NMR-BASED METABOLOMIC APPROACHES TO EVALUATE THE EFFECTS OF *EURYCOMA LONGIFOLIA* EXTRACTS ON RAT SPERMATOGENESIS

by

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To my husband and parents for their unconditional love and support

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LIST OF ABBREVIATIONS

ACN	acetonitrile
APS	ammonium persulfate
CD_3OD	deuterated methanol
CO_2	carbon dioxide
dd H_2O	distilled deionized water
DSA	4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate
EtOH	ethanol
HRP	horseradish peroxidase
KOD	deuterated potassium (kalium) hydroxide
MeOH	methanol
$NaHCO_3$	sodium bicarbonate
NaN_3	sodium azide
NaOD	deuterated sodium hydroxide
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
Tris-HCl	tris (hydroxymethyl) aminomethane hydrochloric acid
TSP	sodium 3- (trimethylsilyl) propionate- 2,2,3,3- d4
AMIX	analysis of mixtures
APCI	atmospheric pressure chemical ionization
BBO	broadband observe

^{13}C	carbon
D1	relaxation delay
Dept-Q	distorsionless enhancement by polarization transfer-quaternary
DS	number of dummy scans
ESI+	electron spray ionization positive mode
FID	free induction decay
FT	Fourier transformation
GC-MS	gas chromatography-mass spectrometry
^{1}H	proton
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography- ultra violet
HSQC	heteronuclear single quantum coherence
LB	line broadening
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-mass spectrometry/mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
MS	mass spectrometry
NOESY	nuclear Overhauser effect spectroscopy
NS	number of scans
PC	principal component
PCA	principal component analysis
PDA	photodiode array detector

RT	room temperature
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIMCA	soft independent modeling of class analogy
SRM	single reaction monitoring
TAQP	Tongkat Ali quassinoid poor
TAQR	Tongkat Ali quassinoid rich
TAW	Tongkat Ali water
TIC	total ion chromatogram

LIST OF SYMBOLS

br s	broad singlet
br d	broad doublet
br t	broad triplet
d	doublet
D1	relaxation delay
dd	doublet of doublet
dt	doublet of triplet
td	triplet of doublet
Hz	Hertz
J	coupling constant
kDa	kilodalton
m	multiplet
m/z	mass over charge
MHz	mega hertz
ng	nanogram
p1	pulse width
ppm	part per million
SSB	shifted sine bell
δ_H	proton chemical shift
δ_C	carbon chemical shift
1D	one dimensional
2D	two dimensional

r^2	coefficient of determination
v/v	volume over volume
w/v	weight over volume
w/w	weight over weight

PENDEKATAN METABOLOMIK BERDASARKAN NMR UNTUK MENILAI KESAN EKSTRAK *EURYCOMA LONGIFOLIA* KE ATAS SPERMATOGENESIS TIKUS

ABSTRAK

Eurycoma longifolia (Tongkat Ali, TA) dikenali untuk meningkatkan kesuburan lelaki dan libido. Suatu analisis metabolomik analisis berasaskan resonans magnetik nuklear (NMR) dikombinasi dengan kaedah kimometrik termaju telah dibangunkan, divalidasikan dan digunakan serentak untuk pengenalan dan kuantifikasi serentak metabolit dalam *E. longifolia* dan cecair mamalia (air kencing dan plasma tikus). Suatu profil umum ekstrak akueus yang diperolehi daripada 30 sampel di Perak, Malaysia telah ditentukan untuk metabolit terutamanya bagi kuasinoid bioaktif eurikomanon, eurikomanol, 13,21-dihidroeuricomanon dan euricomanol-2-*O*-*β*-D-glikopiranosida. Profil rujukan kemudian dibandingkan dengan ekstrak akueus yang lain dari Selangor, Kedah dan Terengganu untuk menyiasat sebarang metabolit diskriminasi berkaitan dengan lokasi dan variasi seperti suhu alam sekitar dan pH tanah. Satu lagi kajian adalah berkaitan dengan analisis NMR pada air kencing tikus untuk menyiasat sebarang korelasi antara metabolit didiskriminasi dengan status bilangan sperma (SC) berikutan rawatan tikus dengan pelbagai kandungan kuasinoid dalam air (TAW, 125 mg/kg), Tongkat Ali kurang kandungan kuasinoid (TAQP, 125 mg/kg) dan ekstrak kuasinoid yang kaya dengan Tongkat Ali (TAQR, 21 mg/kg). Suatu peningkatan 6-kali ganda dos TAW, sama dengan TAQP dalam kepekatan kuasinoid, telah diadministrasi untuk menyiasat sama ada sebatian dalam kedua-dua ekstrak yang bertanggungjawab bagi

peningkatan dalam SC tikus. Tikus-tikus itu kemudian dikategorikan ke dalam kumpulan SC normal dan tinggi berdasarkan nilai median rujukan bagi tikus SC yang normal. Profil NMR air kencing kumpulan SC normal dan tinggi telah diperiksa untuk diskriminasi metabolomik. Tambahan lagi, keputusan rawatan yang sama pada profil NMR plasma tikus telah disiasat. Bahagian terakhir kajian bertujuan bagi penemuan penanda bio kesuburan daripada metabolit air kencing dan plasma. Profil min ekstrak akueus *E. longifolia* mengandungi α-glukosa, alanina, fenilalanina, tirosina, kolina, asid formik, suksinik, metilsuksinik, fumarat, syringik, laktik dan asid asetik dan juga eurikomanon, eurikomanol, 13,21-dihidroeuricomanon dan euricomanol-2-*O*-*β*-D-glikopiranosida. Profil metabolomik kuantitatif akar *E. longifolia* tidak berbeza berdasarkan suhu dan pH tanah. Kuantitatif NMR yang divalidasikan (qNMR) dengan pastinya menentukan tahap kuasinoid dalam ekstrak akueus *E. longifolia*, menunjukkan eurikomanon (% w/w ± SD) dengan kepekatan kuasinoid yang paling tinggi pada julat 5.984 ± 1.949 to 7.752 ± 2.892 dan tidak berbeza secara statistik dalam sampel di empat lokasi. Sebaliknya, sampel dari lokasi yang berbeza menunjukkan kepekatan yang berbeza secara statistik untuk kolina, euricomanol, euricomanol-2-*O*-*β*-D-glikopiranosida, asid laktik dan succinik. Haiwan yang dirawat dengan TAW dan TAQR mempunyai bilangan sperma jauh lebih tinggi dan signifikan berbanding dengan dua kumpulan lain. Nilai SC ($x10^6$/mL/g testis) adalah 25.35 ± 6.42, 65.9 ± 26.11, 28.73 ± 17.42 dan 68.27 ± 14.96 untuk masing-masing kumpulan kawalan, dirawat dengan TAW, dirawat dengan TAP dan dirawat dengan TAQR. Daripada analisis air kencing, paras trigonelina, asid benzoik dan alanina meningkat dengan signifikan dalam kumpulan SC tinggi berbanding dengan yang SC normal, manakala paras etanol

menurun secara statistik dalam kumpulan SC tinggi. Berdasarkan data literatur, perubahan paras metabolit diskriminasi berkorelasi dengan paras testosteron yang berbeza dalam sampel air kencing, selepas rawatan dengan ekstrak *E. longifolia* yang mengandungi kepekatan kuasinoid yang berbeza. Bukti lanjut diperolehi dengan mengukur paras testosteron dalam kencing tikus. Fasa organik (org) air kencing, kaya dengan testosteron, telah diperolehi daripada air kencing yang diekstrak dengan dietileter. Air kencing fasa organik dan berair masing-masing dianalisis dengan LC-MS/MS untuk testosteron dan eurikomanon (sebagai kuasinoid yang paling banyak). Kehadiran eurikomanon (hanya dalam kumpulan SC tinggi) telah terbukti dalam fasa akueus (aq) air kencing dari puncak isotop $[M+H]^+$ pada *m/z* 409.02. Testosteron telah dikenal pasti dalam kumpulan SC normal dan tinggi dengan ion molekul pada *m/z* 289.2 $[M+H]^+$ dan ion yang terserpih pada *m/z* 96.8 and 108.8. Jumlah testosteron (%w/v) dalam kumpulan SC tinggi adalah 2.2 kali ganda lebih tinggi daripada SC normal. Analisis statistik plasma tikus juga mendedahkan peningkatan paras alanina, laktat dan histidina dalam kumpulan SC tinggi berbanding dengan kumpulan SC normal. Walau bagaimanapun, paras etanol menurun dengan signifikan dalam kumpulan SC tinggi. Metabolit diskriminasi yang sama telah ditemui dalam plasma seperti yang ditemui dalam air kencing. Kesimpulannya, profil metabolomik piawai dari sampel Perak telah diperolehi sebagai rujukan. Sampel dari Perak dan yang berada di tiga lokasi yang berlainan telah didiskriminasi mengikut paras metabolit mereka. Keberkesanan kuasinoid pada peningkatan dalam jumlah sperma telah disahkan oleh peningkatan paras kuasinoid dan testosteron air kencing, bersama-sama dengan beberapa metabolit primer seperti yang dinyatakan di atas. Pendekatan metabolomik berdasarkan NMR telah

memberikan penanda bio air kencing dan plasma yang dikaitkan dengan peningkatan jumlah sperma, dan mempunyai potensi untuk penilaian status kesuburan lelaki tanpa memerlukan sampel air mani.

NMR-BASED METABOLOMIC APPROACHES TO EVALUATE THE EFFECTS OF *EURYCOMA LONGIFOLIA* EXTRACTS ON RAT SPERMATOGENESIS

ABSTRACT

Eurycoma longifolia (Tongkat Ali, TA) is known for boosting male fertility and libido. A nuclear magnetic resonance (NMR)-based metabolomics analysis in combination with advanced chemometric methods was developed, validated and applied for the simultaneous identification and quantification of metabolites in *E. longifolia* and mammalian fluids (rat urine and plasma). A general profile of the aqueous extract derived from 30 samples in Perak, Malaysia was established for metabolites especially for the bioactive quassinoids of eurycomanone, eurycomanol, 13,21-dihydroeurycomanone and eurycomanol-2-*O*-*β*-D-glycopyranoside. The reference profile was then compared with other aqueous extracts from Selangor, Kedah and Terengganu to investigate any discriminatory metabolites with respect to location and variations such as environmental temperature and soil pH. Another study dealt with the NMR analysis of rat urine to investigate any correlation of the discriminated metabolites with the sperm count (SC) status following the treatment of rats with varying quassinoid content in water (TAW, 125 mg/kg), quassinoid-poor Tongkat Ali (TAQP, 125 mg/kg) and quassinoid-rich Tongkat Ali (TAQR, 21 mg/kg) extracts. A 6-fold increase in dose of TAW, equal with TAQP in concentration of quassinoids, was administered to investigate whether the compounds in the two extracts were responsible for the increase in rat SC. The rats were then categorized into normal- and high-SC groups following the

reference median value of normal rat SC. The urine NMR profiles of the normal- and high-SC groups were next examined for metabolomic discrimination. In addition, the results of the same treatment on the rat plasma NMR profiles were investigated. The last part of study aimed at the fertility biomarker discovery of the urine and plasma metabolites. The *E. longifolia* aqueous extract general profile contained α- glucose, alanine, phenylalanine, tyrosine, choline, formic, succinic, methylsuccinic, fumaric, syringic, lactic and acetic acids and also eurycomanone, eurycomanol, 13,21-dihydroeurycomanone and eurycomanol-2-*O*-*β*-D-glycopyranoside. The quantitative metabolomic profiles of *E. longifolia* roots were not different with respect to temperature and soil pH. The validated quantitative NMR (qNMR) reliably determined the quassinoid levels in *E. longifolia* aqueous extracts, showing eurycomanone (% w/w ± SD) with the highest quassinoid concentration at a range of 5.984 ± 1.949 to 7.752 ± 2.892 and not statistically different in the samples at the four locations. In contrast, the samples from different locations were statistically different in concentration of choline, eurycomanol, eurycomanol-2-*O*-*β*-D-glycopyranoside, lactic and succinic acids. TAW- and TAQR-treated animals had significantly higher sperm number compared to the other two groups. The SC values ($x10^6$/mL/g testis) were 25.35 ± 6.42, 65.9 ± 26.11, 28.73 ± 17.42 and 68.27 ± 14.96 for control, TAW-treated, TAQP-treated and TAQR-treated groups, respectively. From the urine analysis, trigonelline, benzoic acid and alanine levels significantly increased in high-SC group compared to the normal-SC one, whereas the ethanol level statistically decreased in high-SC group. Based on the literature data, the altered level of discriminatory metabolites correlated with the different levels of testosterone in urine samples, following the treatment with *E.*

longifolia extracts containing different quassinoid concentration. Further evidence was obtained by measuring testosterone level in rat urine. The organic-phase (org) urine, rich in testosterone, was obtained from the diethylether extracted urine. The organic and aqueous phase urine were analyzed by LC-MS/MS for testosterone and eurycomanone (as the most abundant quassinoid), respectively. The presence of eurycomanone (only in high-SC group) was proven in aqueous-phase (aq) urine by the isotope peak $[M+H]^+$ at *m/z* 409.02. Testosterone was identified in normal- and high-SC groups displaying the molecular ion at *m/z* 289.2 $[M+H]^+$ and the fragmented ions at *m/z* 96.8 and 108.8. The testosterone amount (% w/v) in the high-SC groups was 2.2 fold higher than that of the normal-SC. The statistical analysis of rat plasma also revealed the increased levels of alanine, lactate and histidine in the high-SC group compared to the normal-SC group. However, the ethanol level significantly decreased in the high-SC group. Similar discriminatory plasma metabolites as those of urine were found. In conclusion, a standardized metabolomic profile of Perak samples was obtained as a reference. The samples in Perak and those in three other locations were discriminated according to their metabolite levels. The efficacy on the increase in sperm count of the quassinoids was confirmed by the increase in urinary quassinoid and testosterone levels, together with some of the above-mentioned primary metabolites. The NMR-based metabolomic approach has provided urine and plasma biomarkers associated with an increase in sperm count, and has potential for the evaluation of male fertility status without requiring semen sample.

CHAPTER ONE

INTRODUCTION

1.1 Infertility as a public health issue

Infertility, defined as the inability of a couple to conceive after a year of frequent unprotected intercourse, is a worldwide health issue influencing nearly up to one in five couples during reproductive age (Chamley and Clarke, 2007). Male infertility contributes to an almost 50 % of all cases of infertility across the globe and is the most difficult form of infertility to cure (Irvine, 1998).

1.2 Male fertility assessment

The traditional method of male fertility assessment is mainly based on the analysis of sperm count, morphology and motility on the semen sample provided by subjects through masturbation (Guzick *et al*., 2001). Despite the availability and the effectiveness of the advanced conventional methods for fertility evaluation through semen analysis and given the inconvenience felt by subjects during masturbation, there is still a high demand for a fast, non-invasive and accurate approach to evaluate fertility without requiring semen sample.

1.3 Traditional remedies for boosting male fertility

Medicinal plants are non-directed therapy, easy to collect and less expensive compared to prescription medications. Consequently, many people have been attracted to such remedies. There are natural remedies becoming extensively popular in boosting fertility and serving as aphrodisiac supplements (Rowland and Tai, 2003).

Plants such as *Eurycoma longifolia, Smilax myosotiflora*, *Polyalthia bullata, Labisia pumila, Terminalia catappa* and *Rafflesia sp*. have been claimed to possess aphrodisiac properties in Malay traditional medicine. However, the aphrodisiac properties of only *E. longifolia* (Ang *et al*. 2000) and *T. catappa* (Ratnasooriya and Dharmasiri, 2000) have been scientifically proven. Roots of *Eurycoma longifolia* (*E. longifolia*, Tongkat Ali, TA), from the family of Simaroubaceae, have been also traditionally used for treating various ailments including body aches, dysentery and glandular swelling (Darise *et al*., 1982). In general, plant phytochemicals are among either primary or secondary metabolites. A primary metabolite is directly involved in normal growth, development and reproduction. It performs a physiological function such as an intrinsic function. Many organisms or cells contain primary metabolites. The structural variety of such metabolites is rather limited to a set of compound classes such as carbohydrates, amino acids, organic and fatty acids. In contrast, secondary metabolites are not involved in primary life supporting functions. Instead, they usually have important ecological functions. They mainly include nucleosides, peptides, alkaloids, terpenoids and polyphenols (Wink, 2003; Ratcliffe and Shachar-Hill, 2006).

E. longifolia consists of a variety of phytochemicals, one of the most important of which is quassinoids. Quassinoids are highly oxygenated triterpenes and the major secondary metabolites indigenous only to the plants from the Simaroubaceae family which are widely researched. The other secondary metabolites of the plant, however, have not been extensively studied. A wide range of *in vitro* pharmacological properties, such as antimalarial (Chan *et al*., 2005), antiulcer (Tada *et al*., 1991) and also cytotoxic properties against human cell lines (Kardono *et al*., 1991) has been reported on

quassinoids. More importantly, the studies by Wahab *et al*. (2010) and Chan *et al.* (2009) on rat models proved the efficacy of *E. longifolia* root extracts on spermatogenesis and sperm quality. The water-soluble extract of *E. longifolia* root improved sperm quality in infertile patients and led to 14.7% spontaneous pregnancies (Tambi and Imran 2010; Tambi *et al*., 2012). Such findings, in general, cast doubt on the efficacy of *E. longifolia* and quassinoids, especially on male fertility.

1.4 The necessity of a new approach to *E. longifolia* analysis and the management of fertility

Due to the fact that *E. longifolia* has a myriad of pharmacological effects, specially fertility boosting properties, a robust and accurate approach is required for the simultaneous identification and quantification of the plant phytochemicals particulary its active constituent, quassinoids. The simultaneous identification of primary and secondary metabolites of *E. longifolia* will create a general profile which can serve as a reference for the metabolomic comparison of plants being exposed to different variations with respect to the region of origin, age, soil pH and environmental variations such as temperature. Moreover, the overall profile can be referred to in the future studies on *E. longifolia* metabolomic analysis.

The lack of a fast, non-invasive and robust method for the evaluation of sperm quality and male fertility status has created a big challenge in the management of male fertility. To overcome such limitation, a new field of science known as metabolomics has been emerged. One of the functions of metabolomics is to analyze body fluids (e.g. urine and plasma, etc.) to create a cost-effective and informative means of measuring related

metabolites of different health-related issues such as male fertility. Metabolomics is the study of a group of small molecules, known as metabolites, representing functional phenotype in a cell, tissue, organ or organism (Rochfort, 2005; Hollywood *et al*., 2006). There are other branches of "omics" such as transcriptomics or proteomics which regulate metabolic fluxes. However, metabolome, rather than transcriptome and/or proteome, is closer to the phenotype (ter Kuile and Westerhoff, 2001). Although alteration in the level of individual enzymes does not have much impact on metabolic fluxes, it significantly affects the level of individual metabolites. As a result, metabolomics can provide richer and more useful information at a lower cost compared to genomics, transcriptomics and proteomics (Raamsdonk *et al*., 2001).

1.5 NMR-based metabolomics

Nuclear magnetic resonance (NMR) spectroscopy combined with the statistical analysis approaches has been extensively used in the metabolomic analysis of plants and biological fluids including urine and plasma. It has been employed in plant sciences mostly for the purpose of quality control, detecting the biomarkers of plant diseases and studying the effect of different environmental conditions and regions of origin on plant metabolomic profile.

Brassica rapa has nutritional and health benefits due to its high content of antioxidant metabolites (phenolics, flavonoids, vitamins C and E). Abdel-Farid *et al*. (2007) used an NMR-based metabolomic approach to investigate the metabolic discrimination of different cultivars and ages of *Brassica rapa* leaves. In another study, ^{1}H-NMR spectroscopy was used to acquire the metabolite fingerprints of two sunflower genotypes

(Kruger *et al*., 2008). A fast and sensitive ^{1}H-NMR-based metabolomic profiling of deuterated methanol-D_2O buffer extracts of transgenic tomato flesh was performed by Fatma *et al*. (2012). The same approach was used by Kim *et al*. (2010) for the discriminatory analysis of 11 South American Ilex species. A clear separation between species was achieved and finally resulted in four distinct classes with respect to metabolomic similarities. High resolution magic angle spinning-^{1}H-NMR (HRMAS-NMR) spectroscopy was employed by Ritota *et al*. (2012) to analyze red and white varieties of garlic (*Allium sativum L*.) collected in different geographical regions in Italy, in order to address the traceability issue and identify the discriminatory metabolites of different varieties and origins. Plants which have undergone different type of treatments can also be analyzed with NMR-based metabolomics approaches (Liang *et al*., 2006). Pereira *et al.* (2014) performed the metabolomic analysis of lettuce (*Lactuca sativa L*.) leaves to characterize metabolic variations during exposure to mancozeb and the impact of variations on plant metabolism.

Metabolomics studies have been conducted on *E. longifolia* using other technologies except NMR. LC-MS/MS-based secondary metabolomic analysis of *E. longifolia* aqueous extracts from two locations in Malaysia (Perak and Pahang) was investigated by Chua *et al*. (2011). Zaini *et al*. (2016) reported a solid phase extraction-liquid chromatography (SPE-LC) approach for the fingerprinting of *E. longifolia* roots. A more comprehensive and straightforward metabolomics approach using NMR was studied in the current research. Enough number of *E. longifolia* aqueous extracts from Perak were used to establish a metabolomic profile of primary metabolites and major biologically active quassinoids. The aqueous extracts of Perak and three other locations (Selangor,

Kedah and Terengganu) were then compared using chemometric tools for the discriminatory metabolites.

The above-mentioned facts on the health promoting effects of *E. longifolia* (especially fertility improvement) (Section 1.3) and the informative nature of NMR-based metabolomics made us develop a validated approach to establish a mean reference profile of the 30 plant aqueous extracts from Perak for metabolites, particularly the major biologically active quassinoids due to their large number of pharmacological effects discussed in Section 1.3. The potential of the approach for distinguishing *E. longifolia* roots from different geographical locations in Malaysia (Perak, Selangor, Kedah and Terengganu) and of different environmental conditions (soil pH and temperature) was also assessed. A quantitative NMR (qNMR) method was then developed for the simultaneous determination of the discriminatory metabolite levels.

In pursuit of a new method for evaluating male fertility without requiring semen analysis, an animal experimental procedure was designed in which rats were treated with variable content of quassinoids in TA extracts such as the water (TAW, 125 mg/kg), quassinoid-poor TA (TAQP, 125 mg/kg) and quassinoid-rich TA (TAQR, 21 mg/kg) extracts. TAW and TAQR extracts contained same amount of quassinoids, while TAQP extract was purposely depleted of quassinoids. The effect of quassinoids on sperm count (SC) increase was evaluated and the animals were then classified into normal- and high-SC groups considering the reference median value of normal rat SC. An NMR-based metabolomic approach was then applied on urine samples of the normal- and high-SC groups to detect discriminatory metabolites. Furthermore, the discriminatory metabolites of plasma were also investigated following the same treatment on the rats. The

discriminatory metabolites of urine and plasma may be considered as the potential biomarkers for male fertility evaluation without requiring semen analysis. Such an approach may be applicable to human for assessing male fertility status.

1.6 Objectives of the current study

The aims and objectives of the present study are as follows:

1. To develop a standardized mean NMR profile for the identification of primary metabolites and particularly major biologically active quassinoids in crude aqueous extracts of *Eurycoma longifolia* using the Perak samples
2. To develop a validated NMR method for the quantification of major quassinoids in *E. longifolia* aqueous extracts
3. To compare the ^{1}H-NMR profiles of aqueous extracts of *E. longifolia* from four different regions in Malaysia
4. To determine the efficacy of major quassinoids on sperm count increase in rats treated with different extracts of *E. longifolia* containing different quassinoids
5. To evaluate the post-treatment changes of rat urinary metabolites and fertility status without performing semen analysis
6. To evaluate the post-treatment changes of rat plasma metabolites and fertility status without performing semen analysis

1.7 Expected outcomes of the current study

Based on the study objectives the following outcomes were expected:

1. A standardized general NMR profile of *E. longifolia* crude aqueous extracts from Perak on abundant primary metabolites and major quassinoids as a reference for further future research on *E. longifolia*
2. NMR-based validated quantification of major quassinoids in *E. longifolia* aqueous extracts
3. Metabolomic discrimination of aqueous extracts of *E. longifolia* from Perak, Selangor, Kedah and Terengganu
4. The effectiveness of quassinoids (*vs.* glycoproteins) in increasing sperm count in rats and in general improving male fertility
5. A non-invasive approach for evaluating male fertility status through urine metabolites and without requiring semen analysis
6. Confirmation of the accuracy of male fertility biomarkers in urine through plasma metabolomic analysis
7. High potential of NMR spectroscopy combined with chemometric tools in the discovery of male fertility biomarkers

CHAPTER TWO

LITERATURE REVIEW

2.1 *Eurycoma longifolia* Jack

Eurycoma longifolia Jack, *E. longifolia* (locally known as Tongkat Ali (TA) or Penawar Pahit in Malaysia, Pasak Bumi in Indonesia, Ian-don in Thailand and Cay ba binh in Vietnam), is a plant from the family of Simaroubaceae (Kuo *et al*., 2004). It is an unbranched tree or shrub with the height of up to 8 m with a few upright branches which are crowned by umbrella-like rosettes of leaves. It grows in well-drained sandy soils most effectively (Corner, 1952). The biological information of the plant (Ismail *et al*., 1999) together with the plant pictures are included in Fig. 2.1.

2.1.1 Chemical constituents of *E. longifolia*

E. longifolia constituents include quassinoids, squalenes, biphenylneolignan, triterpene-like tirucallanes, canthin-6-one alkaloids, β-carbolines and glycopeptides. Major squalene derivatives of *E. longifolia* include eurylene, 14-deacetyl eurylene, longilene peroxide and teurilene (Itokawa *et al*., 1991a and b; Morita *et al*., 1993). Biphenylneolignan compounds isolated from *E. longifolia* are two isomeric 2,2'-dimethoxy-4-(3-hydroxy-1-propenyl)-4'-(1,2,3-trihydroxypropyl) diphenyl ethers, two isomers of 2-hydroxy-3,2',6'-trimethoxy-4'-(2,3-epoxy-1-hydroxypropyl)-5-(3-hydroxy-1-propenyl)-biphenyl and also 2-hydroxy-3,2'-dimethoxy-4'-(2,3-epoxy-1-hydroxypropyl)-

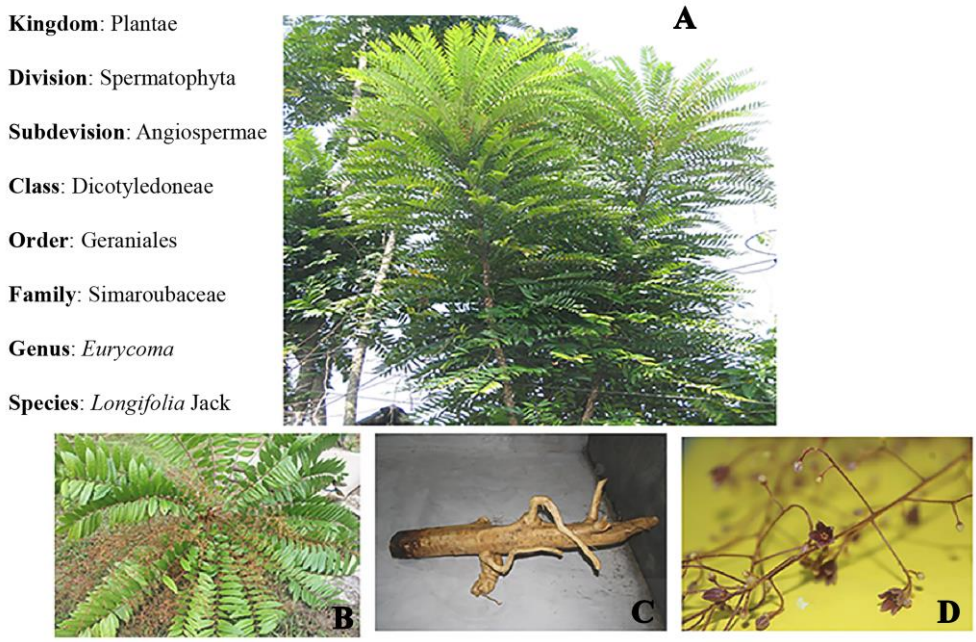

Fig. 2.1 *Eurycoma longifolia* Jack; **A** the whole plant, **B** leaves, **C** root and **D** flowers

hydroxypropyl)- 5-(3-hydroxy-1-propenyl)-biphenyl (Morita *et al*., 1992). The isolated triterpene-like tirucallanes include niloticin, dihydroniloticin, piscidinol A, bourjotinolone A, 3-episapelin A, melianone and hispidone (Itokawa *et al*., 1992). 9,10-Dimethoxycanthin-6-one, 10-hydroxy-9-methoxycanthin-6-one, 11-hydroxy-10-methoxycanthin-6-one and 5,9-dimethoxycanthin-6-one are the isloated canthin-6-one alkaloids from *E. longifolia* (Kardono *et al*., 1991; Mitsunaga *et al*., 1994; Choo and Chan, 2002). β-Carboline derivatives of *E. longifolia* are β-carboline-1-propionic acid and 7-methoxy-β-carboline-1-propionic acid (Kardono *et al*., 1991; Kuo *et al*., 2004). A biologically active glycopeptide (4.3 kDa) has been isolated from an aqueous extract of *E. longifolia* (Asiah *et al*., 2007). Most of the therapeutic properties of *E. longifolia* has been attributed to quassinoids as the indigenous phytochemicals of the plant. On the other hand, although not evidently proven, Sambandan *et al.* (2006) attributed the

fertility boosting properties of *E. longifolia* to its glycoprotein content, although glycoproteins are the ordinary constituents which may be found in many plant families (Galili *et al*., 1998). As a result, these constituents of *E. longifolia* (quassinoids and glycoprotein) are worthy of further literature search included in the following sections (Sections 2.1.1(a) and 2.1.1(b)).

2.1.1(a) Quassinoids

Quassinoids are the bitter constituents of the plants from the Simaroubaceae family and the secondary metabolites characteristic of this family. Nearly 120 quassinoid derivatives have been isolated and identified. The term quassinoids is derived from quassin which is the first identified member of this class of compounds isolated from the specimen Quassia Amara. Quassinoids are chemically defined as highly oxygenated degraded triterpenoids derived from tetracyclic triterpenes. They are categorized into groups based on their basic skeleton. The basic skeletons of quassinoids include C18, C19, C20, C22 and C25 (Fig. 2.2) (Joshi *et al*., 2013; Nuddin *et al*., 2015).

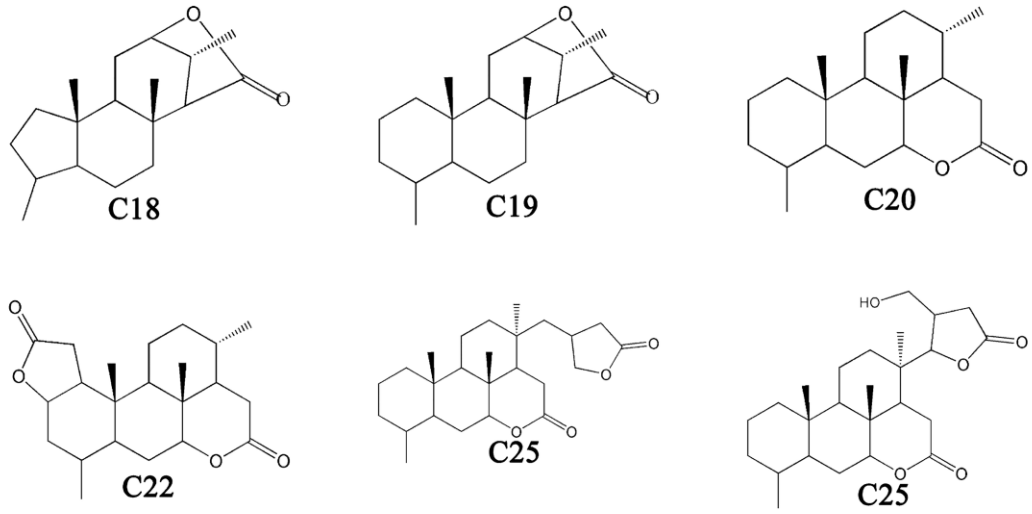

Fig. 2.2 Basic skeletons of quassinoids

2.1.1(b) Glycoproteins

2.1.1(b)(i) Glycoproteins in *E. longifolia* extract

A biologically-active glycopeptide with the molecular mass 4.3 kDa was previously isolated from *E. longifolia* aqueous extract (Patent No. PI 20003988, MAL; 10/362697, USA). According to the patent and some conference presentations, the aphrodisiac and fertility boosting properties of *E. longifolia* aqueous extract has been claimed to be due to the glycoprotein (especially the 4.3 kDa glycopeptide) content of this plant to an extent where the administration of *E. longifolia* to rats was reported to increase testosterone level in Leydig cells (Sambandan *et al.*, 2006). However, such claims only originate from the above-mentioned sources and have not even been published in peer-reviewed journals, yet (Tambi, 2003; Sambandan *et al*., 2006).

As a part of the current research, aqueous extract of *E. longifolia* (TA water extract, TAW) was intentionally depleted of quassinoids to result in a quassinoid-poor TA extract (TAQP). The details on the experimental procedure can be found in Section 3.11. The TAQP extract could be tested using the chromatographic methods such as high-performance liquid chromatography (HPLC) to ensure zero or negligible amount of quassinoids remaining. However, it was also important to analyze the glycoprotein content in both the original extract (TAW) and the one depleted of quassinoids (TAQP) to ensure the glycoprotein content of TAQP extract remained intact as that of the TAW extract. As a consequence, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used for such analysis (Section 2.1.1(b)(ii)).

2.1.1(b)(ii) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used as a powerful technique for the identification and separation of proteins and glycoproteins from plant and tissue samples. In this technique, an electric field is applied and the charged molecules in a gel matrix migrate, in response. Proteins and glycoproteins, with the molecular weight range of 10–200 kDa, in a complex mixture can be separate using this technique.

Useful information can be derived from the SDS-PAGE analysis. It can be an evidence for the presence of glycoproteins in a complex matrix such as plant extract, biological fluids, etc. It is also useful in estimating the molecular weight of the glycoprotein.

A number of staining reagents have been commonly used for the detection of protein bands among which Coomassie Briliant Blue, amido black, nigrosine, Procion Blue and periodic acid-Schiff (PAS) (Koiw *et al.,* 1952) can be mentioned. A very common glycoprotein detection kit is the modification of periodic acid Schiff (PAS) methods which yields magenta bands with a light pink or colorless background (Zacharius *et al*., 1969; Jay *et al*., 1990). The detection limit of this technique has been reported in the range of 25-100 ng for carbohydrates depending on the nature and the degree of protein glycosylation. Horseradish peroxidase has a carbohydrate content of approximately 16% and is used as a positive control in the glycoprotein detection kit.

SDS-PAGE can be performed in the presence of either reducing or nonreducing agents.

Under reducing conditions, proteins are linearized through the dissociation of inter- and intra-chain disulfide bonds. It includes a short heating of the protein sample in a boiling water bath in the presence of a reducing agent. The presence of the anionic detergent

SDS causes proteins to become coated with a negative charge. The proteins migrate in an electric field through the molecular "sieving" action of the gel matrix. As a result, they are separated as discrete bands (Racusen 1979).

2.1.2 Therapeutic effects of *E. longifolia*

E. longifolia has been traditionally used to treat various ailments including body aches, dysentery and glandular swelling (Darise *et al*., 1982). It has been proven *in vitro* that *E. longifolia* serves as an antibacterial agent against Gram-positive and Gram-negative bacteria (Farouk and Benafri, 2007). It also affects libido and sexual function and serves as an aphrodisiac supplement (Ang and Sim, 1997). Furthermore, a wide range of *in vitro* pharmacological properties has been attributed to quassinoids. It includes antimalarial (Kuo *et al*., 2003; Trager and Polonsky, 1981; Kardono *et al*., 1991; Chan *et al*., 2005), anticancer (Okano *et al*., 1981; Wong *et al*., 2012) and antiulcer (Tada *et al*., 1991) properties. Moreover, quassinoids and some triterpenes isolated from *E. longifolia* show anti-tumor activities against some type of cancer cells, *in vitro* (Itokawa *et al*., 1992; Jiwajinda *et al*., 2002). Another *in vitro* assay showed that a eurycomanone-rich fraction of the plant was able to induce cell apoptosis in HepG2 liver cancer (Zakaria *et al*., 2009). In a study performed on human, it was proven that the water-soluble extract of *E. longifolia* root enhanced sperm quality in patients and finally resulted in 14.7% spontaneous pregnancies (Tambi and Imran 2010; Tambi *et al*., 2012). Besides, there are also studies on rat models which prove the efficacy of *E. longifolia* root extracts on spermatogenesis and sperm quality (Wahab *et al*., 2010; Chan *et al*., 2009).

2.2 Male fertility

Infertility is a worldwide health-related issue influencing 10 to 15 percent of the couples (Callister, 2010) in which 8 % of males need to seek medical attention during reproductive years (Esteves *et al*., 2011). Male infertility is among the main leading causes of infertility in young adults (World Health Organization, WHO 1999). In 10-20 percent of the cases, the exact cause of infertility is unidentifiable and unexplained, although in general, it may happen as a result of physiological, endocrinal, environmental and/or genetic mutations. Surgical and medical treatments are available for such conditions. However, the successful treatment of male infertility is difficult and unaffordable, to some great extent (Gupta *et al*., 2013).

In recent years, many patients have gained interest to natural remedies such as herbal supplements to cure infertility. Such supplements are easily obtainable and more cost-effective than prescription drugs (Rowland and Tai, 2003). *Eurycoma longifolia*, *Withania somnifera* and *Terminalia catappa* are among these herbal supplements (Ang and Sim, 1997; Sharma et al., 2011; Ratnasooriya and Dharmasiri, 2000).

Semen analysis is a routine examination of male fertility status through the analysis of sperm count, morphology and motility and also blood monitoring of hormones such as testosterone, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (World Health Organization, WHO 1999; Dohle *et al*., 2004). Fertility evaluation is usually performed on sperm samples obtained through masturbation that may be awkward and inconvenience to the patients. Moreover, blood hormonal investigation is an invasive procedure. All these protocols necessitate considering an alternative to investigate semen profile (especially sperm count) without requiring semen sample.

2.2.1 Physiology of testis and its functions

A testis consists of a large number of coiled seminiferous tubules in which spermatozoa are produced and then transferred to epididymides. The epididymis is connected to vas deferen, which then leads to urethra by passing the prostate gland. Urethra is the last part of the reproductive system which connects the whole reproductive system to the exterior (Guyton and Hal, 1996). Seminiferous tubules contain different types of germinal cells which undergo spermatogenesis. There are spaces between the stacked tubules called interstitial tissue which contains lymph vessels, blood vessels and interstitial or Leydig cells. These cells produce the major amount of steroid hormones, most importantly testosterone.

The testes functions are to produce male gametes or spermatozoa and male sex hormones (androgens). These two functions are closely correlated in that sufficient production of androgens is required for the efficient production of spermatozoa (Setchell, 1978).

2.2.2 Steroidogenesis and spermatogenesis

Steroid hormones (androgens) are produced and secreted by testis and required for a successful spermatogenesis (Ohkura *et al*., 2000). Gonadotropin from the anterior pituitary regulates the production of androgens. These androgens include testosterone **(A)**, dihydrotestosterone **(B)**, 5α-androstan-3β,17β-diol **(C)**, Δ^4-androstenedione **(D)**, 17α-hydroxyperogesterone **(E)**, dehydroepiandrosterone (DHEA) **(F)**, 17α-hydroxypregnenolone **(G)**, pregnenolone **(H)**, progesterone **(I)** and oestradiol **(J)** (Fig.

2.3). The building block of all these androgens is cholesterol (Setchell, 1978; Ohkura *et al*., 2000).

Spermatogenesis is an active replication process which occurs in the seminiferous tubules. The mechanism by which spermatozoon (sperm) is produced, is almost the same in human and other mammals. In rats, which are the main focus of this research, there are roughly 10-20 seminiferous tubules in each testis. Primitive, diploid, stem cell spermatogonia finally produce haploid spermatozoa. This process involves a few mitotic divisions on the spermatogonia. The last mitotic division leads to the formation of spermatocyte. The spermatocyte then undergoes several meiotic cell division, the first part of which is spermatocyte DNA duplication during preleptotene stage, chromosome pairing and condensing during pachytene stage and eventually culminating in two divisions for the production of haploid spermatid. The spermatid undergoes a rapid series of morphological changes. The nuclear DNA then gets very condensed and elongated to a head region covered by a glycoprotein acrosome coat. The cytoplasm, on the other hand, forms a whip-like tail comprising flagellum and mitochondria. The sequential morphological steps in the differentiation of the spermatid (19 steps of spermatogenesis) is included in Fig.2.4 (OECD, 2001; Cheng and Mruk, 2010).

The effect of testosterone on maintaining spermatogenesis has been well-documented (Steinberger, 1974). Setchell (1978) proved that the administration of high-dose testosterone to rats prevented the regression of the seminiferous tubules, a process which takes place after hypophysectomy. Furthermore, Steinberger (1970) proved that the conversion of gonocytes to type A spermatogonia was regulated by testosterone.

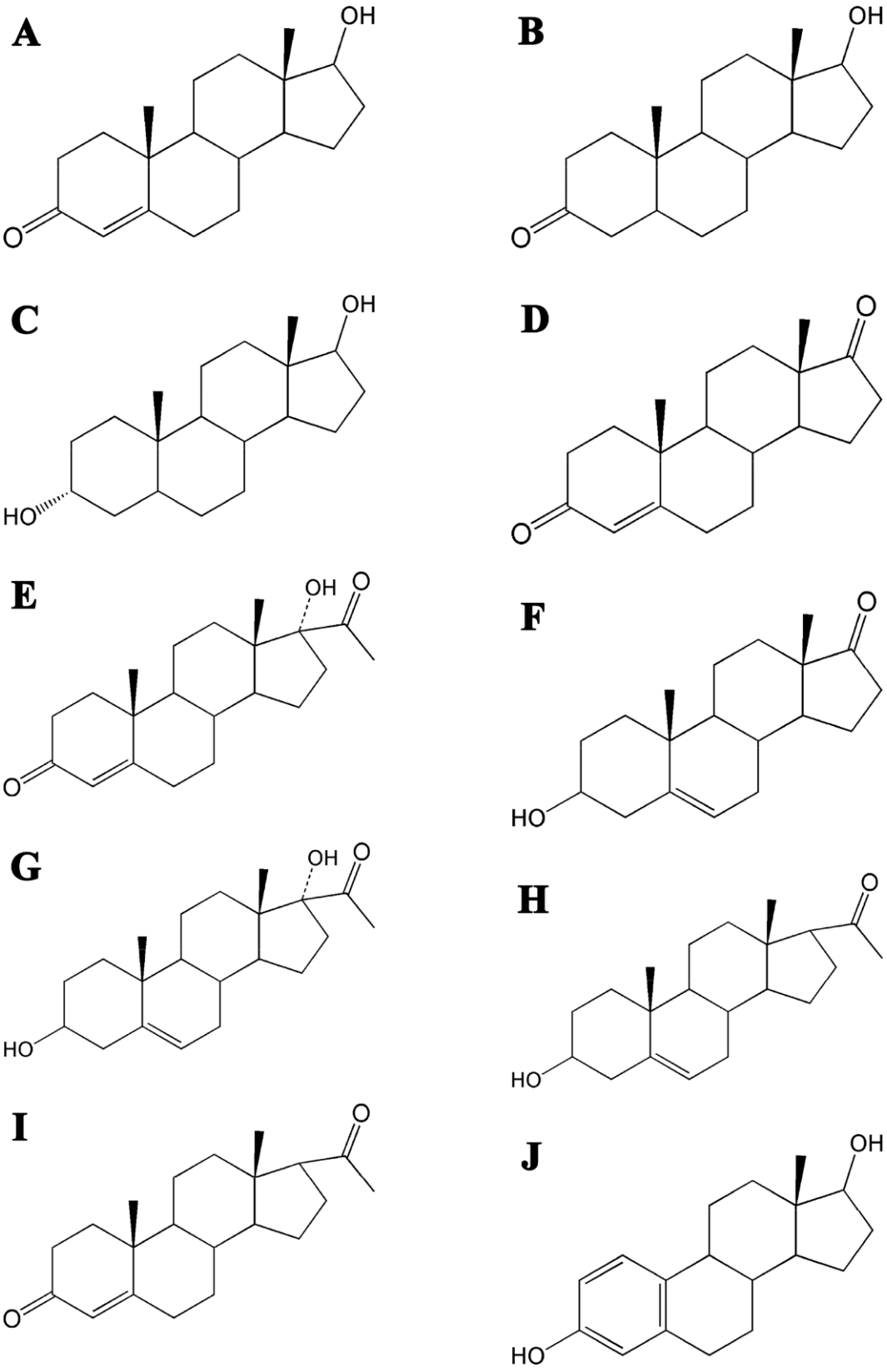

Fig. 2.3 The chemical structure of androgens produced in testis

Testosterone also enables diakinesis, which is the fifth and last stage of the meiosis prophase. Moreover, testosterone plays an active role in the early stages of spermatid formation (Steinberger, 1970).

2.2.3 Sperm count

Sperm count is one of the most critical indicators of male fertility. Any impairments in sperm count, due mainly to lifestyle, infection, occupational and environmental hazards and genetic mutations can cause infertility (Evens 2004; Parekattil and Agarwal, 2012). Normal semen is a mixture of spermatozoa suspended in testicular and epididymal secretions. Semen analysis provides a wealth of information on the clinical status of an individual. That is the reason why proper care must be taken while collecting and analyzing semen. Standardized procedures are available to ensure valid analysis results (World Health Organization, 1999).

There are different categories of semen variables in terms of number of sperm such as normospermia (ejaculate with normal sperm concentration based on the reported value in the references), oligospermia (ejaculate with less sperm concentration than that of reported for normospermia), azoospermia (no spermatozoa in the ejaculate) and aspermia (no cjaculate at all) (Eliasson *et al*., 1970; World Health Organization, 1999; Gupta *et al*., 2013).

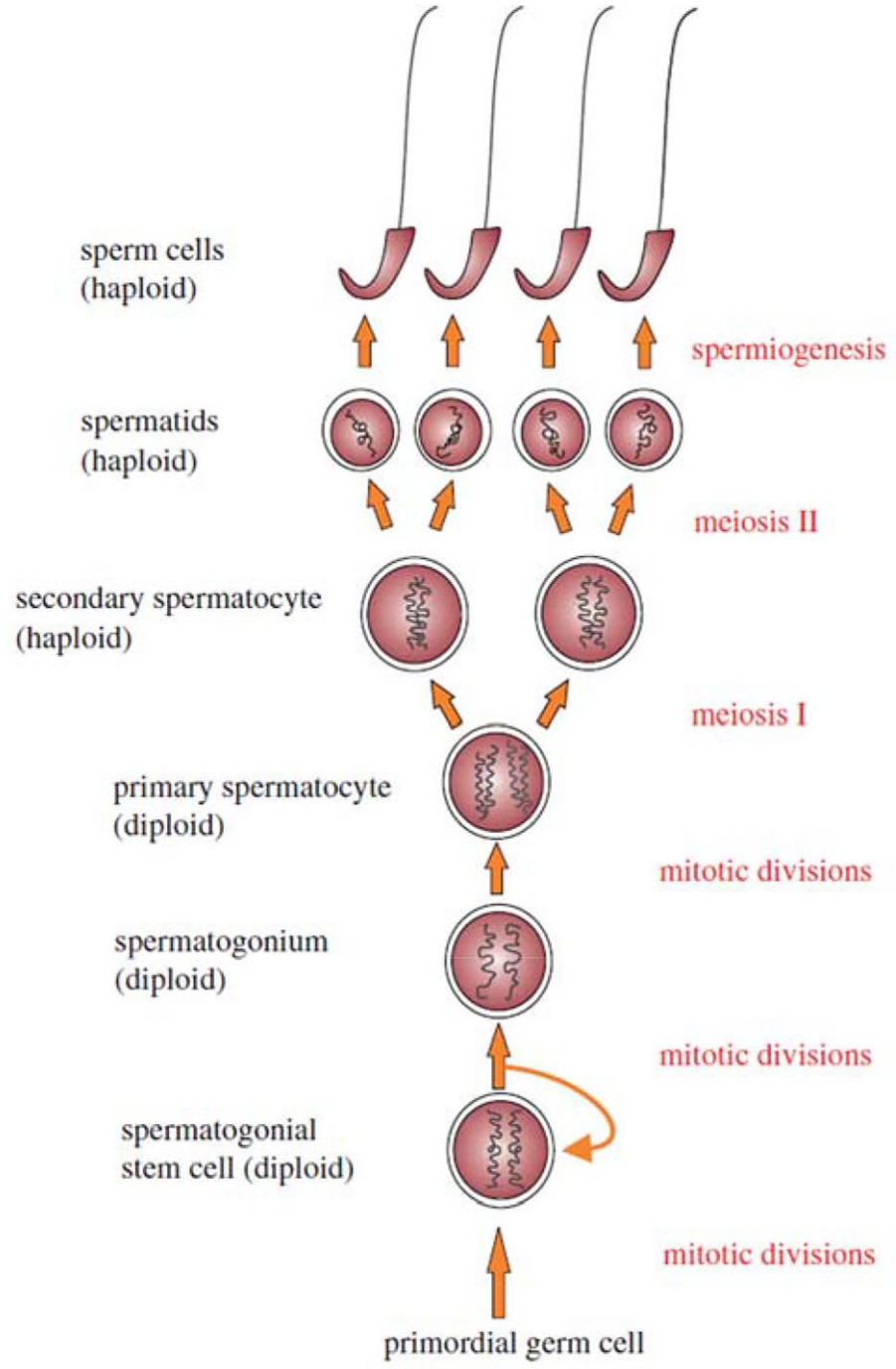

Fig. 2.4 Drawing depicting the development stages of spermatogenesis in rat germ cells (depicted from Cheng and Mruk, 2010)

2.3 Comparison of different 'OMICS' platforms

There are different branches of OMICS technology such as metabolomics, genomics and transcriptomics as a sub-branch of genomics (Lee *et al*., 2005). These three OMICS technologies together with proteomics form 'Systems Biology' (Nicholson and Wilson, 2003). It is possible through genome sequencing to develop new approaches for understanding gene functions. This field of study is known as 'functional genomics' (Ward and German, 2004). Transcriptomics is about profiling the expression of mRNA molecules which are transcribed from the genes. Proteomics, is related to profiling the proteins encoded by mRNA molecules. A fact in genomics is that the regulation of gene activity is performed on the basis of different levels. Chemical modification of the DNA encoding a gene affects its transcription.

In general, metabolomics is concerned with phenotype and deals with the exhaustive profiling of metabolites in an organism. Phenotype is defined as a collection of observable characteristics of an individual cell or organism resulting from the interaction of genes with the environment (Mahner and Kary, 1997). Proteomics and transcriptomics are, however, a flow of media concerning genetic information which do not directly indicate gene function. Metabolomics is advantageous over proteomics and transcriptomics in that it is a stand-alone field of OMICS which does not require genome information. Furthermore, metabolites are a better measure of enzyme activity than genes and proteins (Schad *et al*., 2005; Fukusaki and Kobayashi, 2005).

2.4 Metabolomics

Metabolomics, in general, is defined as the comprehensive evaluation of endogenous low-molecular weight metabolites in a biological system which may provide additional insight into the molecular mechanisms (Yao *et al*., 2014). These metabolites are context dependent and also different based on the physiological and developmental state of the cell, tissue, organ or organism" (Jove *et al*., 2014). Although the terms metabolomics and metabonomics are used interchangeably in the literature, the former is defined as the unbiased analysis of all metabolites in a mixture, whereas the latter is the measurement of metabolic response to stimuli or genetic modifications (Fiehn, 2001; Fiehn, 2002).

2.4.1 Metabolomics technologies

Different metabolomic technologies are available, among the most important of which GC-MS, LC-MS, FT-IR and NMR can be mentioned (Bino *et al*., 2004; Schripsema, 2010).

GC-MS technique was used for the metabolic analysis of potato tubers. The same approach was also employed towards the fast and unbiased comparative multivariate analysis of the volatile metabolite composition of different tomato genotypes (Roessner *et al*., 2000; Tikunov *et al*., 2005). GC-MS technique is also used for the metabolomic analysis of biological fluids. Lu *et al*. (2008) discovered the potential plasma biomarkers of hypertension in a rat model through a GC-MS approach. The same approach was used to identify the plasma biomarkers of hepatocellular carcinoma in a human model (Nezami Ranjbar *et al*., 2015). In general, there are large GC-MS databases available which make the metabolite identification rapid. However, a serious drawback of GC-MS

is the fact that metabolites should be volatile for the analysis. Considering the fact that high temperatures are applied during the analysis, the metabolites should also be stable. As a consequence, less number of metabolites can be detected by GC-MS. The non-volatile compounds should be derivatized to convert to usable volatile adducts for GC-MS analysis (Krishnan *et al*., 2005; Schripsema, 2010).

Metabolic profiling of transgenic rice plant seedlings was performed through an LC-MS method (Mustada *et al*., 2010). An LC-MS-based approach was employed in another study for the global metabolite profiling of grapes (Theodoridis *et al*., 2011). The discriminatory metabolites of colorectal cancer (CRC) patients and healthy subjects were investigated using an LC-MS-based approach (Fukui and Itoh, 2010). LC-MS was employed in another study for detecting low molecular weight metabolite in maternal fluid (Luan *et al*., 2015). In LC-MS analysis, metabolites can be separated by LC and subsequently analyzed by MS. High–resolution spectra can be obtained using modern LC columns. However, the problem with the use of LC-MS is that large and comprehensive databases do not exist, yet. Besides, all the techniques coupled to MS are intrinsically biased against special classes of compounds. For instance, when the MS ionization mode is electrospray (ESI) in LC-MS, simple terpenes, carotenoids and aliphatics are semi-inert and these compounds cannot be detected successfully by the analytical method. The effects of ion suppression due to matrix effect is another drawback which should not be denied (Sterner *et al*., 2000; Choi *et al*., 2001; Weckwerth, 2003).

FT-IR has also been extensively studied in the field of metabolomics. Wu *et al.* (2008) successfully managed to perform a rapid discrimination of two similar natural products,

the extracts of Chinese propolis and poplar buds, by this technique. A FT-IR metabolomics fingerprinting approach was performed to analyze the interactions between structural variants of N-alkyltropinium bromide surfactants with the Gram negative and the Gram positive bacteria. The metabolomic damage exerted by these compounds was then determined (Corte *et al*., 2015). Furthermore, FT-IR was also used for the metabolomic analysis of lyophilized and fresh *Saccharomyces cerevisiae* yeast cells. The FT-IR spectral data provided interesting and informative data on the metabolic status of the whole yeast cell (Correa-García *et al*., 2014). FT-IR has both benefits and drawbacks. It is a non-destructive, simple and fast method with high repeatability. The IR spectrum of a specific chemical compound is unique due to the unique bonding arrangements and functional groups. However, despite the usefulness of FT-IR in the identification of the metabolites in a mixture and its being as a requisite method in the Pharmacopoeia, some limitations exist in the identification of unknown compounds in such a way that the metabolite identification is only possible if the FT-IR spectrum of the pure compound is available as a reference for further analysis. As a result, this technique lacks a comprehensive database of metabolites (Levine *et al*., 1989; Wu *et al*., 2008).

There are myriad of advantages to the use of NMR spectroscopy in the field of metabolomics. One of the major benefits of NMR is its robustness and the lack of sample pre-treatment (separation/fractionation) which reduces the analysis time to a great extent. NMR is a quantitative and non-destructive approach which provides a complete and comprehensive picture of the whole metabolome in a way that no metabolite is lost in the matrix, although some metabolites might be represented by only