# CHROMATOGRAPHIC ENRICHMENT OF BIOACTIVE QUASSINOIDS IN TONGKAT ALI FOR THE IMPROVEMENT OF SPERMATOGENESIS

**CHUNG WAN JIE**

**UNIVERSITI SAINS MALAYSIA**

**2018**

# CHROMATOGRAPHIC ENRICHMENT OF BIOACTIVE QUASSINOIDS IN TONGKAT ALI FOR THE IMPROVEMENT OF SPERMATOGENESIS

**by**

**CHUNG WAN JIE**

**Thesis submitted in fulfilment of the requirements**

**for the degree of**

**Master of Science**

**September 2018**

## ACKNOWLEDGEMENT

First of all, I would like to express my sincere gratitude to my main supervisor, Prof. Chan Kit Lam for his guidance, motivation and advice all the way in my postgraduate study. His passion and persistency towards research inspired me and make this study a success. Next, I would like to thank my co-supervisor Dr. Lee Chong Yew for his constructive suggestions, support and kindness in helping me whenever I face doubt and uncertainties. Many thanks goes to Dr. Vikneswaran, Dr. Yap Beow Keat and Dr. Mahfoudh for their valuable comments and guidance. Not forgetting the laboratory staff in School of Pharmaceutical Sciences, Encik Fisal, Encik Zainudin, Puan Mahani, Encik Anuar, Encik Rosli and Encik Farid for their patience and assistance along the study.

Besides that, I would like to thank Ministry of Agriculture (MOA) for the financial support of the grant 304/PFARMASI650733/K123. Meanwhile, I would like to send gratitude to my marvellous laboratory mates and friends Elaine Lee Hui Chien, Kishantini, Liew Kok Fui, Cheng Lee Chuen, Liew Wai Lam, Ho Yoke Mei, Amiera Khurshid, Sim Xuan Yi, Cheng Yueh Fang, Kang Inn Nee, Foroogh Ebrahim, Saiful, Shahrul, Sultan, Matthew Mathias, Sharon Chiang, Lee Hooi Xian and Khor Boon Keat for the help and encouragement throughout this study. Last but not least, I am grateful to my beloved parents Mr and Mrs. Chung, my sister Chung Yng Jye and betrothed husband Chew Shio Heong for their unconditional love and unwavering support. Without you all, I would have never make it.

Thank you.

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF APPENDICES

## LIST OF ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ANOVA | Analysis of variance |
| br | Broad |
| $^{13}C$ | Carbon-13 |
| $cm^{-3}$ | Cubic centimeter |
| CMC | Carboxymethylcellulose |
| CV | Coefficient of variation |
| d | Doublet |
| dd | Doublet of doublets |
| DHY | 13α,21-dihydroeurycomanone |
| EL | Eurycomanol |
| EN | Eurycomanone |
| EP | 13(α)21-epoxyeurycomanone |
| EtOH | Ethanol |
| FDA | Food and Drug Administration |
| FSH | Follicle-stimulating hormone |
| FTIR | Fourier-transform infrared spectroscopy |
| g | Gram |
| GnRH | Gonadotropin-releasing hormone |
| $^{1}H$ | Proton |
| h | Hour |
| Hz | Hertz |
| $IC_{50}$ | Half maximal inhibitory concentration |
| ICH | International Council for Harmonization |
| IV | Intravenous injection |
| KBr | Potassium bromide |
| kg | Kilogram |
| L | Liter |
| LCMS | Liquid chromatography mass spectrometry |
| $LD_{50}$ | Lethal dose |
| LH | Luteinizing hormone |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| m | Multiplet |
| $m^2$ | Meter square |
| MeOH | Methanol |
| mg | Milligram |
| min | Minutes |
| mL | Milliliter |
| MTT | 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide |

| | |
|---|---|
| m/z | Mass-to-charge ratio |
| NF-κb | Nuclear Factor Kappa B |
| NMR | Nuclear magnetic resonance |
| OECD | Organization for Economic Co-Operation and Development |
| ppm | Parts per million |
| PTFE | Polytetrafluoroethylene |
| $R^2$ | Coefficient of determination |
| RSD | Relative standard deviation |
| s | Singlet |
| SD | Standard deviation |
| SEM | Standard error of mean |
| SPSS | Statistical Package for the Social Sciences |
| TA | Tongkat Ali |
| TLC | Thin layer chromatography |
| µg | Microgram |
| UV | Ultraviolet |
| UV-vis | Ultraviolet-visible |
| v/v | Volume over volume |
| WHO | World Health Organization |
| w/w | Weight over weight |
| α | Alpha |
| β | Beta |
| $\lambda_{max}$ | Maximum absorption |
| °C | Degree Celsius |

**PENGAYAAN KROMATOGRAFI KUASINODE YANG BIOAKTIF DALAM EKSTRAK TONGKAT ALI UNTUK MENINGKATKAN SPERMATOGENESIS TIKUS**

## ABSTRAK

Akar *Eurycoma longifolia* digunakan sebagai ubat herba tradisional untuk meningkatkan libido dan menambahbaik kesihatan mengandungi sebatian kuasinode yang pahit dan alkaloid kantinon, yang hanya boleh dididapati dalam tumbuh-tumbuhan berasal dari keluarga Simaroubaceae. Kuasinode terutamamya dengan tulang belakang karbon 20 seperti eurikomanon (EN), 13α(21)-epoksieurikomanon (EP) dan kuasinoid minor 13,21-dihidroeurikomanon (DHY) mempunyai aktiviti farmakologi anti-malaria, anti-deman dan anti-ketidaksuburan. Dalam kajian ini, tiga jenis resin bermakroliang Diaion HP-20, SP-700 dan SP-207 telah dikaji untuk fraksi EN dan DHY dalam ekstrak metanol *E. longifolia*. Hasil kajian mengesahkan Diaion HP-20 paling sesuai dalam pengayaan EN dan DHY memandangkan fraksi-EN dan fraksi-DHY masing-masing telah diperkayakan sebanyak 30.40 % dan 13.96 % w/w, berbanding dengan 6.13 % and 0.54 % dalam ekstrak asal *E. longifolia*. Sementara itu, satu kaedah kromatograifi cecair berprestasi tinggi yang mudah dan boleh dipercayai dengan pengesanan ultraungu telah dibangunkan untuk penentuan kandungan EN dan DHY dalam fraksi-fraksi tersebut. Seterusnya, fraksi-EN dan fraksi-DHY yang diperolehi ini digunakan dalam kajian farmakokinetik. Didapati bahawa DHY mencapai puncak kepekatan ($C_{max}$) dan jumlah luas di bawah keluk ($AUC_{0-\infty}$) yang lebih tinggi daripada EN selepas pemberian secara oral. Kedua-dua sebatian menunjukkan biokeperolehan mutlak yang rendah (< 5 %). Namun begitu,

biokeperolehan mutlak DHY (1 %) adalah lebih tinggi daripada EN (0.3 %) sebanyak tiga kali ganda. Seterusnya, keupayaan fraksi-EN dan fraksi-DHY dalam penambahbaikan spermatogenesis *in vivo* tikus telah dikaji. Kiraan sperma, motiliti dan morfologi sperma telah dilaksanakan. Kumpulan tikus yang dirawat dengan fraksi-EN mencatat bilangan sperma (48.60 $x10^6$/mL/g testis) yang paling tinggi, diikuti oleh kumpulan rawatan fraksi-DHY (39.24 $x10^6$/mL/g testis) dan kumpulan kawalan biasa (25.87 $x10^6$/mL/g of testis), mencadangkan EN sebagai sebatian berpotensi tinggi dalam meningkatkan spermatogenesis berbanding dengan DHY. Di samping itu, tiada perbezaan signifikan motiliti dan morphologi sperma diperhatikan dalam ketiga-tiga kumpulan tikus tersebut. Di samping itu, satu model tikus oligospermik direka untuk menilai keberkesanan fraksi-EN dan fraksi-DHY dalam peningkatan spermatogenesis yang tidak normal. Tikus-tikus oligospermik yang dirawat dengan fraksi-EN (37.69 x $10^6$/mL/g) dan fraksi-DHY (30.08 x $10^6$/mL/g) menunjukkan peningkatan dalam jumlah sperma ($P$ < 00.5) berbanding kumpulan kawalan negatif. Kesimpulannya, walaupun DHY mempunyai kelebihan biokeperolehan tiga kali ganda lebih tinggi daripada EN, namun fraksi-EN lebih berkesan dalam meningkatkan pengeluaran sperma daripada fraksi-DHY. Ini menunjukkan bahawa bioaktiviti kuasinode bukan sahaja dipengaruhi oleh faktor-faktor farmakokinetik dan fizikokimia tetapi juga dipengaruhi faktor-faktor farmakodinamik seperti interaksi molekul intrinsik dengan tapak tindakan sasaran.

**CHROMATOGRAPHIC ENRICHMENT OF BIOACTIVE QUASSINOIDS IN TONGKAT ALI FOR THE IMPROVEMENT OF SPERMATOGENESIS**

## ABSTRACT

*Eurycoma longifolia* Jack roots, traditionally used as herbal remedies for increasing libido and better health conditions, contain a high content of quassinoids, the bitter substances and alkaloidal canthinones, indigenous in the Simaroubaceae family that are responsible for the plant pharmacological properties. Amongst the quassinoids, the 20-carbon eurycomanone (EN), 13α(21)-epoxyeurycomanone (EP), eurycomanol (EL) and 13α,21-dihydroeurycomanone (DHY), possess anti-malarial, anti-ulcer, anti-pyretic, anti-osteoporosis, aphrodisiac and anti-infertility properties. In the present study, the chromatographic separation employing macroporous resin Diaion HP-20, Sepabeads SP-700 and SP-207 were evaluated for the enrichment of EN and DHY in *E. longifolia* methanolic extract. Results showed that among the three macroporous resin, Diaion HP-20 performed the best enrichment and separation characteristics. After single chromatographic fractionation with Diaion HP-20, the content of EN and DHY were increased from 6.13 % and 0.54 % to 30.40 % to 13.96 % w/w, respectively. Meanwhile, a simple and reliable high performance liquid chromatography with ultraviolet detection method was developed and validated for the determination of EN and DHY. Subsequently, the EN-rich and DHY-rich fractions were subjected to pharmacokinetic evaluation in rats. The present finding indicated that DHY was able to achieve a higher peak concentration ($C_{max}$) and total area under the curve ($AUC_{0-\infty}$) compared to those of EN in oral dosing. The absolute bioavailability of both compounds remained low (< 5 %) but DHY showed a significantly higher bioavailability (1 %) than EN (0.3 %) which may be attributed to

the slightly higher lipophilicity of DHY. Subsequently, the EN-rich and DHY-rich fraction were tested for *in vivo* spermatogenesis activity. Sperm, count, motility and morphology at the cauda epididymal of the rats were carried out. The animals treated with EN-rich fraction scored the highest increase in sperm count of 48.60 x $10^6$/mL/g of testis, followed by DHY-rich treated rats (39.24 x $10^6$/mL/g of testis) and control group (25.87 x $10^6$/mL/g of testis), suggested that EN was more potent than DHY in the improvement of spermatogenesis. However, there were no significant difference in sperm motility and morphology among the treated rats and control group. In addition, an oligospermic rat model was designed by using andrographolide isolated from *Andrographis paniculata* to evaluate the efficacy of EN and DHY in the augmentation of abnormal spermatogenesis. Oligospermic rats administered with EN-rich fraction (37.69 x $10^6$/mL/g) and DHY-fration (30.08 x $10^6$/mL/g) reported significant ($P < 00.5$) increase in sperm count compared to the negative control group given andrographolide only. In conclusion, despite DHY having a three-fold higher oral bioavailability than EN, EN-rich fraction was more potent in enhancing sperm production than DHY-rich fraction. These results indicated that the bioactivity of the quassinoids may be governed not only by the pharmacokinetic and physicochemical factors but also pharmacodynamics factors such as their intrinsic molecular interaction with the target site of action.

# CHAPTER 1

# INTRODUCTION

## 1.1 Infertility overview

Infertility is a reproductive system disease which is the disability of couples to conceive after one year of unprotected sexual intercourse (WHO, 2017). There are 60 to 80 million of people or 8 % to 12 % of couples around the world suffering from infertility problems with the highest rate in middle east countries, followed by Europe and America (Agarwal et al., 2015; Sahay & Reddy, 2006). In Malaysia, fertility rate has gradually declined from 2.78 to 2.06 children per household since year 2005 to 2015 based on the statistics provided by Department of Statistics in Malaysia (Ho, 2017). According to Dr. Agilan Arjunan, an *in vitro* fertilization (IVF) specialist of the Kuala Lumpur Fertility Centre, male infertility in the past ten years had increased approximately 50 % in Malaysia (Bernama, 2015). Infertility has always been alleged as a predominantly female disorder. However, 50 % of infertility problems among couples were attributed to the male partner (Anawalt, 2013). The true extent of male infertility is not well reported and underestimated owing to sociodemographic constraints.

Semen analysis is the basis step in evaluating male fertility disorder where poor semen parameters lower the chances of pregnancy. The normal concentration of sperm count for a healthy man is between 40 million to 300 million per mL, whereas a count below 10 million per mL is considered as low sperm count or oligospermia (Jungwirth et al., 2012). The proposed normal healthy value for semen volume, pH, total sperm

count, motility, viability and white blood cell per ejaculate are ≥ 1.5 mL, ≥ 7.2, ≥ 39 million sperm, ≥ 40 %, ≥ 58 % and less than 1 million white blood cell per mL, respectively (WHO, 2010). Following semen analysis, WHO (2010) recommended andrological investigations when the semen analysis of patients is abnormal in a duplicate test in order to define a diagnosis. Andrological investigations include hormonal assessments (level of testosterone, follicle-stimulating hormone and luteinizing hormone) and testicular biopsy to govern the severity of infertility of a patient.

Clinically, male infertility can be distributed into four main groups: pretesticular disorder (hypothalamic or pituitary disorder), testicular disorder, post testicular (sperm transport disorder) and idiopathic (Sahay & Reddy, 2006). An epidemiology study published that majority of male infertility diagnosis fall into the idiopathic group owing to seminal abnormalities with unknown causes, followed by varicocele, accessory gland infection, immunological factor, ejaculatory inadequacy and genetic abnormality (Irvine, 1998). Pretesticular disorder is related to endocrine problems, and coital disorder such as hypogonadotropic hypogonadism, erectile dysfunction and ejaculatory failure (Kretser, 1997). Hypothalamic or pituitary disease is a less common aetiology in male infertility. Hypogonadism refers to the low physiological level of testosterone in male with associated signs or symptoms of abnormal spermatozoa production such as reduction of libido and erectile dysfunction. Primary hypogonadism is the failure of testes to produce sufficient testosterone to react, causing elevation of FSH and LH level. It can be caused by testicular injury, tumour, chemotherapy, congenital defects or testicular infectious disease. Secondary hypogonadism is the inability of hypothalamus to stimulate GnRH to secrete FSH and

LH (Dandona & Rosenberg, 2010). Insufficient secretion of FSH and LH has a direct impact on secondary sexual development and fertility. Testicular factors include chromosomal abnormalities, congenital disorder and autosomal karyotype abnormalities. The most common example is Kallmann syndrome owing to Y chromosome deletions (de Roux et al., 2003). Heat, drugs, irradiation and torsion will also lead to testicular disease. Post-testicular factors involve sperm transportation disorder in vas deferens and epididymis. It could be due to vasectomy, congenital issues and idiopathic (Kretser, 1997). Apart from that, male infertility may well arise from social and environmental factors such as aging, lifestyle habits, stress relationship, occupational exposure and scrotal temperature.

Existing therapeutic treatment for male infertility includes oral medications, testicular surgery, *in vitro* fertilization and intracytoplasmic fertilization. Synthetic drugs such as sildenafil (Viagra) and tadalafil (Cialis) are commonly prescribed to treat erectile dysfunction by stimulating nitric oxide that activates cAMP to dilate the corpus luteum. Clomiphene citrate is an estrogen inhibitor that has proven to increase the production of FSH and LH in men (Choy & Ellsworth, 2012). Testosterone replacement therapy is another popular alternative to increase testosterone either through injections, oral medication or topical administration. However, some of the drugs bring about undesirable side effects (Tharakan & Manyam, 2005). For instances, Viagra and Cialis are known to cause negative side effects such as headache, muscle pain, blurred vision and exert dangerous drug-drug interactions (Melnyk & Marcone, 2011). Although testicular surgery, *in vitro* fertilization or intracytoplasmic sperm injection may be other alternatives, the surgery does not completely assure pregnancy and is very costly.

Natural herbs which are believed to be less harmful have been sought after as alternatives for increasing men libido by our ancestors. A herb is a plant or plant part used for its scent, flavor or therapeutic properties, and medicinal products made from them are frequently taken to improve health as dietary supplements (Jarukamjorn & Nemoto, 2008). In fact, 50 % of the drugs available in the market are of natural origin. For instances, aspirin, digitoxin, morphine, quinine and pilocarpine are drugs that derived from plant and has been widely used as pain reliever, heart failure medication, pain medication, anti-malarial drug and cholinergic agent, respectively (Balunas & Kinghorn, 2005). Thus, it is practical and important to extract bioactive ingredients from plants for the incessant development of potent drug. For examples, *Tribulus terrestris*, a Mediterranean plant belongs to the family Zygophyllaceae known as "puncture vine" was claimed to be beneficial in improving men virility by regulating male hormone level. The major bioactive compound found in this plant is protodioscin, a steroidal glycoside of furostanol type. Animal study conducted on rabbits and rats feeding on the plant extract reported significant increase in the level of serum testosterone, dihydrotestosterone and dehydroepiandrosterone in a dose dependent manner (Gauthaman & Ganesan, 2008).

In Malaysia, *Eurycoma longifolia* which is known as Tongkat Ali is an indigenous plant found in Southeast Asia. It has been traditionally use as a tonic to improve general health, sexual prowess, to treat fever, syphilis and afterbirth by the locals and aborigines. Earlier phytochemical research showed that the organic root extract of *E. longifolia* increased penis reflexes and the frequency of ejaculation in rats (Ang et al., 2001). This led to the interests in isolation of bioactive compounds from this plant. It was discovered that *E. longifolia* contained mainly quassinoids, alkaloids,

tricullane-type triterpenes, biphenylneolignans and squalene derivatives (Bhat & Karim, 2010). The major bioactive component in *E. longifolia* was identified as quassinoids, degraded triterpenes that were indigenously found in Simaroubeceae plants. The quassinoids in *E. longifolia* is gaining attention owing to their interesting pharmacology activities such as antiplasmodial activity, cytotoxicity, ergogenic effects, anti-diabetic and aphrodisiac properties. It was identified that the major quassinoid, eurycomanone found in *E. longifolia* increased testosterone level in rats by acting as an aromatase inhibitor that inhibited the transformation of testosterone to estrogen (Low et al., 2013a).

Enrichment of bioactive compounds in plant extracts is important as a preliminary step in manufacturing phytochemical-rich products. Additionally, the enrichment method should be established and optimized as a simple, low cost and reproducible process. Although extensive pharmacological and chemical studies have been carried out on *E. longifolia*, further rigorous testing and ratification on the bioactive components in *E. longifolia* is still needed to ascertain their pharmaceutical value as a therapeutic product.

### 1.1.1 Current therapeutic approach on male infertility

Current approach on male infertility focuses on testosterone replacement therapy, oral medications, assisted reproductive technology and *in vitro* fertilization. Beside the oral capsule or tablet testosterone administration, androgen can be boosted via intramuscular injections, transdermal patches, transdermal gel, buccal tablets and subcutaneous pellets. Common anti-estrogen drugs are clomiphene citrate, tamoxifen, toremifen and raloxifen (Dabaja & Schlegel, 2014). It stimulates the production of

testosterone by inhibiting the negative feedback of estrogen to the hypothalamic pituitary axis, thereby increasing the release of FSH and LH that stimulate Leydig cells to produce more testosterone (Choy & Ellsworth, 2012). Although clomiphene citrate showed beneficial outcome, it has not been legally approved by Food and Drug Administration for hypogonadism treatment. The side effects are minor nausea, dizziness, weight gain and fluid retention (Ramasamy et al., 2014). The contradictions of testosterone replacement therapy are risk of male breast cancer, prostate cancer and gynaecomastia, sleep apnoea and erythrocytosis (Dandona & Rosenberg, 2010).

On the other hand, assisted reproductive technology is a technique where a processed semen sample is washed to remove prostaglandins, leukocytes, immotile sperm and then injected via a catheter directly into the upper uterine cavity. This technique allows sperm to bypass cervical mucus, elevating the chances of conception (Talwar, 2012). *In vitro* fertilization is a modified technique of assisted reproductive technique as both the semen sample and oocytes are taken and mixed externally for fertilization to happen. After an embryo is formed, it is placed into the uterus of the female partner. Although the fertilization rate is 70 – 85 %, the success live birth rate is approximately 23 % for women under age of thirty-five years old (Mansour et al., 2014). These procedures are costly, uncomfortable and causing socio economic problem. The technique is further improved in which only a single sperm is required for to be injected into a mature oocytes. This new technique is termed intracytoplasmic sperm injection. This technique greatly relies on the viability of the spermatozoon, quality of the oocyte and the ability of the oocyte to tolerate intracytoplasmic manipulation (Merchant et al., 2011). The reported success lives birth rate for

intracytoplasmic sperm injection (20 %) is about the same with *in vitro* fertilization (Mansour et al., 2014).

## 1.2 Tongkat Ali (*Eurycoma longifolia*)

*Eurycoma longifolia* is an indigenous, slender, evergreen herbal plant found in Southeast Asia region such as Malaysia, Indonesia, Vietnam and Thailand (Bhat & Karim, 2010). It is known as 'Tongkat Ali' or 'Penawar Pahit' in Malaysia, 'Pasak Bumi' in Indonesia, 'Cay ba binh' in Vietnam and 'Ian-don' in Thailand (Kuo et al., 2004). Table 1.1 shows the taxonomy of *E. longifolia*.

Table 1.1: Taxonomy of *E. longifolia* (Hsuan, 1978).

| **Taxonomy** | |
|---|---|
| Kingdom | Plantae |
| Division | Spermatophyte |
| Subdivision | Angiospermae |
| Class | Dicotyledone |
| Order | Geraniales |
| Family | Simaroubaceae |
| Genus | Eurycoma |
| Species | *E. longifolia* |
| Binomial name | *Eurycoma longifolia* Jack |

The plant grows slowly in sandy soil and can grow up to 15 m in height without branching. The leaves are long, spirally arranged and pinnate with numerous leaflets. Each leaf is roughly 5 to 20 cm long containing 30 to 40 lanceolate or obovate-lanceolate leaflets. The brownish red flowers are hermaphrodite with 5 small lobes petals. The fruits are yellowish brown in colour when young and develop to brownish red gradually after two to three years of cultivation (Wizneh & Asmawi, 2014). It is

usually harvested after a period of at least five years by pulling out the whole plant to obtain its taproots which are cylindrical and yellowish-white (Bode & Dong, 2004).

### 1.2.1 Traditional Uses

*E. longifolia* is famous for its aphrodisiac properties especially among the aborigines. The aqueous roots extract of *E. longifolia* has been used locally to improve overall health including sexual prowess and performance, to treat fever, after birth, wounds, syphilis, hypertension and tuberculosis (Erasmus et al., 2012). The locals believe that two teaspoon of *E. longifolia* root decoction per day can improve sexual performance and help in the proper functioning of the male sex organs. The roots are usually cut into small parts, boiled with water and then consumed as a decoction or concoction. Honey, syrup and dates are added sometimes to reduce the bitter taste of the root extract of *E. longifolia* (Bhat & Karim, 2010). This plant has been marketed as a health supplement or fortified in food and beverages claiming to increase overall health. Currently, there are more than 200 products from *E. longifolia* including mixed capsules, tablets, canned or powdered beverages of tea and coffee accessible on the market (Rehman et al., 2016).

### 1.2.2 Chemical constituents

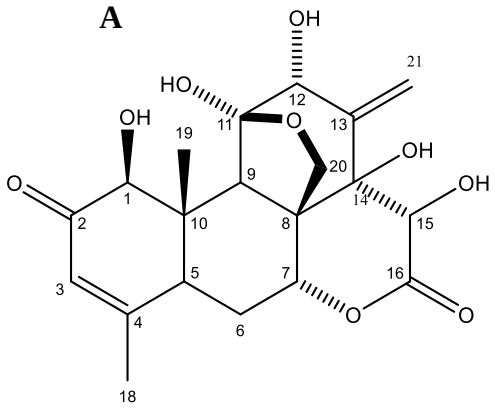
Phytochemical studies on *E. longifolia* lead to the isolation of new bioactive compounds from different parts of the plant. The plant contains mainly quassinoids, canthin-6-one alkaloids, squalene derivatives, tirucallene-type triterpenes, biphenyl-neo-lignans, anthraquinones and polyacetylenes (Teh et al., 2010; Wang et al., 2017). To date, more than 60 compounds have been isolated from this plant and being tested against various diseases in search for bioactive compounds.

### 1.2.2(a) Quassinoids

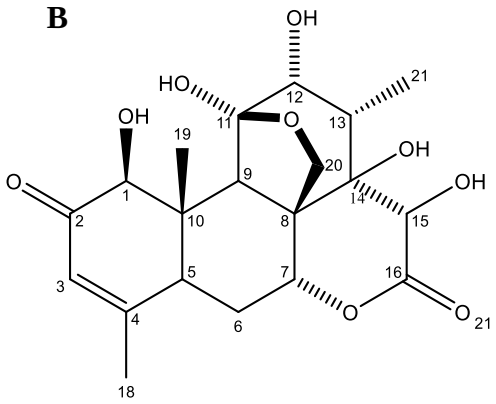
Quassinoids are naturally occurring degraded triterpenes found in the plants of the Simarouboidaea subfamily of Simaroubaceae. They are categorized into different groups according to the number of carbon atoms forming their basic skeleton. Among them, the C-20 quassolidane skeleton is of larger existence and attracting intense attention. In fact, the National Cancer Institute discovered the potency of some C-20 quassinoid compounds in antiluekemia activity since 1970s (Chakraborty & Pal, 2013). On the other hand, C-19 quassinoid displayed a wide spectrum of biological activities such as antiplasmodial, antimalarial, antileukemia, anti-tubercular, antiviral, aphrodisiac properties and insecticidal. Novel quassinoids were reported against antimalarial activity, antifatigue and antiangeogenesis (Al-Salahi et al., 2013; Ang et al., 1995; Gauthaman & Ganesan, 2008; Hamzah & Yusof, 2003). Table 1.2 shows the quassinoids which have been isolated from *E. longifolia* while Figure 1.1 displays the chemical structure of four main quassinoids in *E. longifolia*.

Table 1.2: Quassinoids isolated from *E. longifolia* (Rehman et al., 2016).

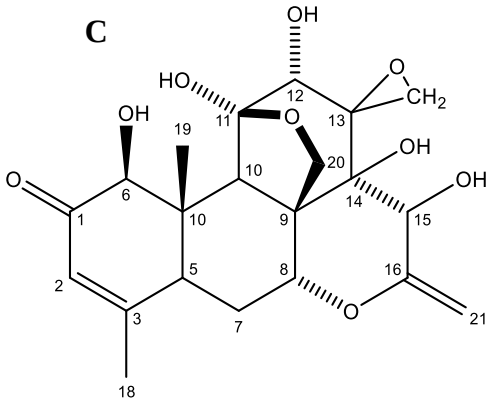
| $C_{18}$ | $C_{19}$ | $C_{20}$ |
|---|---|---|
| Laurycolactone A | 6α-Hydroxyeurycomalactone | Eurycomanone |
| Laurycolactone B | 7α-Hydroxyeurycomalactone | 13α,21-Dihydroeurycomanone |
| Eurycolactone B | 5,6-Hydroxyeurycomalactone | 13α(21)-Dihydroeurycomanone |
| Eurycolactone D | Eurycomalide A | 13β-Methyl,21-dihydroeurycomanone |
| | Eurycomalide B | 12-Acetyl-13,21-dihydroeurycomanone |
| | Eurycomalide C | 15-Acetyl-13α(21)dihydroeurycomanone |
| | Eurycomalide D | 12,15-Diacetyl-13α(21)dihydroeurycomanone |
| | Eurycomalide E | 1β,2α,15β-Triacetyleuryccomanone |
| | 6-Dehydroxylongilactone | Eurycomanol |
| | 11-Dehydroklaineanone | 13β,18-Dihydroeurycomanol |
| | Eurycolactone E | 13β,21-Dihydroxyeurycomanol |
| | Eurycolactone F | 5α,14β,15β-Trihydroxyklaineanone |
| | Eurycolactone G | 11-Dehydroklaineanone |
| | | 12-*epi*-11-Dehydroklaineanone |
| | | 14,15β-Dihydroxyklaineanone |
| | | 15β-Hydroxyklaineanone |
| | | 15β-Acetyl-14-hydroxyklaineanone |
| | | 5-iso-Eurycomadilactone |
| | | 13-*epi*-Eurycomadilactone |
| | | Eurycolactone A |



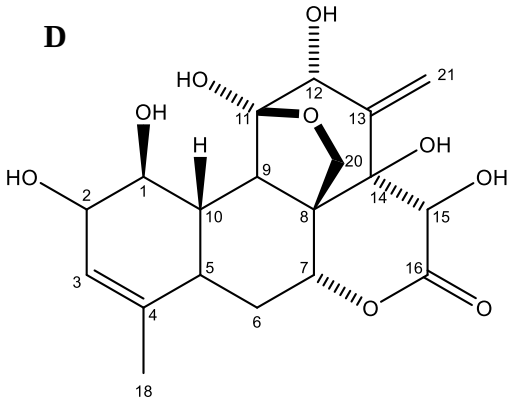

Eurycomanone (EN)




13α, 21-Dihydroeurycomanone (DHY)




13α(21)-Epoxyeurycomanone (EP)




Eurycomanol (EL)

Figure 1.1: Chemical structure of four major quassinoid in *E. longifolia*: (A) eurycomanone, (B) 13α, 21-dihydroeurycomanone, (C) 13α(21)-epoxyeurycomanone and (D) eurycomanol.

### 1.2.2(b) Alkaloids

Alkaloid is a naturally occurring organic compound containing basic nitrogen atom found mainly in plant. Some well-known alkaloid includes morphine, quinine, nicotine and ephedrine. These alkaloid possess important pharmacological activities such as antimalarial, anti-inflammatory and antioxidant. Table 1.3 shows the alkaloids isolated from *E. longifolia*.

Table 1.3: Alkaloids isolated from *E. longifolia*.

| Alkaloids | References |
|---|---|
| Canthin-6-one | (Choo & Chan, 2002; Mitsunaga et al., 1994) |
| 4-Hydroxy-5-methoxycanthin-6-one | (Kuo et al., 2004) |
| 10-hydroxycanthin-6-one | (Chan et al., 1986) |
| 9-Hydroxycanthin-6-one N-oxide | (Lin et al., 2001) |
| 9-Hydroxy-6-one | |
| 11-Hydroxycanthin-6-one | (Mitsunaga et al., 1994) |
| Canthin-6-one-3N-oxide | |
| 11-Hydroxy-10-methoxycanthin-6-one | |
| 9-Methoxycanthin-6-one | |
| 10-Hydroxy-9-methoxycanthin-6-one | |
| 9-Methoxycanthin-6-one 3N-oxide | |
| 5,9-Dimethoxycanthin-6-one | |
| 9,10-Dimethoxycanthin-6-one | |
| 3-Methylcanthin-5,6-dione | |
| 9-Methoxy-3-methylcanthine-5,6-dione | |
| 1-Methoxymethyl-β-carboline | |
| B-Carboline-1-propionic acid | |
| 5-Hydroxymethylcanthin-6-one | (Kuo et al., 2003) |
| Canthin-6-one 9-O-β-glucopyranoside | |
| 5-Methoxycanthin-6-one | |
| 4,5-Dimethoxycanthin-6-one | |
| 10-Methoxycanthin-6-one | |
| 1-Hydroxy-9-methoxycanthin-6-one | |
| 5-Hydroxymethyl-9-methoxycanthin-6-one | |
| 8-Hydroxy-9-methoxycanthin-6-one | |
| n-Pentyl β-carboline-1-propionic acid | |
| Methyl β-carboline-1-carboxylate | |
| 7-Methoxy-β-carboline-1-propionic acid | |

### 1.2.2(c) Squalene Derivatives

Squalene derivatives isolated from *E. longifolia* included eurylene, longilene peroxide (Itokawa et al., 1991), 14-deacetyl eurylene and teurilene (Morita et al., 1993).

### 1.2.2(d) Tricullane-type triterpenes

Tricullane-type triterpenoids isolated from *E. longifolia* included niloticin, dihydroniloticin, piscidinol A, bourjotinolone A, melianone, 3-episapeline A and hispodine (Itokawa et al., 1992).

### 1.2.2(e) Biphenylneolignans

Biphenylneolignans isolated from *E. longifolia* were 2,2'-dimethoxy-4'-(-3-hydroxy-1-propenyl)-4-(1,2,3-trihydroxypropyl) diphenyl ethers, 2-hydroxy-3,2',6'-trimethoxy-4'-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)biphenyl and 2-hydroxy-3,2'-dimethoxy-4'-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl) biphenyl (Morita et al., 1992).

### 1.2.2(f) Anthraquinones and anthraquinones glycosides

Anthraquinones and anthraquinones glycosides isolated from *E. longifolia* were chrysophanol, parietin, emodin, emodin-8-O-β-glucoside, aloeemodin-8-O-β-glucoside, chrysophenol-1-O-β-glucoside, chryso-phenol-8-O-β-glucoside and parietin-8-O-β-glucoside (Lin et al., 2001).

### 1.2.2(g) Polyacetylenes

Polyacetylenes were found in *E. longifolia* recently. The new compounds isolated were longifolione A, B, C, D and E (Wang et al., 2017).

### 1.2.3 Biological activities

Pharmacology studies which have been carried out on *E. longifolia* included aphrodisiac activity, anti-malarial, cytotoxicity and ergogenic effect.

#### 1.2.3(a) Aphrodisiac and anti-infertility activities

*E. longifolia* root extract has been proven scientifically to improve male libido and sexual desire conforming to the traditional belief. Earlier animal studies revealed that sexually unexperienced rats treated with *E. longifolia* extract and fractions displayed an increase in sexual desire and motivation compared to normal control group (Ang & Sim, 1998). Besides that, middle-aged male rats showed prominent aphrodisiac effect after force fed with *E. longifolia* extracts and fractions, twice daily for 10 days (Ang & Lee, 2002). A consecutive *in vivo* study was carried out by evaluating the sperm quality of male rats after feeding on standardized *E. longifolia* methanolic extract that contain mainly eurycomanone (EN), 13α,21-dihydroeurycomanone (DHY), eurycomanol (EL) and 13α(21)-epoxyeurycomanone (EP). It was concluded that the sperm count, morphology and plasma testosterone level increased significantly in a dose dependant manner (Chan et al., 2009). Human trial on *E. longifolia* also reported that 75 sub-fertile patients given 200 mg of *E. longifolia* water extract daily for 3 months showed significant improvement in all semen parameters and 17 % of them had spontaneous pregnancy (Tambi & Imran, 2010). Even patients with late onset hypogonadism have improved sign of ageing male symptoms and increased serum testosterone level after consuming 200 mg of standardized *E. longifolia* water extract for one month (Tambi et al., 2012).

Thus, a more intensive study was continued to determine the active constituents contributing to the aphrodisiac effect in *E. longifolia*. It was proven scientifically that the major quassinoids in *E. longifolia* were the active functional compound in enhancing male libido. A study was conducted by comparing the efficacy of TA water extract and its fraction (TAF2) in increasing rat sperm count. The fraction TAF2 was collected through a patented resin column chromatography fractionation technology (Chan et al., 2006). The TA water extract and TAF2 with adjusted dosage to similar quassinoids content feeding to male rats reported an almost similar increment in sperm concentration and morphology (Low et al., 2013a). Correspondingly, the up-to-date research on *E. longifolia* reported that male rats given *E. longifolia* quassinoid-rich extract orally for 48 days has a significant higher sperm concentration than the other group feeding on *E. longifolia* quassinoid-poor extract (Ebrahimi et al., 2016).

### 1.2.3(b) Antimalarial property

Antiplasmodial activity from *E. longifolia* root extract has been reported since early 1980s. Organic and water extracts of *E. longifolia* together with the isolated compounds 10-hydroxycanthin-6-one, eurycomalactone, eurycomanone and eurycomanol were tested *in vitro* on Thailand strain (K-1) of *Plasmodiun falciparum*. All of the isolated compounds showed inhibition activity towards *P. falciparum* except the alkaloid 10-hydroxycanthin-6-one. It is noteworthy that the inhibition activity of eurycomanone was two-fold greater than the standard drug chloroquine diphosphate which is a known medication to prevent and treat malarial (Chan et al., 1986). Correspondingly, another study on the antimalarial properties of *E. longifolia* reported that eurycomanone was the most active compound among the isolated compounds (β-carboline-propionic acid, 18-dehydro-6α-hydroxyeurycomalactone,

eurycomanol, eurycomanone and chloroquine diphosphate) due to the presence of exomethylene bridge between atom C-8 and C-11 in eurycomanone (Yusuf et al., 2013). The potency of eurycomanone as an antimalarial agent was further supported by continuous investigation. Once more, eurycomanone reported stronger inhibition ($IC_{50}$ = 14.9 ng/mL) against W2 *P. falciparum* clones than chloroquine ($IC_{50}$ = 75.3 ng/mL) but a lower inhibition activity ($IC_{50}$ = 34.0 ng/mL) against D6 *P. falciparum* clones compared to chloroquine ($IC_{50}$ = 2.72 ng/mL) (Kuo et al., 2004).

Tongkat Ali extract containing three main quassinoids, eurycomanone, 13α,21-dihydroeurycomanone and 13α(21)-epoxyeurycomanone were investigated against antiplasmodial activity and showed good inhibition towards *P. falciparum*. The author suggested that it might be due to the synergistic effect between the three quassinoids or presence of other unidentified compound (Wernsdorfer et al., 2009).

**1.2.3(c) Anti-proliferative**

In 1992, six quassinoids and seven tricullane-type triterpenes were isolated from *E. longifolia* extract and tested *in vitro* against P388 (leukemia cells line) and KB (carcinoma cells line) for the evaluation of their cytotoxicity. Interestingly, all of the isolated compounds showed good cytotoxicity activity against the cell line with 6α-hydroxyeurycomalactone being the most potent against P388 cells and 14,15β-dihydroxyklaineanone being the most potent against KB cells (Itokawa et al., 1992). The anti-proliferative activity of 14,15β-dihydroxyklaineanone was further supported by another *in vitro* anti-tumor study induced by Epstein-Bar virus activation where 14,15β-dihydroxyklaineanone displayed $IC_{50}$ of 9.0 µM which was much higher than quercetin (23.0 µM) and β-carotene (30.0 µM) (Jiwajinda et al., 2002). However, it

was suggested that the $C_{19}$ quassinoids were more potent against P388 cells line as the $IC_{50}$ of 6-dehydroxylongilactone and 7α-hydroxyeurycomalactone were 0.11 and 0.66 μg $cm^{-3}$ which were higher than 15-acetyl-13α(21)-epoxyeurycomanone (6.6 μg $cm^{-3}$) and 12,15-diacetyl-13α(21)-epoxyeurycomanone (7.2 μg $cm^{-3}$) (Morita et al., 1993). Twenty-four quassinoids isolated from *E. longifoia* were also evaluated on HT-1080 (human fibrosarcoma cell line) and the result showed that eurycomalactone, 14,15β-dihydroxyklaineanone and 13α,21-dihydroeurycomanone with $IC_{50}$ of 0.98, 1.1 and 0.93 μM were the most cytotoxic quassinoids among the isolated compounds toward HT-1080 cell lines (Miyake et al., 2009).

Later, research focused on various *E. longifolia* extracts and fractions. It was reported that the MeOH, n-butanol and chloroform extracts of *E. longifolia* were cytotoxic against KB (epidermoid carcinoma), DU-145 (prostate cancer), RD (rhabdosarcoma), MCF-7 (breast cancer) and CaOV-3 (ovarian cancer) cell lines but contradictorily, the aqueous extract did not show any sign of toxicity in the respective study (Nurhanan et al., 2005). Then, all fractions (TAF2, TAF3, TAF1 and TAF4) from *E. longifolia* tested against HS27 cells lines showed high toxicity ($IC_{50}$ < 20 μg/mL) against HS27 mammalian cells and moderate cytotoxicity activity against Vero cell lines except fraction TAF3 via MTT assay (Kavitha et al., 2010). Consecutively, study conducted on the three fraction (TAF2, F3 and F4) from *E. longifolia* evaluated against K-562 leukemic cells line *in vivo* and *in vitro* reported that fraction TAF2 showed the most potent anti-proliferative activity (Al-Salahi et al., 2014). Further *ex vivo* and *in vivo* studies on TAF2 revealed its anti-angiogenic potential whereby TAF2 significantly suppressed the development of microvessels in rat aorta and inhibited the angiogenesis of human umbilical vein endothelial cells

(HUVEC) (Al-Salahi et al., 2013). Those studies suggested that TAF2 was worth of further study particularly in investigating the bioactive compounds that cause anti-proliferative activity.

The major quassinoid, eurycomanone from TAF2 fraction was isolated and reported significant suppression on the expression of the A549 lung cancer cells (Wong et al., 2012). The mechanism of action was studied and reported that eurycomanone inhibited 1kBβ phosphorylation and upstream mitogen activated protein kinase (MAPK) signalling, causing regression in the proliferation of Jurkat (human T lymphocyte cell) and K562 cell (human erythroleukemic cell line) (Hajjouli et al., 2014)

### 1.2.3(d) Ergogenic property

Human trial involving male and female elderly between 57-72 years old were carried out to study the ergogenic effect of Physta *E. longifolia* extract. Both male and female participants taking 200 mg of the extracts twice daily for five weeks demonstrated significant increase in serum testosterone concentrations, muscle strength and muscle mass (Henkel et al., 2014). The increase of testosterone level inhibited adipogenesis, thereby increasing lean body mass and physical functions (Herbst & Bhasin, 2004). Earlier study also reported 37 % increase in testosterone level and improvement in psychological mood parameters after supplemented with standardized hot-water extract of *E. longifolia* for four weeks in 63 subjects including 32 men and 31 women (Talbott et al., 2013). This clearly pointed out the effectiveness of *E. longifolia* extract in boosting testosterone level in human and the improvement in exercise performance.

### 1.2.3(e) Toxicity

An acute oral toxicity study showed that the $LD_{50}$ of *E. longifolia* aqueous extract (containing more than 22% eurypeptide, 40 % glycosaponin and 1 % eurycomanone) was more than 5000 mg/kg in rats with no pathological changes (Shuid et al., 2011). Therefore, from this study, it can be postulated that the $LD_{50}$ of EN was equivalent to 50 mg/kg. However, in subacute toxicity study for 28 days, it was reported that some of the animals displayed liver toxicity at 1200 mg/kg and 2400 mg/kg of *E. longifolia* aqueous extract (Shuid et al., 2011). To date, this was the only acute and subchronic toxicity studies on *E. longifolia* aqueous extract. Another acute toxicity conducted by Li et al. (2013) reported that the acute oral $LD_{50}$ of *E. longifolia* root powder was more than 6000 mg/kg. As for subchronic toxicity, the same author reported that rats feeding on 2000 mg/kg of *E. longifolia* root powder did not show significant changes in body weight hematology, serum biochemistry, urinalysis, macropathology or hispathology. Moreover, the treatment with *E. longifolia* root powder for 4 weeks and 13 weeks significantly reduced blood urea nitrogen, lactose dehydrogenase, creatinine, and cholesterol in male rats (Li et al., 2013). The discrepancy in toxicity studies might be due to differences from source of plant materials, extraction methods and experiment designs. From the above studiesan aqueous dose of one tenth of 1200 mg.kg can be considered as relatively safe for experimental studies.

A reproductive and development toxicity study on *E. longifolia* aqueous extract (PHYSTA), a product of Phytes Biotek Sdn Bhd, Malaysia were carried out in compliance with OECD Principles of Good Laboratory Practice and OECD Guidelines for testing of Chemicals, Section 4 No 421. The authors concluded that *E. longifolia* product containing 0.8 – 1.5 % eurycomanone at the dosages of 250, 500 and 1000

mg/kg did not show signs of toxicity in rats after histopathology and pathology studies (Ming et al., 2015). A patented, standardized *E. longifolia* extract, TAF273 (Chan & Low, 2008) was also tested on reproductive toxicity and two-generation teratology studies. Results indicated that the females rats treated with TAF273 showed significant increase in fertility index and litter size than non-treated female rats (Low et al., 2014). In this study, Low et al. (2014) also reported that the $LD_{50}$ and no-observed adverse effect level (NOAEL) of TAF273 1293 mg/kg and 100 mg/kg, respectively.

### 1.3 Macroporous resin

Macroporous resin is a highly cross-linked, lipophilic polymeric adsorbent prepared from the polymerization of styrene-divinylbenzene or acrylic-divinylbenzene. However, most of the macroporous resin is based on polystyrene matrix. There are wide variations in surface area, functionality and porosity among the macroporous resin adsorbents (Mitsubishi Chemical, 2017). The resin beads are essentially solid by having a permanent well developed and homogenous porous structure with pore diameters up to several hundred angstroms (Å). The amount of crosslinking depends on the proportion of different monomers used in the polymerization step, practically ranging between 4 and 16 %. Low level of monomers (< 1 %) is undesirable as it produces mechanically weak networks that could easily damage by heating, handling or solvent shock (Mei & Czarnik, 2002). Resin with very low crosslinking also tends to be more hydrated and changed dimensions markedly depending on which ions are bound. Generally, the surface area of macroporous resin which ranges between 100 to 600 $m^2/g$ offer high adsorption capacities. Macroporous resin has been widely used as a separation medium in chemical synthesis, pharmaceutical research, food industries, mining, agriculture and industrial water treatment. The advantages of using

macroporous resins are high adsorption capacity, relatively low cost, chemically stable, easy regeneration and high recovery (Soto et al., 2011). Macroporous resin can be further categorized as ion exchange resin, chelating resins and non-polar synthetic resins according to the functional group that have been added to the backbone structures during condensation or polymerization. Table 1.4 shows the physical parameters of various macroporous resins.

Table 1.4: Parameters of macroporous polystyrene adsorbent resins (Lailiang, 2017).

| Adsorbents | Area ($m^2/g$) | Average pore diameter (nm) | Manufacturer |
|---|---|---|---|
| AmberliteXAD-2 | ≥ 300 | 18 | Rohm&Haas (USA) |
| AmberliteXAD-4 | ≥ 750 | 10 | Rohm&Haas (USA) |
| AmberliteXAD-16 | ≥ 800 | 15 | Rohm&Haas (USA) |
| AmberliteXAD-761 | 150–250 | 60 | Rohm&Haas (USA) |
| Diaion HP-20 | ≥ 600 | 40 | Mitsubishi Chemical (Japan) |
| Diaion HP-21 | ≥ 570 | 16 | Mitsubishi Chemical (Japan) |
| Diaion HP-2MG | ≥ 500 | 40 | Mitsubishi Chemical (Japan) |
| Sepabeads SP-825 | ≥ 1000 | 11 | Mitsubishi Chemical (Japan) |
| Sepabeads SP-850 | ≥ 1000 | 7.6 | Mitsubishi Chemical (Japan) |
| Sepabeads SP70 | ≥ 800 | 14 | Mitsubishi Chemical (Japan) |
| Sepabeads SP700 | ≥ 1200 | 18 | Mitsubishi Chemical (Japan) |
| Sepabeads SP207 | ≥ 630 | 21 | Mitsubishi Chemical (Japan) |
| NKA-9 | 250–290 | 12-18 | Cangzhou Bonchem (China) |
| NK-110 | ≥ 500 | 16 | Cangzhou Bonchem (China) |
| HA | ≥ 800 | 4-10 | Cangzhou Bonchem (China) |
| X-5 | 500–600 | 29-30 | Cangzhou Bonchem (China) |
| ADS-5 | ≥ 550 | 20-30 | Cangzhou Bonchem (China) |

An ion exchange resin is a polymer with electrically charged sites at which one ion may replace another. The monomers are usually functionalized through chemical reactions before polymerization into beads (Fritz et al., 2010). It can be divided into two broad categories, namely cation exchange resin and anion exchange resins. Strong cation exchange resins containing sulfonic acid groups or the corresponding salts as their functional groups. Weak cation exchange resins contain carboxylic acid groups or the corresponding salts as their functional groups. Strong anion exchange resins containing quaternary ammonium groups that impart higher basicity. Weak cation exchange resins containing ammonium chloride or hydroxide groups (Mitsubishi Chemical, 2017). Ion exchange resins are primarily used in water treatment applications, demineralization and decolourization.

On the other hand, non-polar macroporous resin does not have ionic functional groups and is mainly build-up of diethylbenzene. The adsorption mechanism of polystyrene benzene macroporous resin was dependant on the hydrophobic and electrostatic interactions (Lailiang, 2017). It is usually used as a reverse phase adsorbent eluted with mobile phase in descending polarity. Synthetic adsorbent resin has been developed since 1960 in United State of America. Later on, China and Japan also manufactured macroporous resin according to market requirements. Synthetic resins were widely used because they facilitate a cheap and effective chemical regeneration process (Lin & Juang, 2009). Nonpolar macroporous resin can be build out by different polymers for selective separation of organic compounds. There are adsorbent of styrene body, adsorbent of brominated styrene body and adsorbent of methacryle body (Mitsubishi Chemical, 2017).

Selection of resin for this study is based on the molecule structure of quassinoids and prediction of its possible interaction. Synthetic absorbent were chosen because they were typically used in analytical and preparative chromatographic separation for hydrophilic compounds. Quassinoids are polar, hydrophilic compound with molecular weight around 400. By utilizing the polarity difference of the mobile phase, the impurities could be separated out the compound of interest. Adsorbent resins of styrene body such as Diaion HP series have strong adsorption ability as they form π-π interaction bond with the adsorbed sample. They are commonly used in food and pharmaceutical industry for protein purification, decolourization of various sugar solutions, phenol removal and chemical separation purposes. Therefore Diaon HP-20 was chosen as one of the adsorbent resin in this study.

On the other hand, the modified synthetic resins made up of the brominated styrene backbone (Sepabeads SP series) have a stronger adsorption ability in adsorbing low hydrophobic materials when compared to adsorbent of styrene body. This is due to the high hydrophobicity property of the brominated styrene body in nature that has greater selectivity towards the non-polar molecules (Mitsubishi Chemical, 2017). The electron accepting effect of the added bromine increases the hydrophobicity of the chemically modified synthetic adsorbent. Due to this properties, Sepabeads SP-207 can absorb many compounds very strongly. In this study, the targeted compound DHY was less polar than EN. Therefore, Sepabeads resin was selected as one of the adsorbent in this study seeing that it has a stronger affinity towards the non polar compound.

## 1.4 Spermatogenesis

Spermatogenesis is the development of spermatozoa involving mitosis and meiosis that occur in seminiferous tubules located in the testis. Mitosis is a cell process of division that results in two genetically identical daughter cells developing from parent cells while meiosis involves the division of germ cell into four gametes, each possessing half the number of chromosomes of the original cell. Seminiferous tubules contain an active population of highly dividing and self-sustaining germ cell, an embryonic cell with the potential of developing into a gamete. In human, spermatogenesis take about 65-75 days (Tortora & Derrickson, 2009) while in rat, the complete process from spermatogonia to spermatozoa takes about 48-52 days (Lohmiller & Swing, 2006). Spermatogenesis can be divided into three important phases which are primary spermatocytogenesis, secondary spermatocytogenesis and spermiogenesis (Sharma & Agarwal, 2011).

During primary spermatocytogenesis, the primitive stem cells, spermatogonium proliferate to form diploid spermatocyte. In human, several spermatozoa cell types have been identified (A-0 through A-4, intermediate [IN], and B). Mitosis ends when a B spermatogonium yields two primary spermatocytes. Subsequently, the primary spermatocytes type B spermatogonia enter the prophase of meiosis and divide through to form haploid secondary spermatocytes (Tortora & Derrickson, 2009) (Figure 1.2). During spermiogenesis, the round spermatid, which arises from the secondary spermatocytogenesis meiotic division, undergoes a series of complex cytological events and transformed into spermatozoon. The acrosome is derived from the Golgi apparatus. Centrioles (points of organization of spindle fibers) migrate to a post nuclear region after the completion of meiosis. This transformation

involves nuclear condensation, formation of the acrosomal cap, and development of a tail (Kretser et al., 1998).

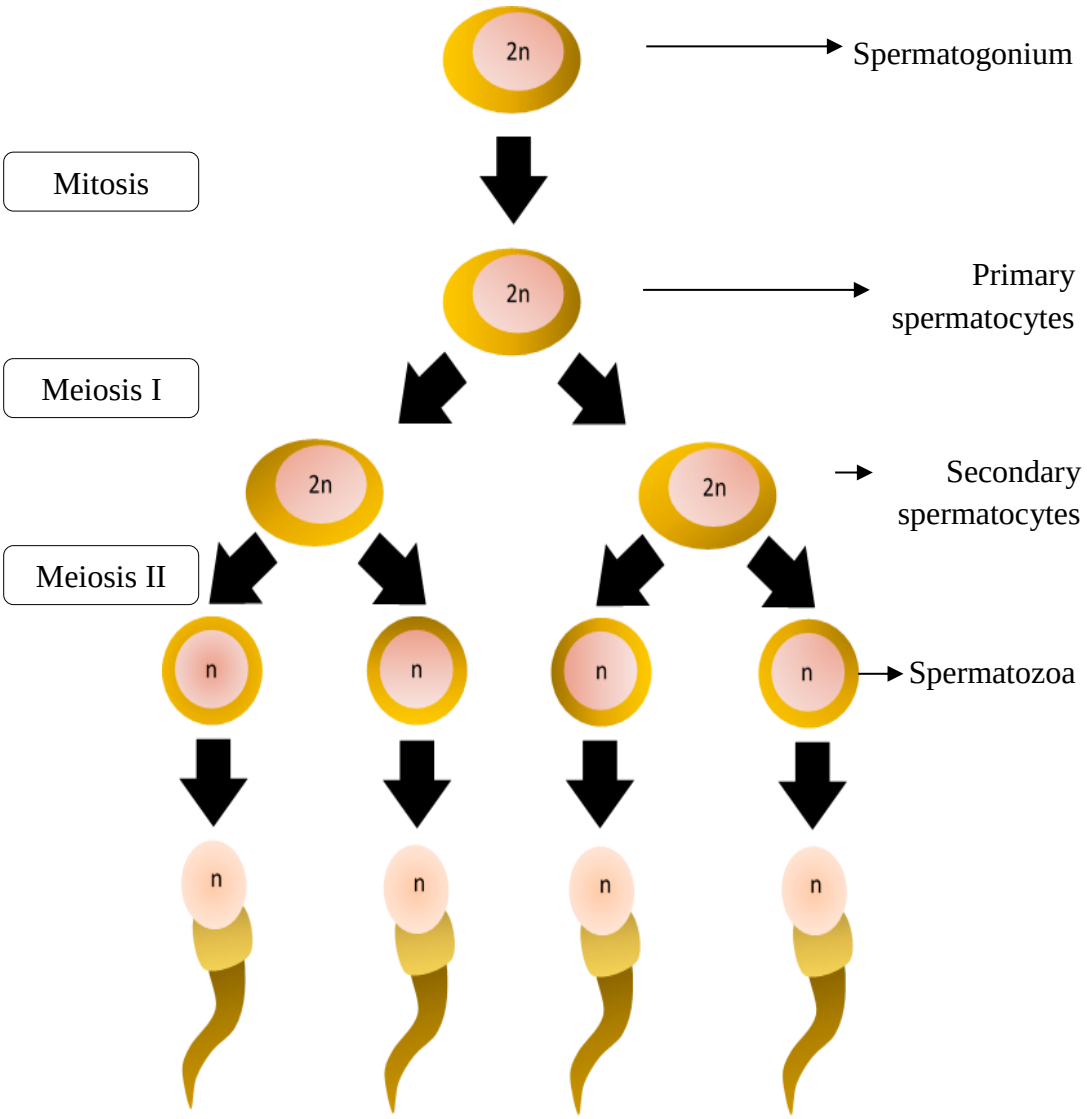



Figure 1.2: The spermatogenesis cycle.