GENOTOXICITY ASSESSMENT OF STANDARDISED ROOT EXTRACT OF EURYCOMA LONGIFOLIA JACK AND SYNTHESIS OF SOME BENZIMIDAZOLE DERIVATIVES AND EVALUATION OF THEIR ANTI-CANCER POTENTIAL

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GENOTOXICITY ASSESSMENT OF STANDARDISED ROOT EXTRACT OF *EURYCOMA LONGIFOLIA* JACK AND SYNTHESIS OF SOME BENZIMIDAZOLE DERIVATIVES AND EVALUATION OF THEIR ANTI-CANCER POTENTIAL

by

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This thesis is dedicated

To,

My beloved parents

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Abstracts

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LIST OF ABBREVIATIONS

µg/mL	Microgram per milliliter
µL	Microliter
µm	Micrometer
µM	Micromolar
µmol/L	Micromol per liter
^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
5-FU	5-fluorouracil
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
AZT	3'-azido-3'-deoxythymidine
bp	Basepair
BER	Base excision repair
BSA	Bovine serum albumin
CA	Chromosome aberration
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
CHO	Chinese hamster ovary
COM	Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment
CP	Cyclophosphamide monohydrate
d	Doublet
DMEM	Dulbecco's Modified Eagle's Medium
DMF	N, N-dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d_6	Deuterated dimethyl sulfoxide
dUMP	20-deoxyuridine-50-monophosphate
dTMP	20-deoxythymidine-50-monophosphate
ECM	Extracellular matrix
ED	13α,21-dihydroeurycomanone
EDTA	Ethylene diamine tetraacetic acid
EL	Eurycomanol
EN	Eurycomanone
EP	13α(21)-epoxyeurycomanone
EPA	Environmental Protection Agency
ESI	Electrospray ionisation

EtOAc	Ethyl acetate
EtOH	Ethanol
EW	Empty well (s)
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GEF	Global evaluation factor
GG	Global genomic
GI_{50}	The molar concentration that inhibit the growth of the cells by 50%
h	Hour (s)
Hex	*n*-Hexane
HIHS	Heat-inactivated horse serum
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HR	Homologous recombination
IARC	International Agency for Research on Cancer
IC_{50}	Inhibitory concentration that cause 50% of cell death
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceutical for Human Use
IMF	Induced mutant frequency
IWGT	International Workshop on Genotoxicity Testing
KCl	Potassium chloride
LC_{50}	The molar concentration required to kill 50% of the cells
LD_{50}	Lethal dose 50
M	Molar
m	Multiplet
MeOH	Methanol
MF	Mutant frequency
mg	Milligram
mg/kg	Milligram per kilogram
mg/mL	Milligram per milliliter
mL	Milliliter
mm	Millimeter
MLA	Mouse lymphoma assay
MMS	Methyl methanesulfonate
MN	Micronucleus
MMP	Matrix metalloproteinase
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

Na_2SO_4	Sodium sulfate
NADP	β-nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NER	Nuclear excision repair
NHEJ	Non-homologous end joining
nm	Nanometer
NPCB	National Pharmaceutical Control Bureau
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE_M	Plating efficiency of mutant colonies
PE_v	Plating efficiency of viable cells
PLC	Preparative layer chromatography
q	Quartet
ROS	Reactive oxygen species
RPE_v	Relative plating efficiency for viability
RPMI-1640	Roswell Park Memorial Institute 1640
RSG	Relative suspension growth
RTG	Relative total growth
s	Singlet
SCE	Sister chromatid exchange
SG	Suspension growth
t	Triplet
TCR	Transcription-coupled repair
TFT	Trifluorothymidine
tk	Thymidine kinase
TLC	Thin layer chromatography
TS	Thymidylate synthase
TSG	Tumour suppressor gene
TW	Total well (s)
UV	Ultraviolet
v/v	Volume to volume
w/w	Weight to weight
WHO	World Health Organisation
XPRT	Xanthine-guanine phosphoribosyl transferase

APPENDICES

PENILAIAN GENOTOKSISITI KE ATAS EKSTRAK PIAWAI AKAR *EURYCOMA LONGIFOLIA* JACK DAN SINTESIS TERBITAN BENZIMIDAZOLE SERTA PENILAIAN TERHADAP POTENSI ANTI-KANSERNYA

ABSTRAK

Dalam kajian ini, potensi genotoksik ekstrak piawai akar, F2 daripada *Eurycoma longifolia* Jack telah dinilai menggunakan cerakin limfoma tikus (MLA) mengikut garis panduan OECD. Kerja ini berfungsi sebagai ujian pertama untuk menilai profil keselamatan *E. longifolia* menggunakan sistem *in vitro* mamalia. MLA berkebolehan dalam mengesan spektrum luas kejadian mutasi termasuk mutasi titik dan aberasi kromosom. Sel L5178Y/tk$^{+/-}$ telah dirawat dengan 1 hingga 50 μg/mL ekstrak akar dalam ketidakhadiran dan kehadiran pengaktifan metabolik S9 bagi tempoh rawatan 3-jam. Satu cerakin rawatan 24-jam telah dijalankan tanpa pengaktifan metabolik S9.

Hasil kajian menunjukkan tiada peningkatan signifikan dan bergantungan kepekatan dalam frekuensi mutan diperhatikan pada kepekatan yang diuji di bawah semua keadaan rawatan. Dalam rawatan 24-jam, jumlah pertumbuhan relatif (RTG) yang merupakan pengukuran sitotoksisiti telah menurun kepada 33.0% dan 21.6% dalam eksperimen 1 dan 2 masing-masing pada kepekatan tertinggi, 50 μg/mL. Tindak balas setiap kultur ujian telah ditentukan sebagai negatif berdasarkan kriteria piawai yang dicadangkan oleh Kumpulan Kerja MLA Bengkel Antarabangsa atas Pengujian Genotoksisiti (IWGT). Frekuensi mutan teraruh (IMF) telah didapati kurang daripada faktor penilaian global (GEF) dan analisis aliran adalah negatif. Penemuan kajian ini menunjukkan

bahawa kepekatan sehingga 50 μg/mL ekstrak akar piawai, F2 adalah negatif untuk potensi genotoksik dalam kehadiran dan ketidakhadiran pengaktif metabolik.

Satu siri terbitan benzimidazole telah disintesis, dicirikan dan dinilai untuk potensi terhadap anti-kanser dalam kajian ini. Semua sebatian telah diuji menggunakan cerakin MTS untuk menilai potensi sitotoksik mereka terhadap titisan sel kanser payudara MCF7 dan MDA-MB-231. Hasil kajian menunjukkan bahawa sebatian-sebatian tersebut tidak mempunyai kesan sitotoksik ke atas kedua-dua titisan sel. Terbitan benzimidazole, 2-(4-fluorofenil)-1*H*-benzimidazole dikaji menggunakan cerakin migrasi "transwell" untuk menilai sama ada sebatian tersebut boleh merencat migrasi sel MDA-MB-231. Menariknya, sebatian tersebut merencatkan migrasi sel MDA-MB-231 ($p < 0.01$) dengan ketara pada 100 μM berbanding dengan kawalan. Kajian lanjut melibatkan analisis biokimia dan fungsian 2-(4-fluorofenil)-1*H*-benzimidazole berfungsi untuk menilai mekanisme tindakannya.

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ABSTRACT

In this study, the genotoxic potential of standardised root extract, F2 of *Eurycoma longifolia* Jack has been assessed using the mouse lymphoma assay (MLA) in accordance with OECD guideline. This work serves to be the first test to evaluate the safety profile of *E*. *longifolia* using the *in vitro* mammalian system. The MLA is capable of detecting a broad spectrum of mutational events include point mutations and chromosomal abberations. The L5178Y/tk$^{+/-}$ cells were treated with 1 to 50 μg/mL of the root extract in the absence and presence of S9 metabolic activation for 3-h. A 24-h treatment assay was performed without the S9 metabolic activation.

The results showed no significant ($p > 0.05$) and concentration-dependent increase in mutant frequency observed at the concentrations tested under all the treatment conditions. In the 24-h treatment, the relative total growth (RTG) which is the measurement of cytotoxicity was 33.0% and 21.6% in experiment 1 and 2, respectively at the highest concentration, 50 μg/mL. The response of each test culture was determined as negative based on the standard criteria proposed by the MLA Workgroup of the International Workshop on Genotoxicity Testing (IWGT). The induced mutant frequency (IMF) was found to be less than the global evaluation factor (GEF) and the trend analysis was negative. The findings revealed that concentrations up to 50 μg/mL

of standardised root extract, F2 did not exhibit genotoxic potential in the presence and absence of the metabolic activator.

A series of benzimidazole derivatives were synthesised, characterised and evaluated for their anti-cancer potential in the present study. All the compounds were tested using MTS assay to assess their cytotoxicity effects towards the MCF7 and MDA-MB-231 breast cancer cell lines. The results showed that the compounds did not have cytotoxic effect on both cell lines. The benzimidazole derivative, 2-(4-fluorophenyl)-1*H*-benzimidazole was studied using the transwell migration assay to evaluate whether the compound can inhibit the migration of MDA-MB-231 cells. Interestingly, the compound at 100 μM significantly inhibited the migration of MDA-MB-231 cells ($p < 0.01$) in comparison with the control. Further investigation involving the biochemical and functional analyses of 2-(4-fluorophenyl)-1*H*-benzimidazole would serve to assess its mechanism of action.

CHAPTER ONE

INTRODUCTION

1.1 Genotoxicology

“Genotoxicity" is a wide term which encompasses mutagenicity, DNA damage, DNA adduct formation, sister chromatid exchange and mitotic recombination. The term "mutagenicity" restricts to gene mutations as well as structural and numerical chromosomal aberrations (Hayashi, 2007). Gene mutations include base pair substitutions and frameshift mutations. Base pair substitutions occur through the substitution of one or several base pairs in the DNA, and frameshift mutations arise from an insertion or deletion of a number of base pairs (Stammberger et al., 2006). Structural aberrations are categorised into two types, chromosome or chromatid aberrations (clastogenicity). Chromosomal mutations and related events are the cause of many human genetic diseases and there is substantial evidence that they are involved in cancer development. Numerical aberrations are referred to the changes in the number of chromosomes of the normal chromosomal content of a cell (aneugenicity) (Stammberger et al., 2006).

1.1.1 DNA damage and repair

DNA damage can either be ‘endogenous DNA damage’ or ‘environmental DNA damage’. Endogenous DNA damage primarily involves hydrolytic and oxidative reactions following the interaction between DNA, reactive oxygen species (ROS) and water within the cells whereas the environmental DNA damage refers to external physical or chemical agents that cause DNA damage (Friedberg et al., 2006). DNA damage can occur in the form of strand breaks, either single strand breaks which involves only one DNA strand or double-strand breaks in which both double helix strands are impaired. The latter is considered more hazardous as it can lead to genome rearrangement (Friedberg et al., 2006).

In response to DNA damage as described above, cells have certain mechanisms to correct the damage. DNA repair is an active process as daily, millions of cells are exposed to various metabolic activities and environmental factors and the majority of this exposure leads to structural damage of the DNA. However, the rate and success of the repair varies based on the severity of the damage and also other factors which include cell types, age of the cells and the extracellular environment. The higher the severity of DNA damage, the higher the possibility of ineffective DNA repair, which could lead to either the cells undergoing senescence (irreversible state of dormancy), cell death (apoptosis) or permanent alterations of DNA structure and function leading to irregular cell division that could ultimately lead to carcinogenesis (Friedberg et al., 2006).

As soon as the damage has been detected, specific molecules are brought to the site of damage and induce other molecules to bind and form a complex for repair. In the event small areas of DNA are affected, such as nearly all oxidative damage as well as single strand breaks, the damage will be repaired by DNA base excision repair (BER). BER is the most active repair process which allows specific recognition of damaged DNA bases

(Friedberg et al., 2006). The second most important DNA repair mechanism is the nuclear excision repair (NER) pathway. NER enzymes recognise damaged lesions by their abnormal structure and followed by excision and replacement mechanisms (Friedberg et al., 2006). There are two sub-pathways for NER, the global genomic NER (GG-NER) and transcription-coupled repair (TCR). Both pathways share the same repair mechanisms but with different recognition steps and use different sets of proteins (Bohr et al., 1985; Hanawalt, 2002). In principle, GGR works by eliminating the lesions from the entire genome whereas TCR repairs the damage at DNA strands that actively transcribe the gene (Altieri et al., 2008).

The occurrence of DNA damage during cell cycle phases such as DNA replication requires correction to avoid permanent mutation in subsequent DNA replications. A repair system called mismatch repair recognises and repairs the incorrect insertion, deletion and mis-incorporation during DNA replications and also recombination. For instance, the mismatches of G/T or A/C pairing bases are repaired by excising the wrong bases and replace it with the right nucleotides (Iyer et al., 2006).

Besides, exogenous DNA damaging agents or endogenous ROS formation can also cause DNA double-strand breaks which promote genome rearrangements and thus initiate carcinogenesis or apoptosis (Hoeijmakers, 2001; Alteiri et al., 2008). Hence, the evolved mammalian system has two mechanisms to repair such damage. The first is by homologous recombination (HR) which uses instructions from sister or homologous chromosomes for a proper repair of the breaks. The second mechanism is known as non-homologous end joining (NHEJ) where the two severed DNA ends are rejoined in a sequence independent manner (Helleday et al., 2007; Weterings and van Gent, 2004).

1.1.2 Carcinogenesis

In general, genotoxicity describes the deleterious action on the cell genome affecting its integrity. Genotoxic chemicals are known to produce mutagenicity (the capacity to induce permanent alteration in the genetic material within living cells) and may proceed to carcinogenicity (formation of cancer). Mutagenesis is important in the carcinogenesis process however not all carcinogenesis is due to mutagens. This is due to the fact that carcinogenesis could also occur via epigenetic (not involving the DNA) mechanisms.

Genotoxins or mutagens can both give rise to carcinogenesis. Irregular cell division during cell cycle due to mutations and ineffective repair processes may lead to this hazardous process. In spite of the significant role of mutations in carcinogenic processes, not all types of mutation may lead to tumour or cancer formation. Mutations of proto-oncogenes will normally modify their normal expression and activity, and they can be transformed to oncogenes via mutation. This can lead the cell to proliferate abnormally. Tumour suppressor gene (TSG), another important gene that regulates the normal cell growth and mitosis also plays a vital role in cancer formation. In cases of cellular stress or DNA damage, the TSG will suppress normal function and promote cell cycle arrest, to allow adequate time for repair and to prevent mutations from passing to new cells. However, in the event the TSG itself has been mutated, the original functions of it can be switched off and DNA damage without repair may lead to mutation. One of the most important TSG is p53. The mutation of p53 has been reported to have high prevalence in human cancers (50 %) and cells that lack this p53 exhibit genetic instability and defects in cell-cycle control (Hollstein et al., 1991; Greenblatt et al., 1994; Soussi and Wiman, 2007).

The formation of tumour or cancer involves a series of complex processes which generally proceeds over years. The multistage process of carcinogenesis comprises of three main stages namely, initiation, promotion and progression (Cohen, 1991; Mehta, 1995; Hasegawa et al., 1998; Trosko, 2001) as depicted in Figure 1.1. DNA damage is the earliest event and has a key role in carcinogenesis. Thus, following DNA damage during initiation stage, the cell undergoes mutations which induce proliferation but not differentiation (Trosko, 2001). Rapidly dividing cells have less time for DNA to get repaired and to remove the DNA-adducts (covalent binding of chemicals with DNA) (Richardson et al., 1986; Frowein, 2000) and these cells may remain dormant over time (Player et al., 2004) until the next stage, promotion. This second stage starts when the influence of promoter increases the cell proliferation in susceptible tissues, which eventually enhance the genetic changes and also the cell growth control (Mehta, 1995; Oliveira et al., 2007). The 'promoted' cells which survived apoptosis may proceed to the final stage, 'progression' which cells are characterised by irreversibility, genetic instability, rapid growth, invasive, metastasize and have various changes biochemically, metabolically and morphologically (Pitot and Dragan, 1991; Butterworth et al., 1998; Dixon and Kopras, 2004; Oliveira et al., 2007).

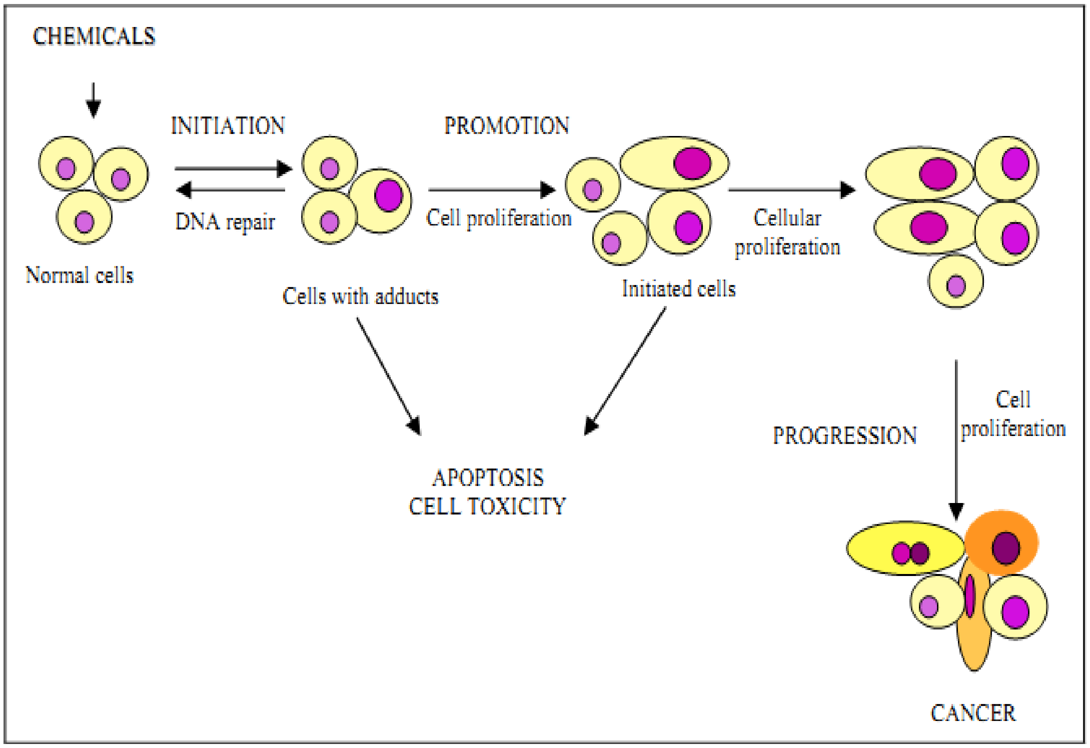


Figure 1.1: Stages of chemical carcinogenesis (Source: Oliveira et al., 2007)

1.1.3 Genotoxicity testing

The field of genetic toxicology began in the 1960s in conjunction with a number of seminal conferences held focusing on chemical mutagens, particularly their effects on germ cells and the risk to future generations. Although germ-cell risk was the initial concern, this was broadened in the 1970s when evidence relating genotoxicity and carcinogenicity began to accumulate. This was further supported by the use of *in vitro* metabolic activation systems capable of producing electrophilic metabolites. From then onwards, various national expert committees were formed to advise on the type of approach that should be taken to screen new chemicals for carcinogenic risk (and any potential heritable effects). Consequently, many guidelines have been prepared over the past 25 years describing the *in vitro* and *in vivo* tests designed to identify compounds that induce genetic damage directly or indirectly through various mechanisms (Gatehouse, 2007; ICH, 1997). Internationally, two main bodies are responsible for providing the guidance and tests methods in assessing genotoxicity which are the Organisation for Economic Cooperation and Development (OECD) and International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use. The guidance and methods are continuously reviewed and refined to improve the approaches for genotoxicity testing and assessment and also to provide a common strategy to increase the harmonisation of the genotoxicity testing and risk assessment due to the difference in legislation between countries (Muller et al., 2003).

As part of the registration requirement, chemicals (natural or synthetic) used for pharmaceutical products or any other consumer products need to be assessed for genotoxic potential. ICH developed a standard approach to conduct the testing using both *in vitro* and *in vivo* methods in predicting the genotoxicity. The following standard test battery has been recommended (ICH, 1997):

1) A test for gene mutation in bacteria
2) An *in vitro* test with cytogenetic evaluation of chromosomal damage with mammalian cells or an *in vitro* mouse lymphoma assay
3) An *in vivo* test for chromosomal damage using rodent hematopoietic cells

The bacterial reverse mutation test (Ames test) utilises strains of *Salmonella typhimurium* and *Escherichia coli* to detect point mutations which involve substitution, addition or deletion of one or a few DNA base pairs (Ames et al., 1975; Maron and Ames, 1983; Gatehouse et al., 1994). Principally, this test measures the reversion of mutations (incapability of synthesizing the essential amino acid required for normal growth) carried by the test strains. The revertant bacteria are detected by their ability to grow and form colonies in the absence of the amino acid required by the parent test strain. This test is commonly used as an initial screen for genotoxic potential (OECD Guideline 471, 1997). A comprehensive evaluation of a database for the Ames test of over 540 chemicals by Kirkland et al. (2005) confirmed that the sensitivity and specificity of the test was around 60 % and 73.9 %, respectively. Even though the Ames test utilizing prokaryotic cells is versatile and rapid, the intrinsic differences between prokaryotic and eukaryotic cells in terms of genome structure and organisation necessitate the use of mammalian test systems within any screening battery designed to detect the broad spectrum of genotoxins (Gatehouse, 2007).

The mouse lymphoma assay (MLA) is one of the tests which employs the mammalian cells in evaluating mutagenesis. The first test was established by Clive and his colleague in 1970's (Clive and Spector, 1975; Clive et al., 1979). Ever since the assay was gradually optimised until it is widely acceptable for genotoxicity testing. In principle, MLA uses the thymidine kinase (tk) gene of the L5178Y/tk$^{+/-}$ -3.7.2C mouse lymphoma cell line as a reporter gene of mutation. The tk gene is located on chromosome 11 in mouse cells and on chromosome 17 in humans (Clive et al., 1987). The tk mutated, tk$^{-/-}$ cells are resistant to the lethal pyrimidine analogue, TFT which is toxic to normal cells (causing inhibition of cellular metabolism and halts the cell division). In MLA, the test agent is assessed with or without exogenous metabolic activation system (post supernatant rat liver S9 induced with Aroclor 1254 or phenobarbital/β-naphthoflavone) for appropriate time points (3 or 4-h with and without S9 and also 24-h without S9). Exogenous metabolic activation system is important as it mimics the *in vivo* metabolism thus converting the compound into its metabolically active form (Prieto-Alamo et al., 1996).

Alternative *in vitro* tests which can be used include the chromosome aberration test or micronucleus test, employing other mammalian cell lines such as Chinese Hamster Ovary cell lines (CHO, V79) or human lymphoblastoid cells (TK6) which detect different end-point of genetic events such as at hypoxanthine-guanine phosphoribosyl transferase (HPRT) or a transgene of xanthine-guanine phosphoribosyl transferase (XPRT) (USFDA/CFSAN, 2006).

1.2 Safety concern on the use of pharmaceutical from plant

Plants have been a source of medicines for thousands of years (Samuelsson, 2004). The World Health Organisation (WHO) has estimated that 65-80 % of the world's population use traditional medicine as their primary health care. Pharmaceuticals from plants represent the majority of this health care and are growing in use especially in developing countries (Drew and Myers, 1997). In Malaysia, the safety of herbal medicines or pharmaceuticals from plants is regulated under a government agency, National Pharmaceutical Control Bureau (NPCB) which is a designated WHO Collaborating Centre for Regulatory Control of Pharmaceuticals.

There is a common belief among us that anything 'natural is safe'. The 'father' of toxicology, Paracelsus has made an important statement 500 years ago concerning safety which was '*All substances are poisons; there is none that is not a poison. The right dose differentiates a poison and a remedy*' (Timbrell, 2001). This statement is applicable to all and includes the natural products as well. Since herbal medicines are commonly supplied as dietary supplements or without prescription, they should be used with caution, as the herbal medicines used in irregular, high doses or in combination with other medications, may pose toxic effects. The toxic effects can range from allergic reactions to cardiovascular, hepatic, renal, neurological and dermatological effects (Pharmar, 2005). Therefore, it is essential to assess the safety of phytotherapeutic compounds because their usage has a major impact on the health of mankind (Marques et al., 2003).

1.3 The Plant *Eurycoma longifolia* Jack

1.3.1 Description of the plant

Eurycoma longifolia Jack is a tropical herbal plant belonging to the Simaroubaceae family (Figure 1.2). This species is indigenous to South-East Asian countries such as Malaysia, Indonesia and Vietnam. This plant is locally known as 'Tongkat Ali' and it is found to be growing wildly in the jungle slopes of Malaysia. It is also well known as 'Pasak Bumi' in Indonesia and 'Cay ba binh' in Vietnam. This plant is an evergreen slow growing tree, which can reach a maximum height of 15-18 metres after approximately 2-3 years of cultivation. The roots harvested after 4 years of cultivation are usually suitable for commercial purposes. The leaves are pinnate, spirally arranged, and can grow 10-15 inches long, while the flowers are produced in large panicles. The fruits appear green in color, and turn to dark red after ripening (Bhat and Karim, 2010).

Figure 1.2: The photo of *Eurycoma longifolia* Jack

1.2.2 Pharmacological properties of the plant

A variety of bioactive constituents have been isolated and characterised from *E. longifolia*, especially from the root. Some of them include canthin-6-one alkaloids, quassinoids, squalene derivatives and biphenylneolignans (Kardono et al., 1991; Morita et al., 1993a; Morita et al., 1993b; Itokawa et al., 1993; Ang et al., 2002; Bedir et al., 2003; Morita et al., 1992). The different parts of the plant are traditionally claimed to have anti-malarial, aphrodisiac, anti-microbial and anti-pyretic activities (Bhat and Karim, 2010).

For instance, the anti-malarial property of *E. longifolia* standardised root extracts (TA164) has been investigated and it was shown that the combination treatment of the extract and artemisinin suppressed *Plasmodium yoelii* infection in the experimental mice (Mohd Ridzuan et al., 2007). In another study, bioactivity-guided fractionation of the ethanolic extract of the roots of *E. longifolia* was studied by Chan et al. (2004) for the possible anti-plasmodial activity on the lactate dehydrogenase activity of *in vitro* chloroquine-resistant Gombak A isolate and chloroquine-sensitive D10 strain of *Plasmodium falciparum* parasites. The results revealed that four quassinoids namely, eurycomanone, 13,21-dihydroeurycomanone, 13α(21)-epoxyeurycomanone and eurycomalactone, and an alkaloid, 9-methoxycanthin-6-one, displayed higher anti-plasmodial activity against Gombak A isolate but were less active against the D10 strain when compared with chloroquine.

Besides that, a pharmacologically active fraction, F16 from *E. longifolia* has been reported to inhibit the proliferation of MCF-7 human breast cancer cells through apoptotic pathway accompanied by specific proteolytic cleavage of poly (ADP-ribose) polymerase-1 (PARP-1). The apoptosis has been shown to occur independently of caspase-9 enzyme and p53 protein (Tee et al., 2007). The methanol, *n*-butanol, and chloroform extracts of the roots of *E. longifolia* have also been shown to have cytotoxic effect on KB, DU-145, RD, MCF-7 and CaOV-3 cancer cell lines while no significant effect was observed on normal kidney cells, MDBK (Nurhanan et al., 2005).

Ang et al. (2004) assessed sexual arousal in sexually sluggish old male rats, (24 months old, retired breeders). The rats were administered with 200, 400, or 800 mg/kg of *E. longifolia* extract (2 times daily, for 10 days) with control rats receiving 3 mL/kg of normal saline. They studied the aphrodisiac effects by visualizing the act of yawning and stretching (alone or with stretching) which has been considered as identity for sexual arousal. The results indicated that rats administered with the extract at various doses increased yawning and stretching, which was highest at the dose of 800 mg/kg. The findings demonstrated the effect of the *E. longifolia* extract on the aphrodisiac activity.

1.4 Cancer

Cancer is a genetic disease resulting from mutations that occur due to the abnormalities in DNA sequence (Futreal et al., 2001). The hallmarks of cancer comprise six biological capabilities include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. As normal cells develop progressively to a neoplastic state, they acquire a sequence of these hallmark capabilities that enable them to become tumourigenic and ultimately malignant (Hanahan and Weinberg, 2011). The International Agency for Research on Cancer (IARC) has estimated about 12.7 million cancer cases and 7.6 million cancer deaths worldwide in 2008 (Ferlay et al., 2010). The increasing burden of cancer constantly demands the discovery of new anti-cancer agents with enhanced therapeutic effect.

1.4.1 Breast cancer

Breast cancer continues to be the most prevalent cancer and the leading cause of cancer death in females worldwide, comprising 23 % (1.38 million) of the total new cancer cases and 14 % (458,400) of the total cancer deaths in 2008 (Ferlay et al., 2010). In Malaysia, a total of 18, 219 new cancer cases were diagnosed in 2007 and registered at the National Cancer Registry published in 2011 (Omar and Tamin, 2011). The most frequent cancer in Malaysia is breast cancer accounting for 18.1 % of all registered cancer cases. The applications of existing knowledge and emerging findings in the area of cancer treatment are essential in preventing a substantial proportion of the worldwide burden of breast cancer.

1.4.2 Estrogen receptor and breast cancer

Estrogen signaling and the estrogen receptor (ER) are associated with breast cancer progression, and the majority of the human breast cancers start out as estrogen dependent and express the estrogen receptor. The binding of estrogen to any of the structurally and functionally distinct ERs (ERα and ERβ) mediates its biological effects (Thomas and Gustafsson, 2011). ERα is the major ER subtype in the mammary epithelium which plays an important role in mammary gland biology (Warner et al., 1999; Curtis Hewitt et al., 2000). The binding of estrogen to ERα translocates the ligand-activated ERα to the nucleus, and eventually stimulates gene transcription (McKenna et al., 1999; McDonnell and Norris, 2002). ER signaling is complex, involving coregulatory proteins and also genomic and extranuclear actions (Barnes et al., 2004).

There are also breast cancers that do not possess ERs and they are known as ER-negative breast cancers. ER-negative tumors are morphologically and phenotypically distinct from ER-positive breast cancer (Putti et al., 2004). Another subtype of breast cancer is the triple-negative breast cancer, characterised by tumors that do not express estrogen receptor (ER), progesterone receptor (PR), or HER-2 genes. This subtype put forward an important clinical challenge because they do not respond to endocrine therapy or other available targeted agents (Hudis and Gianni, 2011).

1.4.3 Cytotoxicity versus cytostasis

DNA damage and the subsequent induction of apoptosis is a primary cytotoxic mechanism of many anti-cancer agents, including alkylating agents, platinum compounds, topoisomerase inhibitors, and the anti-metabolites (Cepeda et al., 2007; Montecucco and Biamonti, 2007). Cytostasis is where the cancer cells are not killed but instead they are stopped from proliferating. A true cytostatic agent should halt the growth of tumours and could prevent the development of metastases without affecting tumour shrinkage (Houghton and Houghton, 1996; Gelmon et al., 2004). It could be argued that all microtubule-targeting agents from vincristine to paclitaxel and, more recently, the epothilones are inherently cytostatic (Johnson et al., 1963; Altaha et al., 2002). None of these agents are intrinsically able to cause cytotoxicity. These agents arrest cells in mitosis and induce cytostasis by interfering with microtubule dynamics. Mitotic arrest is a condition that is poorly tolerated by any cell and must either be escaped or resolved by cellular death, hence the cytotoxic activity of these primarily cytostatic agents. Although these agents are viewed as cytotoxic, they are in fact cytostatic, and the arrest triggers cell death (Blagosklonny and Fojo, 1999).

1.4.4 Cancer cell migration

Cell migration, including the migration of tumour cells during several steps in the metastatic process (Figure 1.3), is a response to environmental cues and results in the orderly rearrangement of adhesive structures that connect the cell to the extracellular matrix (ECM). There are several adhesion receptor families, including selectins, syndecans, the immunoglobulin cell adhesion molecules, cadherins, and integrins. The best-studied adhesion receptors, and of particular interest in migration, are the integrins. These are heterodimeric transmembrane protein structures that bind specific extracellular sites on the ECM and specific intracellular cytoskeleton adapter proteins (Giancotti and Ruoslahti, 1999). As integrins form connections with the extracellular environment, they provide structural support. They also mediate intracellular signaling through the activation of focal adhesion kinase (Guo and Giancotti, 2004). The native ECM of a normal cell is a requisite for survival. Indeed, removal of a cell from its supportive matrix can cause that cell to die, also called “anoikis” (Frisch and Ruoslahti, 1997). In order for a tumour cell to metastasise, it must pass through surrounding stromal elements as it migrates toward a lymph or blood vessel. One important class of molecules within the invasion front is the matrix metalloproteinase (MMP). MMPs can be secreted by neighbouring cells and can become localised and activated on the surface of migrating tumour or endothelial cells (Basbaum and Werb, 1996). Once ECM barriers at the invasion front have been cleared through proteolytic degradation of MMPs, the metastatic cancer cell generates the invasive machinery (Liotta, 1986).

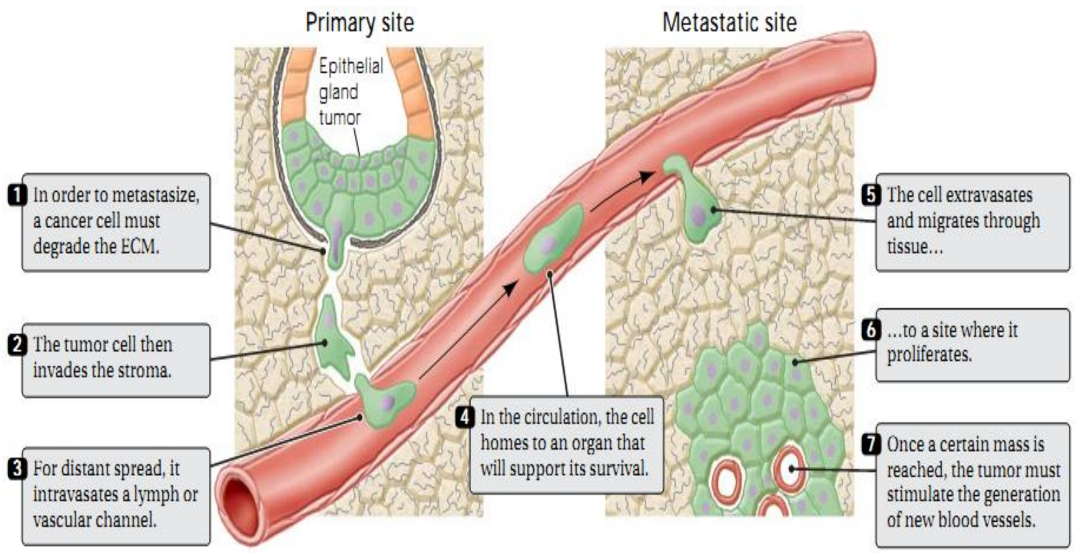


Figure 1.3: The metastatic sequence (Source: Geho et al., 2005)

1.4.5 Limitations of present anti-cancer drugs

The four major modes of cancer treatment include surgery, radiation, chemotherapy and immunotherapy (Gatenby, 2009). Chemotherapy represents one of the major means for cancer treatment, which aims to kill tumour cells or to inhibit their proliferation. Chemotherapeutic agents generally have a narrow margin of safety, and are used in combination usually given at a maximum tolerated dose to achieve maximum cancer cell killing (Chabner and Roberts, 2005). They kill tumour cells by direct cytotoxicity, or activating host immune response, inhibiting the proliferation processes of tumor cells, and inducing apoptosis (Cotter, 2009). For most anti-cancer drugs, there is a large inter-individual variability in their pharmacokinetics and this can result in unpredictable toxicity and variable anti-tumour effects (Undevia et al., 2005). Drug resistance and dose-limiting toxicities are the major problems for the success of cancer chemotherapy (Yague and Raguz, 2005).

Heidelberger et al. (1957) synthesised 5-fluorouracil (5-FU), as an anti-metabolite. 5-FU and its derivatives are potent mechanism based inhibitors of thymidylate synthase (TS), an enzyme which converts 20-deoxyuridine-50-monophosphate (dUMP) to 20-deoxythymidine-50-monophosphate (dTMP) (Peters et al., 1995). An understanding of 5-FU mechanism of action has resulted in major therapeutic advances in the past 15 years. Thus, inhibition of TS by anti-metabolites remains a classic approach and a key strategy for suppressing cell division in cancerous tissue (Lehman, 2002). 5-FU has been used extensively in the treatment of skin cancers and a variety of solid tumours, such as breast, colorectal and gastric cancers. However, the treatment has been found to cause neurotoxic and cardiotoxic side effects due to the lack of selectivity of the drug towards tumours (Mader et al., 1998).

Anti-estrogens are well established in the treatment of hormone-dependent breast cancer (Santen and Harvey, 1999). Tamoxifen (Nolvadex), a non-steroidal triphenylethylene derivative is long known to exhibit anti-estrogenic properties *in vivo* (Studemann et al., 1997). Although tamoxifen has been very successful in the treatment of breast cancer, it is associated with an increased risk of endometrial hyperplasia. In addition, the use of tamoxifen is limited by the development of drug resistance (Osborne et al., 1995). Therefore, the discovery of new, safe and effective anti-cancer agents is still very much in demand.

1.5 Benzimidazole

The benzimidazole nucleus is encountered as a constituent of a variety of bioactive compounds that exhibit diverse pharmacological properties. Thus, benzimidazole is continuously being an important pharmacophore in drug discovery. The general structure of benzimidazole consists of a benzene ring fused to an imidazole ring (Figure 1.4).

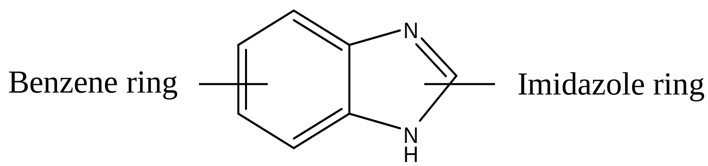
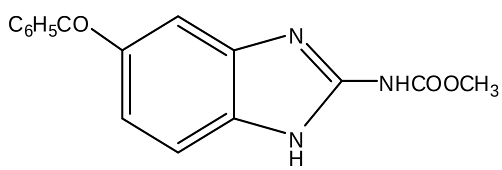


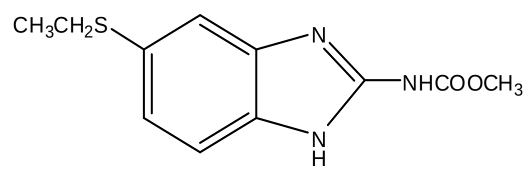
Figure 1.4: Benzimidazole

1.5.1 Pharmacological properties

Albendazole (1) and mebendazole (2), the 2-carbamate benzimidazole derivatives possess anti-helmintic activity and they were being employed in clinical use for 70 decades (Grover et al., 2001; Kohler, 2001). These drugs act by binding to helmintic tubulin and eventually disrupting the microtubule structure and functions (Lacey, 1988).

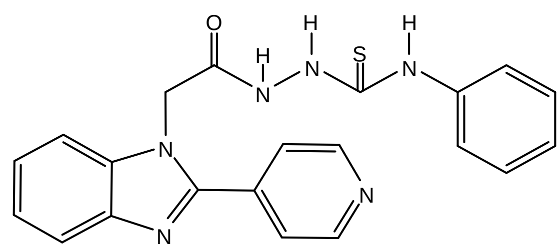


(1)

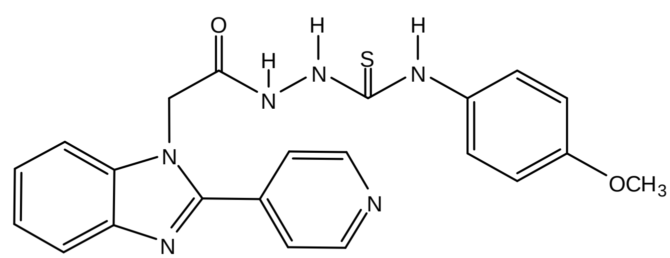


(2)

In another study, the antioxidant evaluation of some novel benzimidazole derivatives showed that compounds 3 and 4 exhibited very good antioxidant capacity with IC_{50} values of 1.3×10^{-5} M and 1.2×10^{-5} M respectively, through the interaction with the 2,2-diphenyl-1-picrylhydrazyl (DPPH). It was 17-18 fold more potent than the standard substance, butylhydroxytoluene (BHT) which showed IC_{50} value of 2.3×10^{-4} M (Ayhan-Kilcigil et al., 2005).



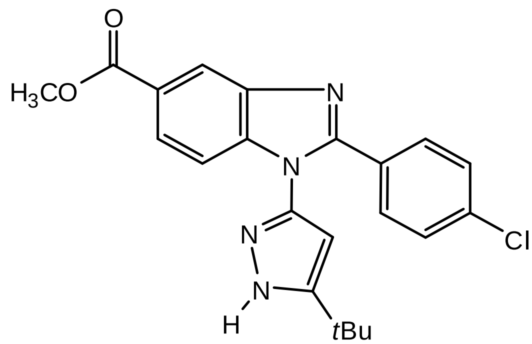
(3)



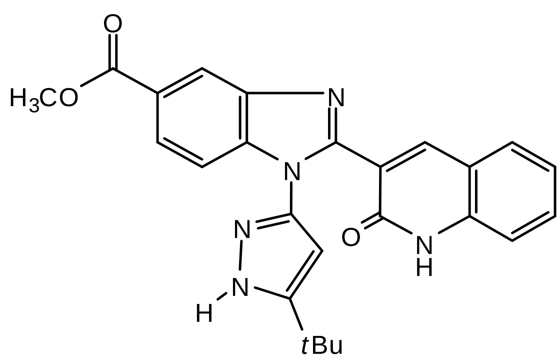
(4)

1.5.2 Benzimidazole derivatives and cancer

In a previous study, the cytostatic activity of compounds 5 and 6 against a panel of cancer cell lines was evaluated. The results were expressed as GI_{50}, the molar concentration of the compounds needed to inhibit the growth of the cell lines by 50 % and LC_{50}, the molar concentration required to kill 50 % of the cells (Boyd and Paull, 1995). The highest activity was demonstrated in panels of non-small cell lung cancer, melanoma and leukemia, with GI_{50} in the range of 1.15-7.33 µM and 0.167-7.59 µM, respectively, and suitable LC_{50} with values greater than 100 µM (Abonia et al., 2011).

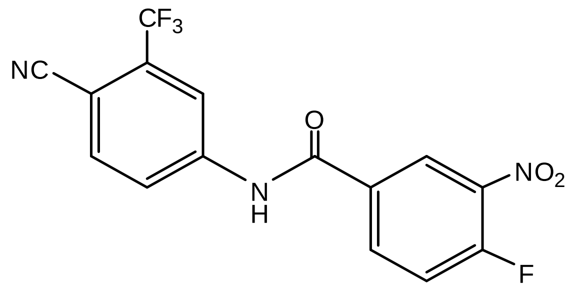


(5)



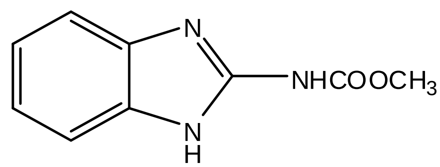
(6)

In another study, the precursor of trisubstituted benzimidazole, compound 7 has been shown to inhibit the proliferation of MDA-MB-231 human breast cancer cells with a maximum inhibition of 60 % (Thimmegowda et al., 2008).



(7)

The compound 8 has been reported to act as potent anti-tumour agent against both the murine B16 melanoma (IC_{50} = 8.5 μM) and human HT-29 colon carcinoma (IC_{50} = 9.5 μM) cell lines by inducing apoptosis (Hao et al., 2002).



(8)