakan sebagai penanda dalam proses pengesahan ini. Had pengesanan (LOD) HPLC adalah pada 24.41 ng/mL dan had kuantifikasi (LOQ) adalah pada bacaan 195.31 µg/mL. Ketepatan dan kejituan proses pengesahan tersebut pada ujian antara hari adalah diantara 95.4 % dan 104.7 % manakala ujian dalam tempoh sehari adalah diantara 0.7 % and 8.2 %. Kandungan lycorine telah dianggarkan menggunakan kaedah pengesahan HPLC dan ianya menunjukkan bahawa umbisi (2.54 ± 0.02 µg/mg), bunga (2.43 ± 0.17 µg/mg) dan anter (2.13 ± 0.35 µg/mg) mempunyai kandungan yang tinggi. Kultur kalus *in vitro* yang dihasilkan menggunakan hormon 2,4-D (4.5 µM) sahaja menunjukkan kandungan lycorine yang tertinggi (2.58 ± 0.35 µg/mg) manakala kultur kalus dengan kombinasi dan kepekatan 2,4-D (4.5 µM) dan BAP (4.5 µM) menunjukkan kandungan lycorine sebanyak 2.45 ± 0.15 µg/mg. Penemuan ini membuktikan bahawa pembentukkan kalus secara *in vitro* mampu memberikan jumlah lycorine yang setanding berbanding pokok liar. Pembentukkan kalus mengunakan teknik mikropropagasi ini adalah sangat cekap kerana penggandaannya yang pesat dan mempunyai kandungan genetik yang hampir sama dengan pokok liar. Penemuan ini berjaya membuktikan kepentingan terapeutik pokok hiasan *Hymenocallis littoralis* dalam bidang farmaseutikal. Oleh itu, penemuan ini dapat membantu dalam pembentukkan ejen anti-Candida, antioksidan dan antikanser yang murah dan lebih berkesan.

Pharmacological Studies and Establishment of Tissue Culture for *Hymenocallis littoralis*

ABSTRACT

Hymenocallis littoralis an ornamental plant exhibits numerous therapeutic properties such as anti-Candida, antioxidant, cytotoxicity and wound healing activities. *Hymenocallis littoralis* parts including anther, bulb, flower, leaves, stem and roots were extracted via sonication technique using methanol solvent. The wild plant extracts was subjected to anti-Candida activity and it shows promising activity against *Candida albicans* strain (ATCC 10231). The flower and root methanolic extracts exhibits better anti-Candida activity at 6.25 mg/mL as minimum inhibitory concentration (MIC). Scanning and transmission electron microscopy analysis reveals the cells lost its cell wall contact integrity and also nucleus properties at 36 h of treatment. The flower and anther methanolic extracts shows ferric reducing antioxidant power (FRAP) at 555.12 ± 1.67 and 568.09 ± 0.42 µmol ($Fe2^{+}$)/g. Beside FRAP, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay carried out and it expresses inhibitory concentration at 50 % (IC_{50}) for flower: 1.29 mg/mL, leaves: 5.07 mg/mL, stem: 1.33 mg/mL, bulb: 5.42 mg/mL, root: 3.68 mg/mL and anther 0.31 mg/mL. Finally, the phenolic and flavonoid contents of all the extracts were analyzed. The phenolic content in ascending order was root < leaves < stem < bulb < flower < anther, while flavonoid content was bulb < root < leaves < flower < stem < anther. In conclusion, the methanolic extract of anther exhibits better antioxidant activity with high amount of phenolic and flavonoid contents. Brine shrimp lethality assay was carried out to determine the cytotoxicity activity and the methanolic

leaves extract exhibits 100 % death for all the nauplii at 12 h of treatment point. The median lethality concentration (LC_{50}) was analyzed and the LC_{50} concentration for bulb, root, anther, stem and flower are 8.50 µg/mL, 11.73 µg/mL, 15.95 µg/mL, 16.92 µg/mL and 64.58 µg/mL, respectively. Leaves and bulb methanolic extracts have strong cytotoxicity activity. The pharmacology activity was further carried out to focus on wound healing properties. Interestingly, the bulb, root, stem and anther methanolic extracts demonstrated active wound healing activity at 1 µg/mL at 36 h of treatment. Since the wild plant extract exhibits prominent pharmacological activities, *in vitro* micro-propagation techniques was carried out to establish callus and shoots production by using combinations of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Benzylaminopurine (BAP) hormones at different concentrations. Highest percentage and earliest induction of callus was observed from 13.5 µM of 2,4-D and 4.5 µM of BAP treatment with 93.75 % of callus induction at 15^{th} day. The 2,4-D hormone at 13. 5 µM concentrations induced shoot at earlier time period (10^{th} day) with length of 6.2 cm. Meanwhile, BAP at 13.5 µM produced higher number of shoots with the average of 4.2 shoots per bulb meristematic tissue. Subsequently, the extracts were subjected to high performance liquid chromatography (HPLC) ultraviolet (UV) analysis to validate the extracts with lycorine. The limit of detection (LOD) and limit of quantification (LOQ) of lycorine was 24.41 ng/mL and 195.31 µg/mL. The accuracy and precision of the validation for between-day and within day was between 95.4 % and 104.7 % and between 0.7 % and 8.2 % respectively. The content of lycorine compound was estimated through HPLC validation method and it demonstrated that the bulb (2.54 ± 0.02 µg/mg), flower (2.43 ± 0.17 µg/mg) and anther (2.13 ± 0.35 µg/mg) to have high amount of

lycorine content in wild plant extracts. *In vitro* callus initiated through best combination hormones of 4.5 µM of 2,4-D and 0 µM of BAP has highest amount of lycorine content at 2.58 ± 0.35 µg/mg and 4.5 µM of 2,4-D and 4.5 µM BAP combination displays 2.45 ± 0.15 µg/mg of lycorine. *In vitro* callus establishment technique express comparable amount of lycorine as wild plant extracts. The micropropagation technique are very efficient for callus production because of the rapid propagation with genetically alike properties as wild plant. These findings prove the therapeutic importance of an ornamental plant in pharmaceuticals facet. These could aid in the development of new cost effective and proficient anti-Candida, antioxidant and anti-proliferation agents.

CHAPTER 1.0

GENERAL INTRODUCTION

Plants, plant parts, and isolated phytochemicals are widely used for the prevention and treatment of various health ailments from time immemorial (Sahoo et al., 2010). Demand for herbal medicines, herbal health products, nutraceuticals, food supplements, herbal pharmaceuticals and cosmetics are increasing globally (Sen et al., 2011). This is all contributed to the recognition of these products as mainly non-toxic, less side effects, better compatibility with physiological flora and availability at affordable price (Dubey et al., 2004).

Plants have a great history as the basis of potential therapeutic agents with evidences of plant secondary metabolites such as reserpine, vinblastine, vincristine, taxol, ginkgolides, lectinan, deserpidine, morphine, codeine incorporated into modern medicine (Mukherjee et al., 2010). Thus, approximately 5-15 % of the total 250 000 species of higher plants have been systematically investigated and identified to be a good source of novel bioactive compounds (Cragg and Newman, 2005).

Spider lily or scientifically known as *Hymenocallis littoralis* is one of the well-known plant species for its medicinal properties. *Hymenocallis littoralis* is grouped under *Amaryllidaceae* family. This plant species has high amount of alkaloidal content. As been reviewed by Abou-Donia et al., (2008a) *Hymenocallis salisb* genus was first phytochemically studied in 1920, which resulted in the isolation of lycorine (alkaloid). Numbers of alkaloids such as lycorine, narciclasine, lycoricidine (bulb), pancratistatin (bulb and roots in small extent), trisphaeridine and

others were isolated from this plant (Lin et al., 1995). This plant has antineoplastic, antiviral activity (Renard-Noiaki et al., 1989), cytotoxicity for 60 human cancer cell lines (Pettit et al., 1993a) and antitumor properties (Idso et al., 2000). Therefore, to explore more on the pharmacological effects, antifungal, antioxidant, wound healing activities and brine shrimp toxicity studies of this plant were explored. Moreover, establishment of plant tissue culture system in *Hymenocallis littoralis* also was carried out this study.

Most of the immune-compromised patients become victim for the fungal infection. *Candida* spp. are the most common pathogens that kills immune-compromised patients (Yigit et al., 2008). The candidiasis infection is often severe, rapidly progressive, difficult to diagnose and refractory to therapy (Yigit et al., 2008). Among the various species, *Candida albicans* is the dangerous causative agent associated with serious fungal infections (Edwards, 1995; Douglas, 2003; Naeini et al., 2009). There are numerous problems in the management of the invasive fungal infection due to modern medicines (Naeini et al., 2009). These difficulties necessitate the discovery of new antifungal agents in order to increase the spectrum of activity against *Candida* spp. There is an increasing acceptance of herbal medicine as an alternative form of health care (Shai et al., 2008). Abundant herbs or plant base isolated drugs are widely used as antibacterial or antifungal agent (Katerere et al., 2003). Therefore, these caused an interest to evaluate the antifungal activity of *Hymenocallis littoralis* in this study.

The importance of antioxidant to human's health was realized in 1960s with the publications of vitamins, flavonoids and ascorbic acids' effects on cancers and common cold (Matough et al., 2012). Currently, there is an upsurge of interest in

phytochemicals as a potential new source of natural antioxidant (Tawaha et al., 2007). Innumerable herbal medicine have been reported to exhibit antioxidant activity, including *Ocimum sanctum, Piper cubeba* Linn., *Allium sativum* Linn., *Terminalia bellerica, Camellia sinensis* Linn., *Zingiber officinale Roscoe* and several Indian and Chinese plants (Khalaf et al., 2008). However there is no report on the antioxidant property on *Hymenocallis littoralis* plant. Therefore, antioxidant activity was carried out for this plant using ferric reducing antioxidant power (FRAP), total phenolic assay, total flavonoid content and DPPH radical scavenging capacity activity protocols.

Wound healing property of *Hymenocallis littoralis* extract was undertaken in this study. Fascinatingly, this exploration was carried out because *Aloe vera* is the only plant which use for the study of wound healing using cultured-based, animal and human based studies (Krishnan, 2006). Currently, there are reports on anticancer property of *Hymenocallis littoralis* extract (Idso et al., 2000; Ingrassia et al., 2008) but there are no reports on the wound healing properties. Thus, bulb, anther, stem, leaves, flower and root's crude extracts were subjected to the wound healing assessment using human foreskin fibroblast cell line (Hs27). Fibroblast cells are mainly involved in the synthesis and deposition of the extracellular matrix in wound healing process (Thakur et al., 2011). Hence, the human fibroblast *in vitro* model is essential to correlate the contractile events of wound (Margaret et al., 1998; Thakur et al., 2011).

Brine shrimp lethality assay is well known assay for the determination of toxicity in various materials including plant extracts (McLaughlin et al., 1998), pesticides (Barahona and Sanchez-Fortun, 1999), cytotoxicity testing of dental

materials (Pelka et al., 2000) and others (Carballo et al., 2002). There are numerous data presented for the correlation between brine shrimp and cytotoxicity assay (McLaughlin et al., 1998). *Hymenocallis littoralis* possess effectual anticancer activity in various cancerous cell lines (Gabrielsen et al., 1992; Ivanov et al., 2009). In this study, the bulb, anther, stem, leaves, flower and root's crude extracts were used for the brine shrimp assay.

In natural product research, large amount of plant biomass is required to provide enough bioactive compounds (Sidik, 2008). Due to natural variability, it is an increasing exertion to obtain adequate plant material from single source (Sidik, 2008). Many efforts have developed feasible method for the production using micropropagation technique. Micropropagation is a technique using plant meristematic tissue to propagate the plant asexually under *in vitro* condition (Yew et al., 2010). The significant advance in micropropagation led to the establishment of callus and cell suspension culture of various plants species. This promote for the evaluation of the biological activities and production of selected valuable secondary metabolites. Consequently, the bulb was used to propagate callus and shoot of this plant via *in vitro* technique.

High performance liquid chromatography (HPLC) is a superior precision, high resolution and has the capacity to analyze thermally labile and non-volatile compounds from plant extracts (Naumovski et al., 2010). Lycorine is the compound with the widest spectrum of biological activities isolated from *Hymenocallis littoralis*. It has antiviral, cytotoxicity, antimalarial and anti-inflammatory activities (Abou-Donia et al., 2008a). In this study, lycorine compound was used as a standard marker to compare the quantity in both wild plant and tissue culture samples. This is

to demonstrate the utility of micropropagation techniques in current plant tissue culture epoch.

Concisely, the bulb, anther, stem, leaves, flower and root's crude extracts were subjected to various standard protocols to determine the pharmacological properties of the plant extracts. These pharmacological findings may assist in the development of an herbal based therapy in the future.

1.1 OBJECTIVES

The objectives of this research project are:

- To identify of the anti-Candida activity of the *Hymenocallis littoralis* plant extracts against *Candida albicans*,
- To determine of the antioxidant properties of the *Hymenocallis littoralis* plant extracts,
- To detect the cytotoxicity level of the *Hymenocallis littoralis* plant extracts using brine shrimp lethality assay,
- To study the wound healing properties of the *Hymenocallis littoralis* plant extracts,
- To establish of the *in vitro* shoot and callus initiation for the *Hymenocallis littoralis*,
- To develop HPLC method for the determination of lycorine content in the wild and tissue culture *Hymenocallis littoralis* plant.

1.2 RESEARCH FLOW

Hymenocallis littoralis's bulb, flower, anther, leaves, stem, roots were extracted using sonication method via methanol solvent to obtain the crude methanolic extracts. This crude extract was subjected to few pharmacological screening using various standard methods (Chapter 2: Materials and Methods) for anti-Candida, antioxidant, brine shrimp lethality evaluation and wound healing activity. As for the *in vitro* micropropagation technique, *Hymenocallis littoralis*'s bulb meristematic tissue was used to establish the callus and shoot cultures. Finally, high performance liquid chromatography (HPLC) was used to develop a method to detect lycorine content in *Hymenocallis littoralis* extract and compared the presences of lycorine in the wild and tissue cultures extracts. Lycorine is one of the major phytochemical substances isolated from the *Hymenocallis littoralis* plant. The research flow for this study is briefly illustrated in Figure 1.1.

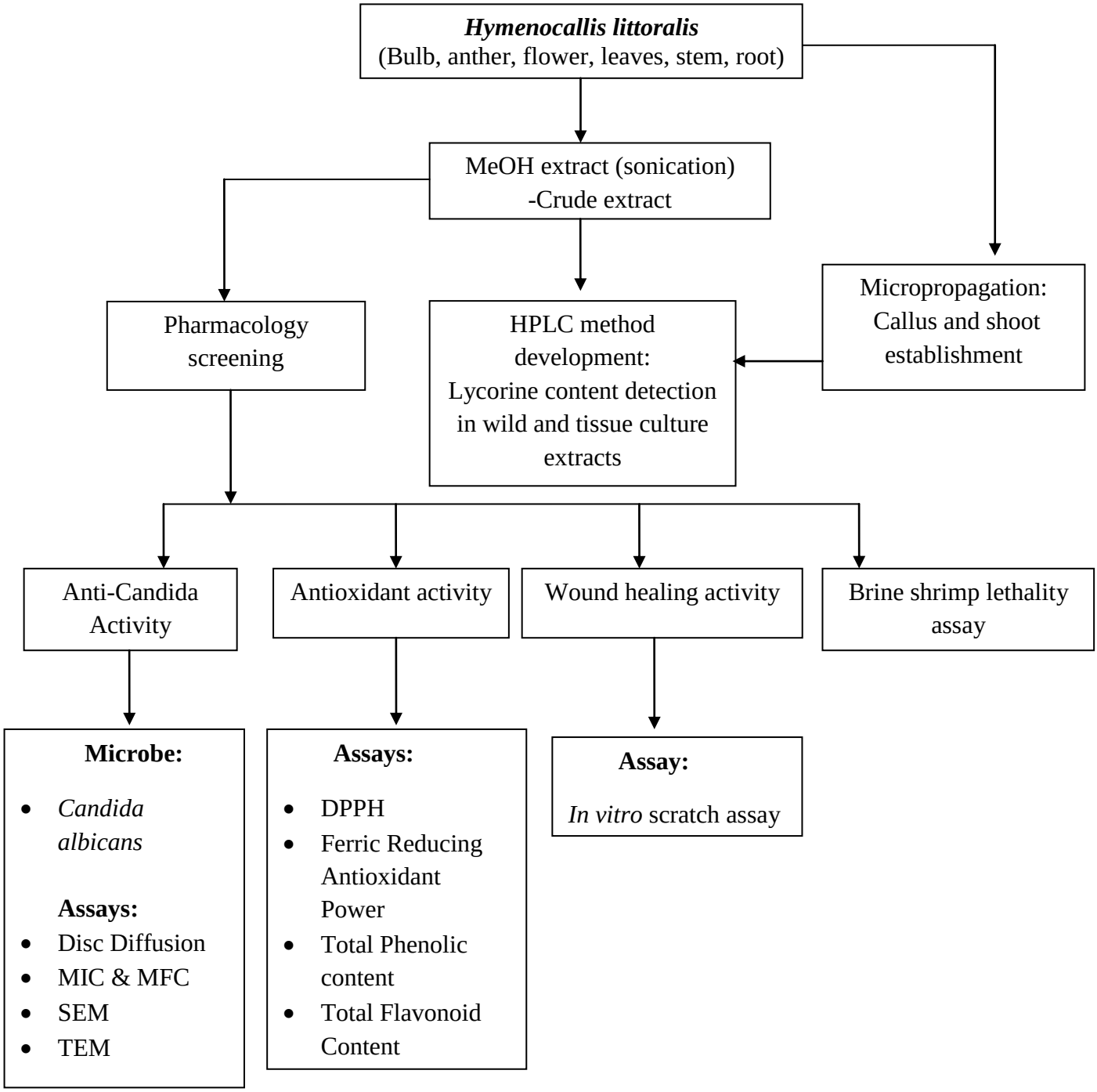

Figure 1.1: Research flow for the pharmacology evaluation, establishment of tissue culture and lycorine determination using HPLC method on *Hymenocallis littoralis* plant.

CHAPTER 2.0

LITERATURE REVIEW

2.1 Importance of Plant Secondary Metabolites

In the 21st century, herbal medicines are gaining importance in mainstream human healthcare (Sen et al., 2011). A greater number of people are seeking herbal remedies which are free from side effects compared to synthetic chemical medicines (Dubey et al., 2004). The western population are considering for natural medications due to the adverse side effects of synthetic drugs (Gijtenbeek et al., 1999; Johnson and William, 2002; Dubey et al., 2004).

It is estimated that about 80 % of the world population have faith in traditional medicine particularly from plant based drugs (Dubey et al., 2004). This is due to the unmatched availability of secondary metabolites diversity in natural products, either pure compounds or as standardized plant extracts (Cos et al., 2006). Total of 20,000 plant species were used for medicinal purposes are reported by World Health Organization (WHO) for the year 2005 (Gullece et al., 2006). Sahoo et al. (2010) reported that 25 % of the drugs prescribed worldwide are derived from plants with total of 121 active compounds. There are 252 drugs in WHO's essential medicine list and out of it 11 % is entirely from plant origin (Sahoo et al., 2010).

From 2000 to 2005, annual sales for traditional medicines increased from US$ 385 million (RM 1 billion) to US$ 1.29 billion (RM 4.5 billion) in globally (Aziz and Tey, 2009). This transformation is because the herbal products are non-

toxic, at affordable prices, have less side effects, (Dubey et al., 2004; Sharma et al., 2008) possess better tolerance and are globally competitive (Sen et al., 2011) compared to modern synthetic medicines.

Plants have wide range of secondary metabolites with abundant pharmacological properties. Numbers of plant secondary metabolites such as flavonoids, alkaloids, saponins, phenolics and terpenes have been used widely as antifungal, antibacterial, anticancer, antimalarial and antiviral agents (Ncube et al., 2008). The flavonoid rich extracts from *Scutellaria baicalensis* roots have been proven to exhibit anti-proliferative effects on various cancer cell lines (Scheck et al., 2006). Papavarine is a benzylisoquinoline alkaloid that has inhibitory effect on several viruses and idoquinoline alkaloid from *Cryptolepsis sanguinolenta* exhibits activity against number of gram negative bacteria and yeast (Silva et al., 1996; Ncube et al., 2008).

Plant-derived products became a primary choice for biological and pharmacological researchers to investigate the medicinal properties of the herbal plants. Wide category of researchers including ethno-pharmacologist, botanist, microbiologist, and natural product chemist are extensively searching for pharmacologically active phytochemicals from plants (Tanaka et al., 2006). In India, only 7500 species from 17 000 species of higher plant are known for their medicinal values (Sen et al., 2011).

Malaysia has been awarded as the world's 12^{th} Mega Biodiversity which is rich in flora and fauna (Ang, 2004) and blessed with an abundance of varied medicinal plants. The most common Malaysian plants undergoing extensive research

are *Eurycoma longifolia, Labisia pumila, Andrographis paniculata, Orthosiphon stamineus, Centela asiatica* and so on (Jamal, 2006). However, there are numerous species especially under ornamental plants which are not yet proven scientifically for its pharmacological activities. Thus, it will be of great benefit for the society at large to study the biological and pharmacological properties of these plants especially *Hymenocallis littoralis*.

2.2 *Hymenocallis littoralis* plant

Hymenocallis littoralis Salisb. is from *Amaryllidaceae* family. The plant's description is as bellow:

Kingdom : Plantae

Division : Angiospermae

Class : Monocotyledoneae

Order : Asparagales

Family : Amaryllidaceae

Genus : *Hymenocallis*

Species : *littoralis*

Alkaloid is a member of a large group of secondary metabolites which is produced in *Amaryllidaceae* plant species. Many alkaloids possess potent pharmacologic effects. From ancient time, numbers of alkaloids from *Amaryllidaceae* plants extracts have been widely used in scientific investigation. The phytochemical constituents of *Hymenocallis salisb*. genus was studied during 1920 and cause for the isolation of lycorine compound. This plant is common but it possesses great importance especially to mankind. The odor of *Amaryllidaceae* plant flowers has been highly valued in the fragrance industry. Volatile compounds from these plants are given more interest because of their allelochemical importance as defensive compounds, insect repellents, attractants, and their role in ecological balance (Abou-Donia et al., 2008b).

Figure 2.1: *Hymenocallis littoralis*. Scale bar (1:5cm)

2.2.1 Physiological Characteristics of *Hymenocallis littoralis*

Hymenocallis littoralis is a bulbous perennial herb which can grow up to 60 to 70cm (36 inches). The leaves of this plant are fleshy, dark green and glossy. The shape of the leaves is narrowly lanceolate. It is 0.5 to 1.0 meter long and 6 to 7 cm wide (Shields, 2006). The plant's scape is erect, solid and slightly compressed. It is about 0.5 m tall. At the top of the scape, there are few to many sessile and umbellate flowers. The bulb of the plant is 7-10 cm (3-4 inches) in diameter. With age, the bulb develops a neck that reaches 4-5cm in diameter (up to 2 inches) (Shields, 2006). The flowers are large, white, vanilla scented and sessile flowers. *Hymencallis littoralis* grows well in normal room temperature for most of the year and needs high humidity (Grossi, 2007).

2.2.2 Phytochemistry of *Hymenocallis littoralis* perennial herb

Hymenocallis littoralis is rich in alkaloids. A chromatographic study of the qualitative composition of the total material isolated using thin-layer chromatography in a fixed layer of type KSK silica gel with various systems of solvents showed the identical qualitative compositions of the alkaloids of the epigealand hypogeal part of the plant (Lin et al., 1995). Lycorine is the dominant in *Hymenocallis littoralis* plant extract. These alkaloids are contributing as major role in the pharmacological effects (Lin et al., 1995).

There are also other alkaloids that have been isolated from this plant. The names of the isolated compounds are Lycorine (dominant in *Hymenocallis littoralis'* bulb), Narciclasine (bulb), Lycoricidine (7-deoxynarciclasine) (bulb), Pancratistatin (bulb and roots in small extent), trisphaeridine (from bulb with roots), Tazettine,

galanthamine, 7,4'-dihydroxy-8-methylflavan (stem), Hippeastrine (bulb), Littoraline (bulb), Haemanthamine (bulb), pretazettine (bulb), macronine (bulb), homolycorine (bulb), lycorenine (bulb), O-methyllycorenine (bulb), Lycoramine (bulb), Demethylmaritidine (bulb), Vittatine (bulb) and 5,6-dihydrobicolorine (Ismine) (bulb) (Abou-Donia et al., 2008b).

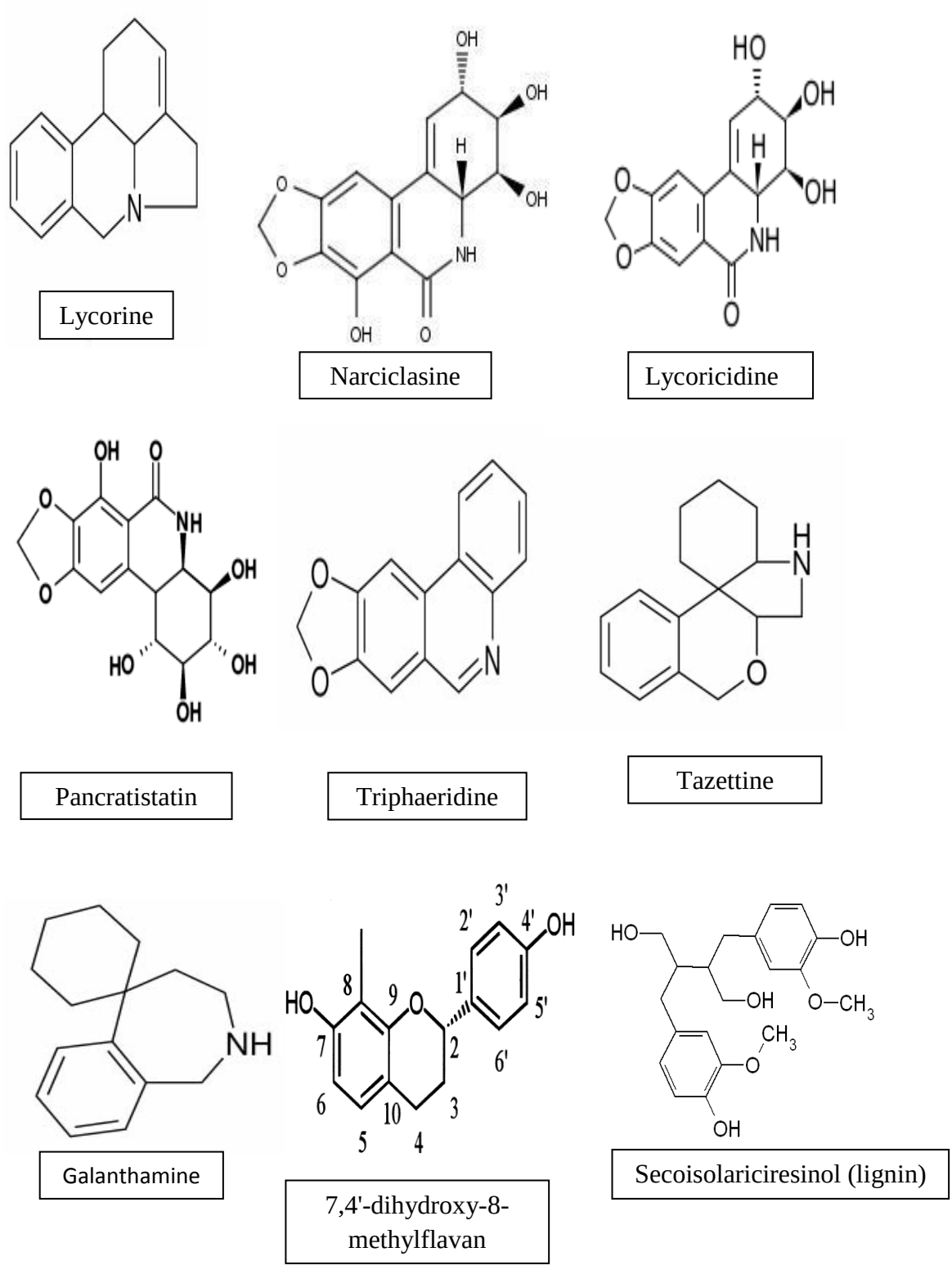

Figure 2.2: Few isolated compounds from *Hymenocallis littoralis* (Abou-Donia et al., 2008a).

2.2.3 The Therapeutic Importance of *Hymenocallis littoralis*

2.2.3.1 Free radical scavenging activity

Free radicals increases the risk for occurs more than 30 different diseases in human body system (Aruoma, 1998). A compound known as 7, 4'-dihydroxy-8-methylflavan was isolated from *Hymenocallis littoralis* stem. The compound was subjected to free radical scavenging activity using DPPH spectrophotometric assay. The radical scavenging activity of 7,4'-dihydroxy-8-methylflavan (320 μM) was found to be weaker than quercetin but equipotent to butylhydroxytolune (BHT) (Ioset et al., 2001).

2.2.3.2. Antiviral properties

Two important alkaloids, lycoricidine and pancratistatin that were isolated from *Hymenocallis littoralis* show strong RNA anti-viral activity (Gabrielsen et al., 1992). Pancratistatin demonstrated significant *in vitro* antiviral activity against Japanese encephalitis, yellow fever and dengue-type 4 (Gabrielsen et al., 1992). Moreover, Gabrielsen et al. (1992) reported the plant activity against RNA-containing flaviviruses and bunyaviruses namely Punta Toro and Rift Valley fever. Lycorine exerts antiviral effects on several RNA and DNA viruses (Fennell and Staden, 2001). The effect is accomplished via delaying virus production and decreasing the amount of virus by blocking viral protein synthesis (Ieven et al., 1983; Fennell and Staden, 2001), possibly at the level of termination (Vrijsen et al., 1986; Fennell and Staden, 2001). Lycorine pronounces antiviral activity against poliomyelitis, coxsackie and herpes type I viruses (Harborne and Baxter, 1993; Fennell and Staden, 2001). This compound has stopped the production of the

poliovirus via inhibiting the precursors of poliovirus and poliopeptidase (Ghosal et al., 1985; Fennell and Staden, 2001). Littoraline alkaloid from this plant shows an inhibitory action towards HIV reverse transcriptase (Lin et al., 1995).

Lycorine exhibit positive effects against many viruses such as human immunodeficiency virus (HIV-1), severe acute respiratory syndrome-associated coronavirus (SARS-CoV), poliovirus, coxsackie virus, measles virus, herpes simplex virus type I (HSV-I) (Ieven et al., 1983; Szlavik et al., 2004; Li et al., 2005) and vaccinia small pox virus (Deng et al., 2007; McNulty et al., 2009). However, Littoraline shows weakly inhibited HIV-1 reverse transcriptase (IC_{50} = 142.0 mg/mL) activity (Lin et al., 1995).

2.2.3.3. Anticancer properties

The tropical spider lily has been known to has antitumor property since in ancient time (Idso et al., 2000). Pancratistatin exhibits a vast anticancer activity against various cell lines (Griffin et al., 2007).

Pancratistatin, a natural compound isolated from the Hawaiian spider lily (*Hymenocallis littoralis*) displayed potent cytotoxicity against human tumor cell lines (Pettit et al., 1993b; Griffin et al., 2007). Pancratistatin is proved to be effective (38-106% life extension at 0.75-12.5 mg/kg dose levels) against the murine P-388 lymphocytic leukemia. PST also markedly inhibited (ED_{50}, 0.01 µg/mL) growth of the P-388 *in vitro* cell line and *in vivo* murine M-5076 ovary sarcoma (53-84% life extension at 0.38-3.0 mg/kg) (Pettit et al., 1986; Idso et al., 2000).

Further investigation reveals that, pancratistatin possess cytotoxicity against 60 human cancer cell lines, demonstrating highest efficiency against melanoma subpanel lines and active for certain brain, colon, lung and renal cancer cell lines (Pettit et al., 1993b; Idso et al., 2000). The pancratistatin compound exhibits selective toxic effects to cancer cells and normal cells at micro-molar doses (Kekre et al., 2005; Griffin et al., 2007). This compound reacts to the cell by affecting mitochondrial function and inducing apoptosis in malignant cell lines (McLanchlan et al., 2005; Griffin et al., 2007).

Two other alkaloids that also play an important role in anticancer properties are lycorine and haemanthamine. Both were potent *in vitro* cytotoxicity against a battery of human cancer cell lines such as human breast cancer, human colon cancer and human lung cancer (ED_{50} values of 0.2-5.0 µg/mL) (Lin et al., 1995). Lycorine inhibits the *in vivo* growth of a murine ascite tumor and reduce the viability of *in vitro* grown tumor cells (Ghosal et al., 1985). Lycorines also inhibits the synthesis of DNA and proteins in murine cells (Ghosal et al., 1985).

2.2.3.4. Antibacterial and antifungal properties

Antibacterial and antifungal screenings of *H. littoralis* were carried out using the agar-diffusion technique against the Gram-positive bacterium *Staphylococcus aureus*, the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, and the fungus *Candida albicans*. The petroleum ether extract of *H. littoralis* showed antibacterial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Pseudomonas aeruginosa*, but no activity against *Escherichia coli* and *Candida albicans* (Abou-Donia et al., 2008b). The corresponding minimum-

inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were 15.6 and 62.5 mg/mL (*S. aureus*), and 250 and 500 mg/mL (*P. aeruginosa*), respectively (Abou-Donia et al., 2008b). Lycorine, pseudolycorine, narciclasine and pretazettine compounds exhibit antifungal activity against *Saccharomyces cerevisiae* (Giudice et al., 2005) and inhibiting the protein synthesis and blocking peptide bond formation in eukaryotic cells (Cordell, 1981; Ghosal et al., 1985; Fennell and Staden, 2001).

2.2.3.5 Anti-parasitic properties

Pancratistatin and lycoricidine showed an *in vitro* anti-parasitic effect against a microsporidium causing infections in humans (Quarzane-Amara et al., 2001). The anti-parasitic effect of a collection of compounds with anti-mitotic activity has been tested on a mammalian cell line infected with *Encephalitozoon intestinalis*, a microsporidian causing intestinal and systemic infection in immune-deficient patients (HIV patients) (Weber and Bryan, 1994).

The anti-parasitic effect was evaluated by counting the number of parasitophorous vacuoles detected by immunofluorescence. Two out of 526 compounds tested show inhibition activity of the infection without affecting the host cell. These two compounds are PST and 7-deoxynarciclasine. The 50% inhibitory concentrations (IC_{50}) of PST and 7-deoxynarciclasine for *E. intestinalis* were 0.18 µM and 0.2 µM, respectively, approximately eightfold lower than the IC_{50} of these same compounds against the host cells (Quarzane-Amara et al., 2001). Electron microscopy confirmed the gradual decreases in the number of parasitophorous vacuoles and showed that of the two life cycle phases, sporogony was more sensitive

to the inhibitors than merogony. Furthermore, the persistence of meronts in some cells apparently devoid of sporonts and spores indicated that the inhibitors block development rather than entry of the parasite into the host cell. The occurrence of binucleate sporoblasts and spores suggests that these inhibitors blocked a specific phase of cell division (Quarzane-Amara et al., 2001).

2.2.4. Other pharmacological effects of *Hymenocallis littoralis*

The locals believed that the underground stem when boiled and the decoction drunk could increase appetite. The whole plant is also pounded for poulticing swollen joints and broken bones (Idso et al., 2000). Lycorine proved to be a powerful feeding deterrent against highly poyphagous insect, the desert locust (Singh and Pant, 1980) and insect anti-feedant activity (Evidente et al., 1986).

Lycorine suppressed tumor cell growth and reduced cell survival via cell cycle arrest and induction of apoptosis for the *in vitro* mode of action in a leukemia (HL-60) cell line model (Liu et al., 2004; McNulty et al., 2009). Lycorine also investigated on severe combined immune-deficiency (SCID) mice inoculated with HL-60 cells and suppressed tumor cell growth and increased survival rate of treated animal without any adverse effects (Liu et al., 2007). This compound also arrest the cell cycle progression and exhibit apoptosis on multiple myeloma (KM3) cells (Li et al., 2007b; McNulty et al., 2009).

2.3 Antifungal Activity

2.3.1 Rise of Fungal Infections in Humans

Fungal infection in human beings is remains a therapeutic problem despite there is availability of antifungal ointments, lotions, capsules, powders and paints (Yigit et al., 2008). The infection has increased dramatically over the past few decades. The fungal infections are often challenging to diagnose, severe, swiftly progressive and refractory to the therapy (Yigit et al., 2008).

2.3.2 Common Fungal Infection

Yeast fungal infections became quite common in immuno-compromised hosts, especially in HIV-infected individuals, or in patients given immunosuppressive or broad-spectrum antibiotics (Sanglard, 2002). In recent years, Candidiasis become a serious infectious disease commonly in immunologically compromised patients (Duarte et al., 2005).

Candidiasis disease can be acute or chronic, superficial or deep infection to human beings (Prabhakar et al., 2008). This disease mostly knocks in patients who are predisposed to an overgrowth of their own yeast flora (Prabhakar et al., 2008). The oropharyngeal candidiasis occurs to diabetes mellitus patients, to those receiving antibacterial antibiotics and infected with HIV 1 and HIV 2 (Kwon-Chung and Bennet, 1992). Among the various species, *Candida albicans* is the most causative agent for the candidiasis infection (Edwards, 1995; Douglas, 2003; Naeini et al., 2009).

2.3.3 *Candida albicans*

Candida is a thin walled small cell and grouped under yeast family (Najafi and Sadeghi-Nejad, 2011). *Candida albicans* is an opportunistic pathogen that can cause local and systemic infections in those undergoing persistent antibiotic treatment (Duarte et al., 2005). It is a yeast fungus that resides on skin and mucosa of immune-competent individuals (Fringuelli et al., 2002) and causes oral, vaginal and systemic infections (Lyons and White, 2000).

There are several antifungal drugs are available in the markets for the treatment of *Candida* infections. Amphotericin B (AmB), 5-flouorocytosine (5-FC), Azoles group antifungal agent such as fluconazole, itraconazole, ketoconazole, miconazole and clotrimazole, are the common antifungal drugs for the treatment of *Candida* illnesses (Sanglard, 2002). These drugs are divided into two groups as topical antifungal (miconazole, nystatin, tioconazole and clotrimazole) and oral drugs (fluconazole and AmB) (Najafi and Sadeghi-Nejad, 2011).

2.3.4 Failure of The Current Antifungal Drugs

Currently a lot of antifungal drugs are available yet there are rising out breaks for *Candida* species infections. There are few causes for the failure of the suppression of *Candida* infections. Certainly the toxic effects of the available antifungal drugs, the high cost of drugs, limited number of antifungal drugs for the illness, resistance and relapse of *Candida* infections are the main causes for the failure (Klepser, 2001; Khan et al., 2003; Runyoro et al., 2006; Naeini et al., 2009).

Currently, the widespread and incorrect use of azole group and Amphotericin B antifungal has led to emergence of resistance in common pathogenic fungi (Graybill, 1996; Masoko et al., 2007). Several studies shows the *Candida* species resistance towards amphotericin B, fluconazole, flucytosine, itraconazole and ketoconazole (Hawser and Douglas, 1995; Chandra et al., 2001; Lewis et al., 2002; Al-Fattani and Douglaas, 2004). This led to the increases of opportunistic infections especially with Acquired Immunodeficiency Syndromes (AIDS) patients and individuals on immunosuppressive chemotherapy (Maregesi et al., 2008).

2.3.4.1 Amphotericin B

The widespread and over uses of these drugs lead *Candida* to evolve resistance mechanism against to the antifungal medications (Najafi and Sadeghi-Nejad, 2011). AmB is a strong fungicidal drug and its primary mode of action is to bind ergosterol in the membrane bilayer. This leads to the production of pore-like structure on the membrane and the leakage of vital cytoplasmic components such as mono and divalent cations (Najafi and Sadeghi-Nejad, 2011). Nonetheless, clinical isolates of *Candida* from AIDS patient shows resistance towards this drug (Kelly et al., 1994; Najafi and Sadeghi-Nejad, 2011). The drug resistance often associates with the alteration of membrane lipids and lack of ergosterol content associate in cell membrane (Dick et al., 1980; Najafi and Sadeghi-Nejad, 2011).

2.3.4.2 5-Fluorocytosine (5-FC)

5-FC displayed a good *in vitro* and *in vivo* antifungal activity (Polok, 1990). Cytosine deaminase in *Candida* cells deaminated the 5-FC into 5-fluorouracil (5-FU). This 5-FU can be incorporate into RNA and lead to miscoding protein.

Moreover, can be converted to a deoxynucleoside which inhibits thymidilate synthase and thereby DNA synthesis (Coleman et al., 1998; Sanglard, 2002). Conversely, the lack of cytosine permease enzyme implication in the metabolism of 5-FC and deregulation of the pyrimidine biosynthetic pathway could lead to the resistance of the 5-FC drug (Sanglard, 2002).

2.3.4.3 Azole groups

Azole groups antifungal (ketoconazole, miconazole, fluconazole, clotrimazole and itraconazole) have a cytochrome P450 (*ERG11* gene) as a common cellular target in yeast or fungi (White et al., 1998). The unhindered nitrogen ring in the drug binds the heme iron of Erg11p and inhibiting the enzymatic reaction. The first case of resistance was reported on *Candida albicans* due to the prolonged therapy with ketoconazole and miconazole (White et al., 1998). The extended treatment cause mutation in the gene encoding for Erg11p and trigger conformational changes in the binding azole drugs (Lamb et al., 1998; Sanglard, 2002).

2.3.5 New Antifungal Exploration

The different morphological forms and differential sensitivity necessitating enhanced doses of antifungals for *Candida albicans* (Mukharjee et al., 2003). Many antifungal drugs have limited use due to the side effects and emergence of antibiotic resistant to human pathogenic fungi (Maregesi et al., 2008). Thus, there is a need of searching more new substances which could act host friendly (Zore et al., 2011).