

**THE EFFECTS OF DIFFERENT ELICITORS SUPPLEMENTED
IN CELL SUSPENSION CULTURE OF *Eurycoma longifolia* Jack.
ON CYTOTOXIC ACTIVITIES AGAINST HUMAN COLON
CANCER CELL LINE**

KWAN LI SEE

UNIVERSITI SAINS MALAYSIA

2015

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by

KWAN LI SEE

**Thesis submitted in fulfilment of the requirement for the
degree of Master of Science**

June 2015

ACKNOWLEDGEMENT

I am grateful to have plenty of encouragements and supports from many people. They let me know that I am not alone on the long journey in obtaining my Master degree. Unfortunately, some are not with us anymore and I wish to dedicate this thesis to my beloved grandmother whom I lost in year 2013. ‘Po Po, you are always in my heart!’.

A quote from Galileo, an Italian astronomer and mathematician – ‘You cannot teach a man anything; you can only help him discover it in himself.’ Good teacher help in enabling this discovery. Therefore, I would like to express my greatest and sincere appreciation to my ‘good teacher’ who is my supervisor, Professor Chan Lai Keng. She always guides me to the right track when I was confusing and lost in my research pathway. Her encouragement, knowledge, professional experience and passion in research are the most valuable treasurer I have discovered throughout my study. Even though she has retired, she continues to review my thesis, give useful suggestions and made corrections. I will never forget the effort she put on me.

Nevertheless, I wish to say a big ‘thank you’ to my co-supervisor, Dr. S. Sreeramanan for offering me care and help. I am also extremely indebted to my field supervisor, Professor Shinichi Uesato from Department of Biotechnology, Faculty of Engineering, Kansai University, Japan for providing me great hospitality and valuable guidance during my attachment at Kansai University. My sincere thanks go to my lovely and caring lab-mates from Kansai Univesity, particularly Xue Fang and Hirata-san. Without the translation from Xue Fang and helps from Hirata-san, I would not finish my project smoothly and successfully. In addition, I would like to express my sincere thanks to Associate Professor, Dr. Amin Malek from School of

Pharmaceutical Sciences, USM for providing laboratory facilities and resources to accomplish my research on cytotoxicity study. I am also thankful to members of EMAN Centre, especially, Mr. Fuaod and Asif for guiding me to complete my cytotoxicity test.

I gratefully acknowledge School of Biological Sciences and School of Pharmaceutical Sciences, USM for their resourceful infrastructure. Huge thanks to Kak Afida from Botany Laboratory who always give a helping hand whenever needed. My sincere thanks also go to USM-RU-PGRS and Prof. Chan's RUI grants for funding my research project and JPA Sabah Scholarship for providing me financial aid to further my study.

I take this opportunity to say a big thank you to all my lovely lab-mates from Plant Tissue and Cell Culture Laboratory (PTCCL), USM, Chee Leng, Zainah, Melati, Novi, Salmee, Hasmah, Kiah Yann, Song Jin, Nadia, Taufiq, Christine, Nisa, Shu Ying, Eugene, Suganthi, Laleh, Ahmed, E Shuen, Samantha and King Wey. I will never forget the advices, suggestions, encouragements and considerations you all have given to me.

A big hug and thank-you from the deepest of my heart to my beloved family, daddy, mummy, Lui Chyi and Zhi Sheng, for their constant moral support, caring, understanding and consideration since the early day of my study. I hope the completion of my Master of Science degree is one of their most meaningful gifts from me. I would like to send a special appreciation to my love, Randolph Teong and Mike Kwan for keeping me company and being patience all the time. They are always beside me during my toughest moment to push me and motivate me. Last but not least, my deep sense of gratitude goes to Master Lu who had always given me spiritual support and moral aspiration. Thank you!!

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
WHO	World Health Organization
GI	Growth Index
DW	Dried weight
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
rpm	Rotation per minute
SA	Salicylic acid
YE	Yeast extract
NaH ₂ PO ₄	Sodium dihydrogen phosphate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
EtOAc	Ethyl acetate
CO ₂	Carbon dioxide
LN ₂	Liquid nitrogen
UAE	Ultrasound assisted extraction
IC ₅₀	50% growth cell proliferation inhibitory concentration
ATCC	American Type Culture Collection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
ANOVA	Analysis of variance

LIST OF PUBLICATION

- Kwan, L. S.** and Chan, L. K. (2011). Cell growth kinetics for two cell lines of *Eurycoma longifolia* Jack. cell suspension culture. *6th International Symposium in Science and Technology at Kansai University 2011*. Osaka: Kansai Univerisy, pp. 240. (Appendix 1)
- Kwan, L. S.** (2011). Influences of different biotic and abiotic elicitors on eurycomanone production in cell suspension culture of *Eurycoma longifolia* Jack. *Proceeding of the 5th PPSKH Postgraduate Biocolloquim*. Penang: Universiti Sains Malaysia, pp. 42. (Appendix 2)
- Tan, S. Y., **Kwan, L. S.** and Chan, L. K. (2011) *In vitro* culture technology as an approach for the conservation of Tongkat Ali (*Eurycoma longifolia* Jack). *Proceeding of The Role of the Penang Botanical Gardens: Meeting the Challenge of The 21th Century*. Penang: Universiti Sains Malaysia, pp. 123-127. (Appendix 3)
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**KESAN-KESAN PENAMBAHAN PELBAGAI JENIS ELISITOR KE DALAM
KULTUR AMPAIAN SEL *Eurycoma longifolia* Jack. TERHADAP AKTIVITI
SITOTOKSIK TITISAN SEL KANSER KOLON MANUSIA.**

ABSTRAK

Kajian ini dijalankan untuk menyiasat kesan elisitor biotik dan abiotik untuk meningkatkan aktiviti sitotoksik sel-sel *E. longifolia* Jack. terhadap titisan sel kanser kolon manusia.

Kalus rapuh digunakan untuk memulakan kultur sel ampaiian *E. longifolia* Jack. dalam medium cecair MS yang ditambahkan dengan 0.5 mg/L NAA dan 0.25 mg/L 2,4-D. Corak pertumbuhan sel didapati mengikut suatu lengkuangan sigmoid tipikal. Pelbagai fasa pertumbuhan sel, fasa lag, log dan pegun, dengan jangka masa tertentu telah dikenalpastikan berdasar kepada indeks pertumbuhan sel dan berat sel kering. Di samping itu, tiga hari inokulasi elisitor yang berbeza and hari penuaian telah ditentukan berdasarkan corak pertumbuhan sel. Hari inokulasi: Hari 0 (permulaan pengkultran), Hari 13 (pertengahan fasa log) dan Hari 17 (pengakhiran fasa log); Hari penuaian: Hari 18 (permulan fasa pegun) telah ditentukan.

Tiga elisitor biotik (ekstrak yis, pektin dan valin) dan lima elisitor abiotik (metil jasmonat, asid salisilik, kasein hidrolisat, natrium dihydro fosfat dan natrium kloride) telah diuji kesannya dalam kultur sel *E. longifolia*. Kepekatan elisitor yang berbeza ditambahkan ke dalam kultur sel ampaiian pada hari yang berbeza. Keputusan yang diperolehi menunjukkan terdapat perencatan pertumbuhan sel yang bererti apabila kepekatan metil jasmonat, acid salisilik dan natrium kloride yang tinggi ditambahkan ke dalam kultur ampaiian sel *E. longifolia*. Ini boleh disimpulkan

bahawa peningkatan dan perencatan pertumbuhan sel adalah bergantung kepada dos dan hari inokulasi elisitor.

Semua elisitor menunjukkan keberkesanan dalam menghasilkan aktiviti sitotoksik yang lebih baik terhadap sel kanser kolon manusia, HCT116, terutamanya elisitor pektin. Telah didapati bahawa penambahan sebanyak 2.0 g/L pektin pada hari 17 dalam kultur sel ampaiian *E. longifolia* boleh menghalang proliferasi sel kanser HCT116 dengan IC_{50} bernilai 19.4 $\mu\text{g/ml}$.

Bagi kajian pengekstrakan sel tumbuhan, penggunaan kaedah pengekstrakan berbantuan ultrasound (UAE) adalah lebih berkesan daripada kaedah rendaman dalam meningkatkan hasil ekstrak dan aktiviti apoptosis sel *E. longifolia* Jack terhadap sel kanser HCT116. Lapisan etil asetat perolehi daripada kaedah pembahagian menunjukkan aktiviti sitotoksik yang tinggi tetapi hasil ekstrak yang rendah. Oleh itu, kaedah pengekstrakan yang optimum telah dibangunkan dan digunakan dalam pengekstrakan sel yang telah dipereliskan. Kaedah pengekstrakan yang optimum boleh diringkaskan sebagai (1) 0.5 g serbuk sel *E. longifolia* Jack. direndamkan dalam campuran pelarut metanol dan kloroform (1:1). (2) Campuran itu disonatkan selama 60 minit pada 40 kHz. (3) Ekstrak mentah dibahagikan dalam air dan etil asetat. (4) Lapisan etil asetat disejatkan sehingga kering dan diuji atas HCT116 untuk mendapatkan nilai IC_{50} .

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ABSTRACT

This study was carried out to investigate the effects of biotic and abiotic elicitors in enhancing the cytotoxic activities of *E. longifolia* Jack. cell extract against human colon cancer cell line.

Friable callus was used to initiate cell suspension culture of *E. longifolia* Jack. in modified MS liquid medium supplemented with 0.5 mg/L NAA and 0.25 mg/L 2,4-D. The cell growth pattern was found to follow a typical sigmoid curve. Various growth phases, the lag, log and stationary phases, with their specific durations were identified based on the cell growth index and dried cell mass. In addition, three different inoculation days of elicitor and harvesting day were determined based on its cell growth pattern. Inoculation day: Day 0 (beginning of culture), Day 13 (middle of log phase) and Day 17 (end of log phase); harvesting day: Day 18 (beginning of stationary shape) were then determined.

Three biotic elicitors (yeast extract, pectin and valine) and five abiotic elicitors (methyl jasmonate, salicylic acid, casein hydrolysate, sodium dihydro phosphate and sodium chloride) were tested on their effects in the cell culture of *E. longifolia*. Different concentrations of elicitors were supplemented into cell suspension culture inoculated on different days. Results obtained indicated there was a significant suppression of cell growth with the addition of high concentration of methyl jasmonate, salicylic acid and sodium chloride into the cell culture of *E.*

longifolia. It could be concluded that the increment and reduction of cell growth response to elicitor were dose and inoculation day dependent.

All elicitors showed their effectiveness in producing better cytotoxic activity against human colon cancer cell line, HCT116, particularly pectin elicitor. It was found that supplementation of 2.0 g/L pectin on day 17 in cell suspension culture of *E. longifolia* could inhibit the proliferation of HCT116 cancer cell line with IC_{50} of 19.4 μ g/ml.

For plant cell extraction study, ultrasound assisted extraction (UAE) method was more effective than maceration method in improving the extract yield and apoptosis activity of *E. longifolia* Jack. against HCT116 cell line. Ethyl acetate layer obtained from partitioning method showed a high cytotoxic activity but low extract yield. Therefore, an optimised extraction method was established and applied in all elicited cells extraction. The optimised extraction method was summarized as (1) 0.5 g powdered cells of *E. longifolia* Jack. were soaked in combined solvents of methanol and chloroform (1:1). (2) The mixture was sonicated for 60 minutes at 40 kHz. (3) The crude extract was fractionated in water and ethyl acetate. (4) Ethyl acetate layer was evaporated to dryness *in vacuo* and tested on HCT116 to obtain IC_{50} .

CHAPTER 1

INTRODUCTION

1.1 Research background

Cancer is a type of cell disease where the cells grow abnormally fast and become a lump known as tumour. This cell disease occurred when the genes in the cell mutated and the mutated gene then encoded the cell-regulatory proteins in the cell. Abnormal cells differentiated in higher rate than the normal cells and eventually resulted in wrong functioning in vital human organs. Hence, this potential fatal disease can also be called as genetic disease (Alison, 2001; Finn, 2008).

Currently, cancer becomes one of the major fatal diseases in Malaysia as well as worldwide. Based on 2012 statistic obtained from World Health Organization (WHO), there were approximately 14.1 million new diagnosed cancer cases and 8.2 million people lost their lives due to cancer. Compared to year 2008, the new diagnosed cancer cases was 1.4 million higher and 0.6 million more people died from cancer. In view of the increasing trend, WHO predicted that new cancer cases would be raised to 19.3 million in the year 2025. The sharp increased of new cancer cases becomes a huge social burden for many countries, especially those developing countries, like Malaysia (Gerard, 2002; WHO, 2012). According to National Cancer Registry of Malaysia (NCR), every four citizens in Malaysia, there will be one person threaten with cancer during the advanced age and the number of patient is increasing every year. This indirectly resulted in an extra and increased of public health cost (Ariffin and Saleha, 2011).

There are more than 200 different kinds of cancer that could be developed in human body. Specific treatment is used and applied to a particular type of cancer. Basically, several options of treatments could be used to treat cancer. These include

surgery, chemotherapy, radiation therapy, and drug therapy. Currently, many cancer sufferers are resorted to herbal therapy. However, most of the cancer treatment or therapies resulted in many side effects (Partridge et al., 2001). Hence, herbal therapy making used of plant extracts or plant-derived products as the treatment agents becomes a famous alternative for cancer treatment, especially in reducing the side effect during the post-therapy stage (Qi et al., 2010; Yin et al., 2013). From the ancient time, people in many civilizations, such as China, Indian and Egypt had found that plants could be used as remedies (herbals) to heal cancer (Umadevi et al., 2013). In this new century, many cancer related studies were carried out and had been scientifically proven that plant extracts or plant-derived products were having anti-cancer properties as well as played a positive role in disease prevention (Kaneshiro et al., 2005; Nassr-Allah et al., 2009; Aditya et al., 2013; El-Sharkawy et al., 2013).

The most popular medicinal plant in Malaysia, *Eurycoma longifolia* Jack., commonly known as Tongkat Ali, had been proven by many researchers its effectiveness in cancer healing (Razak and Aidoo, 2011; Al-Salahi et al., 2014). *E. longifolia* Jack. is an evergreen shrub with 15 to 18 meters in height when mature. It is a native plant of Southeast Asia region including Malaysia. It becomes popular in Malaysia as many believe it has aphrodisiac properties (Hsuan, 1978). Currently, the extract of *E. longifolia* Jack. available in the market is mostly extracted from the mature plant parts, particularly the roots (Bhat and Karim, 2010). Unfortunately, the plant extract normally contain low amount of the valuable secondary metabolites. The medicinal properties couple with these constraints have resulted in increased value of this species in therapeutic purposes and high demand in market. This has

also lead to uncontrolled harvesting of *E. longifolia* Jack. that eventually lead to near extinction risk of this species (Fernando et al., 2008).

To overcome this problem, *in vitro* plant culture technique could be used as an alternative for production of the useful secondary metabolites. The totipotent ability of plant cell helps the cell generates similar set of genetic information as that of the mother plant (Verdeil et al., 2007). Hence, plant cell suspension culture is a good alternative for mass production of cell biomass and induced for the production of targeted secondary metabolites. The controllable culture conditions can provide the cells a favourable environment for cell growth in shorter duration as compared to the mature plants. The large scale production of cell mass and the targeted active compounds could possibly meet the market demand of *E. longifolia* Jack. without the huge number of mature trees to be sacrificed. Besides, the optimised cell suspension culture system can enhance the secondary metabolites production in a sustainable manner and quality of products could be guaranteed (Smetanska, 2008; Chan et al., 2010; Veeresham and Chiti, 2013).

Plant secondary metabolites were produced and acted as a defence system to protect the plant against harmful agents or external stresses. Plants usually released little amount of secondary metabolites when needed because plant could not tolerate high concentration of secondary metabolites. Thus, to enhance the production of secondary metabolites, stresses or stimulations would be given in cell suspension culture (Smetanska, 2008). Elicitor is one of the stimulators that can be used for this purpose. Applying elicitor to cell suspension culture, called elicitation, was discovered under the theory of plant defence mechanism. Generally, elicitor can be divided into two categories, the biotic elicitor and abiotic elicitor. A suitable amount of elicitor can stimulate the cells to synthesize higher amount of secondary

metabolites, otherwise, over-dose elicitor would create toxicity and cause cells to die. In addition, the effectiveness of each elicitor in stimulating secondary metabolite production was different when it was applied in different plant species because there was variation of plant defence mechanism in each species. Hence, selection of suitable elicitor with suitable concentration is important for plant secondary metabolites enhancement (Mulabagal and Tsay, 2004; Goyal and Ramawat, 2008; Korsangruang et al., 2010). Plant cell culture of *E. longifolia* Jack. was proposed with suitable elicitation for the production of secondary metabolites with cytotoxicity activities. Thus, this study was carried out with the following objectives.

1.2 Research objectives

The present study was carried out with the following objectives:

1. To study the growth pattern of cell suspension culture of *E. longifolia* Jack.
2. To determine an effective extraction method from *E. longifolia* Jack. cultured cells with cytotoxicity activities.
3. To determine the effectiveness of biotic and abiotic elicitors for better production of *E. longifolia* Jack. cell biomass with cytotoxicity activities.

CHAPTER 2

LITERATURE REVIEW

2.1 *Eurycoma longifolia* Jack.

2.1.1 Background

Eurycoma longifolia Jack. is one of the most famous tropical medicinal plants. It is native to the tropical forests of some Southeast Asia countries which include Malaysia, Indonesia, Thailand, Philippines and Vietnam. Hence, there are many common names/vernacular names for *E. longifolia* Jack. It is commonly known as Tongkat Ali by Malaysian locals. In Malay language, ‘Tongkat’ means crutch/walking stick due to its long straight woody root. It is also known as Lempedu pahit and Bidara laut in Malay; Malaysian Ginseng in English; Pasak Bumi and Bedara Pahit in Indonesia; Lan-don in Thailand and Cay Ba Binh in Vietnam (Ali et. al., 2010; Zakaria and Mohd, 2010).

In the Plantae kingdom, *E. longifolia* Jack. is classified under *Eurycoma* genus of Simaroubaceae family. Simaroubaceae, a family which consists of around 30 genera and 200 species, mostly are shrubby trees found in the tropical and subtropical forests. *Eurycoma*, with very small group of flowering plants (four species), consists of *E. apiculata* Benn., *E. harmandiana* Pierre. and *E. latifolia* Ridl as well as *E. longifolia* Jack., all native to Southeast Asia jungles with big panicles but small flowers. Those species prefer to grow on sandy soil and near the foot of hills (Bhat and Karim, 2010).

Below is the scientific classification of *E. longifolia* Jack.:

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Sapindales

Family: Simaroubaceae

Genus: *Eurycoma*

2.1.2 Plant Habitat

E. longifolia Jack. is an evergreen tropical plant with slim woody stem that can be reached the maximum of 15 to 18 metres height after 15 years of cultivation. The green and spirally arranged leaves with 15 to 30 leaflets with length range from 25 to 40 cm (Bhat and Karim, 2010).

June and July of every year is the flowering season of this plant. Its red flower, each with five to six petals are produced in big panicles. *E. longifolia* Jack. is a dioecious and each tree only produce one type of flower, either male or female flowers. The tree starts to produce fruits after two to three years old and September is its fruiting season. There are 200 to 300 fruits produce on each bunch. The unripe fruits are normally green in colour and turn to dark red when ripe. The fruits are 1 to 2 cm in length and with a diameter of 0.5 to 1 cm. The seeds can be germinated after 43 days sowed in sand and soil mixture (1:1) and the seed germination period was about 99 days (Chan et al., 2002). The plant may take about 25 years to reach maturity. Thus, *E. longifolia* Jack. can be considered as a slow growing plants.

The radicals of the germinated *E. longifolia* Jack. seeds can develop into woody tap root system which can be grown to more than 1 metre deep into the soil. The root length can be taller than a mature man after 20 years of cultivation. In the wild, the tap root can only be harvested after 4 to 5 years for the preparation of traditional medicine. There are many secondary roots and rootlets formed

horizontally from the taproot to enhance the absorption of nutrients from the soil. Due to the long straight downward-growing form of root, it is very difficult and time consuming to harvest the root manually, especially in the tropical forest (Zanoli et al., 2009).

2.1.3 Traditional uses of Tongkat Ali

Tongkat Ali (*E. longifolia* Jack.) is popular for its medicinal properties since ancient time especially in Southeast Asia region. Almost all the plant parts of *E. longifolia* Jack. can be used for preparation of traditional medicine or folk remedy (Kuo et al., 2003).

Roots of *E. longifolia* Jack. are considered the most precious plant part as compared to other plant parts due to its high healing capabilities of various illnesses which include sexual insufficiency, malaria, hyperglycaemia, dysentery, schistosomiasis, persistent fever, mouth ulcers and headache. The roots also consider as a health tonic and are consumed daily by some to maintain good health. Hence, Tongkat Ali has been named as Malaysian Ginseng. In Malaysia and Indonesia, the roots of *E. longifolia* Jack. have gained popularity in the man community as they believed the roots possess aphrodisiac property that can improve the man sexual performance. The woody roots are copped into small pieces and boiled and served like tea. The addition of sugar or honey and repeating boiling can lessen the bitterness of the decoction (Zakaria and Mohd, 2010).

The leaves of *E. longifolia* Jack. were found to have anti-ulcer, antimalarial, gum protection agent and as the remedy for some venereal diseases (Bhat and Karim, 2010).

2.1.4 Chemical components of *Eurycoma longifolia* Jack.

Various secondary metabolites have been isolated and identified from this plant. They were summarized as in Table 2.1.

Table 2.1 Different group of secondary metabolites isolated from *E. longifolia* Jack.

Chemical component	Plant parts	References
Quassinoid		
- eurycomanone (C ₂₀ H ₂₄ O ₉)	Root	Chua et al. (2011)
- 13 α (21)-Epoxyeurycomanone (C ₂₀ H ₂₄ O ₁₀)	Root	Chua et al. (2011)
- 12-Acetyl-13,21 dihydroeurycomanone (C ₂₂ H ₂₈ O ₁₀)	Root	Chua et al. (2011)
- 13,21-Dihydroeurycomanone (C ₂₀ H ₂₆ O ₁₁)	Root	Chua et al. (2011)
Alkaloid		
- hydroxyl Methyl β -Carboline propionic acid (C ₁₄ H ₁₂ O ₃ N ₂)	Root	Chua et al. (2011)
- 9-methoxycanthin-6-one (C ₁₅ H ₁₀ O ₂ N ₂)	Leaf Petiole	Mahmood et al. (2011)

Stem

Root

Rachis

Cotyledons

Embryo

Terpenoid

- 3-methyl-1-oxo-2,3-dihydro-1H-	Stem	Rahmalia et al., (2011)
pyrazola [4,3-c][1,10]	Root	
phenanthroline		
Biphenylneolignan	Root	Chua et al. (2011)
Squalene-type triterpene	Root	Morita et al. (1993)
		Chua et al. (2011)

2.1.5 Therapeutic properties of *Eurycoma longifolia* Jack.

2.1.5.1 Aphrodisiac activities

E. longifolia Jack. has attracted public attention worldwide due to its aphrodisiac activities. Study of Zanolini et al., (2009) had shown that the mating activities of sexually sluggish male rats were improved after consumed root powder of *E. longifolia* Jack. Treated sexually sluggish rats performed better in sexual activities with their shorter ejaculation latencies, higher percentage of mounting and shorter post-ejaculatory interval period compared to the control rats. Besides, the testosterone levels of treated rats also increased significantly after taken 500 mg (root powder) per kg (body weight of rats) per day continuously for a total of six days.

A study conducted by Tambi et al., (2012) also showed that the consumption of water-soluble extract of *E. longifolia* Jack. could be used to heal late-onset of hypogonadism (LOH). LOH was also known as andropause or male menopause where there was a constantly reduction of testosterone level in men after reached middle-age and above (Schubert and Jockenhovel, 2005). Eight hundred and fifty male LOH patients were given 200 mg of water-soluble extract per day for 30 days and the results showed a significant 46.8% increased of testosterone level among the treated LOH patients. The effectiveness of *E. longifolia* Jack. in testosterone enhancement was due to the presence of eurycomanone (quassinoid) which prevent alteration of testosterone to oestrogen in Leydig cells of human body (Low et al., 2013).

Success rate of pregnancy is highly depended on the qualities of sperms, number of sperms, normality of sperm morphology and motility of sperm. Studies showed that *E. longifolia* Jack. extract played a significant role in enhancing the performance of sperms in both laboratory *in vitro* test and clinical test (Tambi and

Imran, 2010; Erasmus et al., 2012). Al-Qarawi (2005) found that the coumarin is a sperm quality enhancer and the present of 2H-1-Benzopyran-2-one, 3-phenyl, a coumarin derivative in *E. longifolia* Jack. extract is the evidence that this plant possesses the ability to improve the performance and quality of sperm (Rahmalia et al., 2011).

2.1.5.2 Anti-malaria properties

Malaria is caused by *Plasmodium* sp. parasite and transmitted via infected mosquitoes. According to World Malaria Report (2013) from World Health Organization (WHO), malaria being a deadliest illness resulted in approximately 627 000 deaths globally in 2012. More than 75 % of the victims were children under five years old. Currently, many reports showed that the failures of antimalarial treatment were due to *Plasmodium* sp. resistance to antimalarial drugs, such as chloroquine and azithromycin (Marfurt et al., 2010). Hence, discovery of potential antimalarial medicines is needed urgently to reduce the mortalities. *E. longifolia* Jack. extract is considered as one of the potential antimalarial drugs (Wernsdorfer et al., 2009). A study conducted by Hout et al. (2006) showed that the water extract of *E. longifolia* Jack. actively inhibit the activity of chloroquine resistant *Plasmodium falciparum* strain (W2) with IC_{50} less than 4 $\mu\text{g/ml}$. Chan et al. (2004) isolated five active compounds, eurycomanone, 13,21-dihydroeurycomanone, 13 α (21)-epoxyeurycomanone, eurycomalactone and 9-methoxycanthin-6-one, from the roots of *E. longifolia* Jack. and these five compounds showed activity against chloroquine resistant Gombak A Isolate, especially, eurycomanone and 13 α (21)-epoxyeurycomanone. Kuo et al. (2004) also found that the eurycomanone and pasakbumin B were potential antimalarial drugs against drug resistant *P. falciparum*.

Besides eurycomanone, its derivatives, 1,15-di-*O*-isovaleryleurycomanone, 1,15-di-*O*-benzoyleurycomanone and 1,15-di-*O*-(3,3-dimethylacryloyl)-eurycomanone were also showed strong cytotoxicity activity and inhibited the activities of chloroquine resistant Gombak A parasites (Chan et al., 2005).

2.1.5.3 Antioxidant and anti-inflammatory activities

E. longifolia Jack. has been used traditionally as a health supplement because many people believe it can delay aging and maintain body health. Oxidation activities in human body are the main killers of human youthfulness. Hence, antioxidant products are famous and being demanded against aging. Varghese et al. (2013) determine the antioxidant activity of *E. longifolia* Jack by testing its free radical scavenging activity using the hydroalcoholic root with a Diphenyl Picryl Hydrazine (DPPH) scavenging assay. Result showed that the DPPH scavenging activity was directly proportional to the concentration of extract. This indicated that the antioxidant activity of *E. longifolia* Jack. became more active with the increased concentration of the extract. Anti-inflammatory activity of *E. longifolia* Jack. was determined using human red blood cell membrane stabilization method under same study. Purwantiningsih et al. (2011) indicated that the antioxidant agents in *E. longifolia* Jack. extract were the phenolic compounds.

2.1.5.4 Anticancer activities

Numerous *in vitro* and *in vivo* studies showed that *E. longifolia* Jack. is effective against cancer cells. The *in vitro* study of Tee and Azimahtol (2005) showed that four gram of methanolic root extract tested on MCF-7 (breast cancer cell line from American Type Culture Collection, ATCC) was able to terminate the

proliferation of MCF-7 cells with IC_{50} $7.80 \pm 0.45 \mu\text{g/mL}$. The extract was also found to be less toxic on MCF-10A (normal breast cell line). Kaewpiboon et al. (2012) proved both dichloromethane and ethanol root extracts of *E. longifolia* Jack. were potential apoptosis inducer on four human cancer cell lines, A549 (human lung cancer), MDA-MB-231 (human breast cancer), KB3-1 (human cervical cancer) and SW480 (human colon cancer). The result showed that the dichloromethane and ethanol extracts were most cytotoxic against MDA-MB-231 cell line with great IC_{50} values, $1.6 \mu\text{g/ml}$ and $1.2 \mu\text{g/ml}$ respectively.

The root extract of *E. longifolia* Jack. does not only induced apoptosis on solid tumour cell line, it also showed strong cytotoxic activities against 'liquid tumour', such as leukemia. Findings from Al-Salahi et al. (2012) showed the root extract of *E. longifolia* Jack. possessed anti-leukemia activity with IC_{50} value of $15.2 \mu\text{g/mL}$ against HL-60 cell line (human promyelocytic leukemia cell line). Five cancer inhibitor phenolic compounds were isolated by GC-MS system and identified as 2,6-dimethoxy-phenol, 4-(2,3-dihydro-7-methoxy-3-methyl-5 propyl-2-benzofuranyl)-2-methoxy-phenol, 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol, 3,4,5-trimethoxy-phenol and 2-methoxy-4-(1-propenyl)-(E)-phenol under the study.

An *in vivo* study was carried out by Al-Salahi et al. (2014) by injecting 10^7 K-562 (chronic myelocytic leukemia cell line) cells into nude mice to test the anti-proliferation potential of methanolic root extract of *E. longifolia* Jack. Result showed the extract successfully inhibited the growth of the tumour and the size of the tumour was significantly reduced by 85% compared with the control.

Rich in active secondary metabolites is the main reason *E. longifolia* Jack. can be a potential anticancer drug in market. Eurycomanone (quassinoid), eurycomalactone (quassinoid), 9-methoxycanthin-6-one (alkaloid), 9-

methoxycanthin-6-one-N-oxide(alkaloid), 9-hydroxycanthin-6-one (alkaloid), 9-hydroxycanthin-6-one-N-oxide (alkaloid), 14,15 β -dihydroxyklineanone (alkaloid) and β -carboline (alkaloid) from different plant parts of *E. longifolia* Jack. have been scientifically proven that they are effective in inhibiting the growth of cancerous cells and less cytotoxic on normal human cells (Kardono et al., 1991; Jiwajinda et al., 2003; Cheah and Azimohtol, 2004; Nurhanan et al., 2005; Li et al., 2007; Miyake et al., 2009; Zakaria et al., 2009; Miyake et al., 2010; Al-Salahi et al., 2014).

2.2 Cancer

2.2.1. Theory on cancer formation

Cancer is a potential deadly disease and believed to be a genetic disease. Initially, this disease started in a single cell. The gene plays an important role in synthesis of substances required by human body needed, particularly protein. The alteration of deoxyribonucleic acid (DNA) sequences and configuration of proteins would result in gene mutation and the mutated gene encoded the vital cell regulatory protein. Error of protein coding leads to the abnormality in cell division and cell characteristic. The abnormal cell multiplied in rapid rate and eventually developed into a solid mass of tissue, and this mass of abnormal tissue is called cancer or tumour (Loeb and Loeb, 2000; Alison, 2001). Currently, over 300 genes, more than 1% of the total number of genes in human genome, have been identified as ‘cancer genes’. The mutant cancer genes play a significant role in cancer formation and they can be inherited from one generation to next generation (Futreal et al, 2004).

Cancer can be divided into two types according to their behaviours, they are benign cancer and malignant cancer. Benign cancer is non-invasive, slow growing and does not have spreading ability. However, it may be precancerous and can be

developed into malignant cancer if left untreated. Malignant cancer is cancerous and invasive. It has a higher growing rate compared to benign cancer and normal cells. This cancer will spread very fast from one part of body to another, the cancer is clarified as stage 2 when metastasis happen, means the cancer started to spread to another organ (Loeb and Loeb, 2000; Tahmasbi et al., 2011; Lee et al., 2013).

2.2.2 Brief history of cancer

According to paleopathologic findings, cancers were found in ancient Egypt mummies and some fossilised bone. There were evidences to prove that cancer was not a newly discovered disease but it had been existed since ancient time. The first written manuscript of cancer was recorded by Edwin Smith Papyrus 3000 years ago. From the written description, there were eight cases of breast diseases which included tumours and ulcers of breast, and no treatment was recorded in the manuscript (Breasted, 1930). This lead people to believe that cancer was an incurable illness at that time. Some other records of cancer could also be found in other civilizations, such as Sumerians, Indians, Chinese and Persians about 2000 years ago (Rehemtulla, 2010).

About 2400 years ago in Greece, Hippocrates (460 – 375 BC) recorded the symptoms of various cancers and concluded this disease was caused by natural factors. In his imagination, he connected the growth pattern of cancer cell with a moving crab and this inspired him to give this disease the terms *Carcinor* (tumor) and *Carcinoma* (malignant tumor), both meaning as ‘crab’ in Greek. In Roman period, a physician called Celsus translated both Greek terms to Latin term, *Cancer* also means a crab. The word ‘Cancer’ has been preserved to name this disease until today (Adams, 1886; Smith, 2002).

The studies about cancer progressed slowly until the middle of 18th century. With the development of high technology equipment, the understanding of human cancer has entered to new milestone. In 1863, Rudolf Virchow successfully identified cancer as a cellular origin and Theoder Boveri discovered that the chromosomal mutations were implicated in cancer formation in 1914. In the 21th century, the human genome sequences were drafted and more than 300 cancer genes were determined. All these studies provided us a more understanding about cancer and they were useful for discovery of new treatments and prevention of cancer (Futreal et al., 2004; Devita and Rosenberg, 2012).

2.2.3 Statistics of cancer

According to the statistic of World Health Organization (WHO), cancer is the top killer in developed countries and second leading death causing illness worldwide including the developing countries, ranked just after heart diseases. In 2012, 14.1 millions new cancer cases were diagnosed, 1.4 million increased cases compared to cases recorded in 2008 (12.7 million cases) and 36.1 million people worldwide were suffering from this illness for the past five years. Forty percent of the diagnosed cancers were lung, female breast, bowel and prostate cancers and they were the top four cancers occurred worldwide. In term of mortality, 8.2 millions of people lost their life due to cancer globally in 2012, about 9000 people had sacrificed their lives every minute in battle against cancer. The cancers that caused major deaths globally, included lung cancer (1.59 million deaths), liver cancer (0.75 million deaths), stomach cancer (0.72 million deaths), colorectal cancer (0.69 million deaths), female breast cancer (0.52 million deaths) and oesophageal cancer (0.40 million deaths). In term of gender, the cancer incidence rate in men is 25% compared to women. Data

showed that the cancer incidence rate of men was highly varied from one region to another where the range is 0.8:1000 in Western Africa to 3.7:1000 in Australia and New Zealand. However, the range of incidence rate among women was in the range from 1:1000 in South-central Asia to 3:1000 in North America. Lung cancer is the most happened cancer for men, more than 16 % of diagnosed cancer cases and caused highest cancer-related deaths among men. For women, the most common cancer and top killer is breast cancer, with 25.2 % cases and 14.7 % of deaths. There are no age boundaries for development of cancer. Cancer can be formed in every age level. However, people in advance age have higher chance to suffer from cancer (WHO, 2012).

In Malaysia, cancer (10.59 %) was the third leading death causing disease, ranked just after septicaemia (death caused by microorganism infection in human bloodstream) and heart diseases, with 16.87 % and 15.70 %, respectively. There are 21 773 new cancer cases diagnosed in Peninsular Malaysia and recorded by National Cancer Registry. This figure was made up of 9974 cases of men and 11799 cases of women. Breast cancer, colorectal cancer, lung cancer, cervix cancer and nasopharynx cancer were the most common cancer in Malaysia with the age range from 15 to 50 and above. Children with age range from 1 month old to 14 years old were threatened by leukaemia. Results showed that the age-standardized incidence rate of Malaysian was 131.3 per 10000. In term of gender, female had a higher incidence rate than the male, with 128.6 per 10000 for male and 135.7 per 10000 for female. The cancer patients among Chinese were higher than Malay and Indian population (Omar et al., 2006).

Cancer is a public disease which burdened economic and social aspects worldwide. In 2008, the impact of death and disability due to cancer to global

economic was \$ 895 billion (US Dollar) and this was 1.5 % of the world GDP (gross domestic product). According to WHO (2012), the cost of treating cancer was 19 % exceeded the cost of heart diseases (\$ 753 billion). Lung cancer, colorectal cancer and breast cancer gave the biggest impact on global economic, with the treatment cost of \$ 188 billion, \$ 99 billion and \$ 88 billion, respectively. Three quarter of the world population was concentrated in middle and lower-income countries with less than \$ 3255 per capita income. However, statistic showed that over 50 % of new cancer cases and more than 70 % of cancer caused deaths occurred worldwide were in the developing countries and underdeveloped countries. The low GDP and less investment in national medical infrastructures resulted in more social and economic costs. Besides, individual and household could also be burdened by the loss of income due to the death and working disability from cancer and caused a vicious cycle of poverty in those regions (John and Ross, 2010).

2.2.4 Causes of cancer development

Carcinogen is the substance or agent that interacted with human cells and eventually enhanced the development of cancer in human and animal bodies. Generally, carcinogen can be divided into three categories: physical carcinogen, chemical carcinogen and biological carcinogen (WHO, 2014).

Physical carcinogens consisted of a wide range of substances or agents, such as solid and gel materials (non-water soluble or partially water soluble), various levels of temperature, serious mechanical injury, alpha and beta radiation and electromagnetic radiations (Maltoni et al., 2000). The most common cancer stimulation agent from physical carcinogens is radiation. About 40 decades ago, people already understood the relationship between ionizing radiation and cancer

induction as well as the mechanisms of cancer formation (Little, 2000). High-frequency radiation with powerful energy could penetrate human body to destroy human DNA and eventually resulted in cell mutation. Nucleus radiation is a fearful carcinogen. The long half life of nucleus radiation can lead to a long term contamination on the environment (soil, water, food and etc.) and its high energy could easily alter the characteristics of cell DNA to promote cancer induction. Statistic showed that the food and water contamination caused by the release of radioactive iodine-131, cesium-137 and cesium-134 from Fukushima Daiichi nuclear plant in Fukushima tsunami resulted an additional 130 cases of cancer-related death and 180 cases of cancer-related morbidity (Hoeve and Jacobson, 2012). Radiation released from medical therapy and diagnosis could also be the source of physical carcinogen. Study showed that the risk of cancer incidence rate increased due to exposure to the low ionizing radiation from cardiac imaging after acute myocardial infarction (Elsenberg et al., 2011). Ionizing radiation (IR) led to the development of breast cancer, thyroid cancer, lung cancer and leukaemia (Jennifer et al., 2002). Besides, an estimated 99% of non-melanoma skin cancer and 95% melanoma skin cancer were caused by the excessive absorption of ultraviolet (UV) radiation, type of non-ionizing radiation, sourced from the sun and artificial sunlight, such as sunbeds. Report showed that people who frequently expose to artificial sunlight to tan their skin could increase the potential of skin cancer development (Levine et al., 2005; Carkson, 2012).

Chemical carcinogen is referred to various types of chemicals that found in the earth which play a role in induction of cancer in men and animals. Examples of chemical carcinogens are substances or products contained tobacco, asbestos, mycotoxin, alcohol and chromium. Chemical transformation plays an important role

in carcinogenesis. Chemical carcinogens were always active in claiming the metabolic mechanisms of both activation and detoxication after entered into human body. Cancer development initiated by an interaction between chemical and DNA which then caused the genetic alteration (Guengerich, 2000; Weston and Harris, 2000; Holmes et al., 2008; Linos et al., 2011). According to statistic of WHO, approximately 20% of global cancer-related mortalities and 70% of lung cancer-related mortalities worldwide were caused by inhalation of tobacco and related products (WHO, 2012). Tobacco smoke comprises of a few thousands of chemical substances and 69 of the chemicals were carcinogens. International Agency for Research on Cancer (IARC) stated that tobacco could also promote development of other cancers, such as upper aero-digestive tract, pancreas, stomach, liver, bladder, bowel, kidney cancers and leukaemia. Other than first hand smoke, secondary smoke, either released by the end side of cigar, cigarette or exhaled by smoker, was extremely harmful to environment as well as lead to high risk of developing cancer among non-smoker (Besaratinia and Pfeifer, 2008; Daher et al., 2010).

Biological carcinogens consist of living organisms, such as viruses, bacteria and parasites. Generally, cancers caused by biological carcinogens are infectious type of cancer. More than a quarter of cancer death worldwide was due to infectious cancer, especially in developing countries (Adnand et al., 2008). Viruses are the main infectious agent which causes cancers in human bodies. Hepatitis viruses, such as Hepatitis B Viruses (HBV) and Hepatitis C Viruses (HCV) are the major agents to induce liver cancer worldwide. The viruses lead to chronic viral infection which can weaken human immune system and caused DNA alteration (Perz et al., 2006). Cervical cancer is one of the death leading cancers among women and this cancer mainly due to infection of human papillomaviruses (HPV) (Smith et al, 2007). Other

microorganism are found to link with typical type of cancers such as human polyomaviruses is linked with potential brain cancer, Epstein-Barr virus with nasopharynx cancer, Kaposi's Sarcoma Herpesvirus cause skin cancer, human T-cell Leukemia Virus-1 with leukemia and *Helicobacter pylori* is linked with gastric carcinoma (Pagano et al., 2004). Activation nuclear factor NF- κ B played a significant role in inflammatory diseases. Currently, researches showed that almost all the viruses associated with cancer formation were active in stimulating nuclear factor NF- κ B and concluded that the infectious-virus-inflammation could be a candidate of carcinogen (Tak and Firestein, 2001; Guan et al., 2008; Rial et al., 2012).

2.2.5 Plant derived anticancer drugs

Curative surgery, palliative surgery, chemotherapy, radiation therapy, hormonal therapy and combinational therapy are the mainstream treatments that are widely practised in modern medical field to treat cancer or prolong patient lifespan (Hadju, 2005; Long et al., 2013). However, existing of serious side effects caused by conventional therapies, such as hair fall, severe pain, blood clots, fatigue, infection, vomit and nausea become a nightmare to patients and seriously affect their daily lives (Amin et al., 2009). Hence, alternative cancer therapy becomes an option to patient instead of conventional treatment due to its lower degree of side effects and less toxic to mankind (Charterjee et al., 2005).

Currently, many researches and clinical tests have highlighted the important and effectiveness of plant derived natural remedy as alternative cancer therapy, such as Chinese herbal medicine therapy and homeopathy therapy (Cassileth and Deng, 2003; Sun et al., 2012). During the past two decades, approximately 25% to 30 % of the anticancer drugs in market are directly isolated from plants and 25% are

chemically modified natural product (Chartterjee et al., 2005). There are 19 plant based natural products which have been launched as anticancer drugs and used clinically between year 2006 to 2010 (Newman and Cragg, 2012). Hence, plants are the main sources of anticancer drugs in pharmaceutical field.

Paclitaxel, also be called as Taxol, is a tetracyclic diterpene which derived from the bark of *Taxus sp.*, such as *Taxus brevifolia* and *Taxus baccata* and it has been scientifically verified as an outstanding anticancer drug (Cragg, 1998; Malik et al., 2011; Howat et al., 2014). Research showed that paclitaxel is strongly cytotoxic against lung cancer and breast cancer (Fukui et al., 2009; Zhou et al., 2013). Luteolin (3',4',5,7-tetra-hydroxyflavone), grouped under flavonoid, can be found in various kinds of plants, fruits and vegetables (celery, green pepper and perilla leaf). It acts as an enhancer for paclitaxel to improve apoptosis ability of paclitaxel in human breast cancer therapy (Yang et al, 2014). Luteolin, possess antioxidant property, is believed to inhibit the growth of mast cell while N-nitrosodiethylamine induced carcinogenesis in liver. Hence, luteolin is effective in hepacellular carcinoma treatment (Balamurugan and Karthikeyan, 2012).

2.3 *In vitro* plant Culture Technique

2.3.1 General requirements

Generally, *in vitro* plant culture is a fundamental technique of regenerating plant cells, tissues, organs and plantlet under aseptic condition with controlled chemical and physical environments, such as nutrients composition of culture medium, plant growth regulator, temperature, humidity and illumination to produce pathogen-free plants (Thorpe, 2007). Plant tissue culture technique was developed under the concept of cellular totipotency theory which suggested that every single

plant cell equipped with regenerative power to assist plant cell grows and differentiates into a genetically similar adult plant (Vasil and Vasil, 1972).

The introduction of totipotency theory fuelled the development of plant cell and tissue culture technology for the past decades. After hundred years of hard work, plant *in vitro* culture techniques are considered well established. Callus culture (Perez-Jimenez et al., 2014), cell suspension culture (Yang et al., 2014), hairy root culture (Fattahi et al., 2013), micropropagation technique (Willyams and Daws, 2013), embryogenesis culture (Varhanikova et al., 2014) and large-scale cell suspension culture in bioreactor (Zhang et al., 2013) are the well-known techniques of plant *in vitro* culture (Vasil, 2008). One of the functions of the *in vitro* plant culture techniques is to produce rapid uniform pathogen-free plantlets in shorter time within a limited space. The plant tissue and cell culture technology is considered as one of the important tools for sustainable global food security strategy for crop improvement via genetic modification and large scale commercial plant species production (Nalawade et al., 2012; Arthikala et al., 2014), endangered and rare plant species conservation (Roy et al., 2011; Patel et al., 2014), alternative germplasm storage (Schmale et al., 2006) and sustainable source of valuable secondary metabolites (Moyo et al., 2013).

To apply this technology successfully, a few factors need to be considered. The chemical composition of the culture medium is the key success for proper growth of cells, tissues and other organs in the culture vessels. Commonly, culture medium is comprised of macronutrients, micronutrients, vitamins, minerals, carbon source and plant growth regulators. Murashige and Skoog culture medium (MS medium) (Murashige and Skoog, 1962) is the most common basal medium used in plant tissue culture (Gago et al., 2011). Supporting agents, such as agar, isubgol and

gelling gums (Gelrate) played a role as sand or soil in nature by solidifying the culture medium into a gel to provide a better growing support base (Ozel et al., 2008). Besides the commonly used gelling agents for solid medium, other materials also can be used as the supporting agent for liquid medium to improve the aeration of culture medium, such as polyester, ceramic, wood pulp and cotton fiber (Ichimura et al., 1995).

The second factor that influences the success of *in vitro* plant culture is the aseptic controlled condition. Artificial controlled environment can provide the cultures a favourable condition by adjusting the humidity, illumination and temperature. Reduction of competitive effects by providing sufficient survival resources is important to ensure the culture grows healthily and rapidly (Hoopen et al., 2002; Ascencio-Cabral et al., 2008; Smith, 2012).

Establishment of aseptic explants is important to initiate and influence the success of the *in vitro* cultures. After the selection of plant material (explants) from the healthy mother plant, the selected explants need to be surface-sterilized before they were cultured on a particular nutrient medium. Generally, every plant part of mother plant can be used as explants, such as leaf, shoot, root, inter-node, stem, petiole and seed. Diluted commercial bleach and ethanol are the common disinfectants used in surface-sterilization process and the success rate of sterilization could be increased by combined application of bactericide and fungicide. In order to obtain virus-free explants, shoot meristem culture is a better choice as this organ is usually free from virus infection (Colgecen et al., 2009; Thomas and Kumari, 2010; Sahin et al., 2013).